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Increased hepatic apoptosis during short-term caloric restriction is not associated with an enhancement in caspase levels

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Abstract

Long-term caloric restriction extends lifespan, probably through a reduction in radical production and attenuation of oxidative stress. In addition, caloric restriction is associated with a reduction and incidence in tumor pathology, probably, in part, via an enhanced rate of apoptosis. We examined whether short-term (2-month) caloric restriction (40% reduction compared to ad libitum controls) increased hepatic apoptosis and if this was associated by an enhancement in various proteolytic caspase (-3, -7, -9, -12) levels and/or a decrease in two potential inhibitors of apoptosis (the x-linked inhibitor of apoptosis protein XIAP and heat shock protein 70). Short-term caloric restriction resulted in a significant decline, compared to ad libitum controls, in both body mass (30%) and liver mass (46%). While hepatic apoptosis (DNA fragmentation) was significantly higher in the caloric restricted rats, this was not associated with any increase in caspase (-3, -7, -9, -12) levels in the liver. Indeed, the levels of caspase-3, -7 and -12 were significantly lower in the caloric restricted group compared to the ad libitum controls and no differences were observed between groups in either XIAP or HSP70 levels. These findings suggest that enhanced hepatic apoptosis observed after 2-months of caloric restriction is not a result of elevated caspase levels at this time, thereby suggesting that an alternative, caspase-independent pathway may be involved.

Keywords: Caloric restriction; Apoptosis; Caspase; DNA fragmentation; Oxidative stress

1. Introduction

The most conventional and reproducible experimental manipulation resulting in an extension of lifespan is caloric restriction (CR), which involves a reduction in energy consumption without malnutrition (Barja, 2002; McCarter, 1995; Sohal and Weindruch, 1996). The exact mechanism through which CR acts to extend lifespan, however, has proven difficult to uncover, although there is a growing body of evidence suggesting that a reduction in radical generation and a resultant attenuation in oxidative stress may play a pivotal role (Barja, 2002; Gredilla et al., 2001a,b; Lass et al., 1998; Leeuwenburgh et al., 1997; Lopez-Torres et al., 2002; Sohal and Weindruch, 1996). One additional and highly reproducible clinical manifestation of CR is the reduction in the incidence, severity and age of onset of several spontaneous and experimentally induced tumors (Frame et al., 1998; Grasl-Kraupp et al., 1994; James and Muskhelishvili, 1994; Shimokawa et al., 1996). This reduction in tumor pathology and incidence during CR in rodents appears linked to an elevated rate of apoptosis and a decreased rate of cellular proliferation (Grasl-Kraupp et al., 1994; Hursting and Kari, 1999; James and Muskhelishvili, 1994; James et al., 1998).

Apoptosis (programmed cell death) appears crucial to organ morphogenesis, successful embryonic development and cellular homeostasis through the removal of deleterious cells, e.g. pre-neoplastic, infected or senescent cells (Kagan et al., 2002; Mattson et al., 2002; Papa and Skulachev, 1997; Pollack and Leeuwenburgh, 2001; Ravagnan et al., 2002; Van Loo et al., 2002; Wachsman, 1996). While the exact induction mechanism for apoptosis remains equivocal

Abbreviations: CR, caloric restriction; AD, ad libitum; Caspase, cysteine-dependent aspartic acid specific endoprotease; Xiap, x-chromosome-linked inhibitor of apoptosis protein; APAF, 1, apoptotic protease activating factor 1; AIF, apoptosis inducing factor; HSP70, heat shock protein 70.

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(Van Loo et al., 2002), several signals including reactive oxygen and nitrogen species, TNF-α, increasing intracellular Ca^{2+} levels, insulin-like growth factors and DNA damage (Dunn et al., 1997; Papa and Skulachev, 1997; Moschos and Mantzoros, 2002; Ravagnan et al., 2002; Wang, 2001) appear important in activating various proteases, such as the caspases (cysteine-dependent aspartic acid specific endoproteases). The caspase-dependent pathway generally involves mitochondrial permeability, cytochrome-c release and apoptosomal formation resulting in the cascade of various enzyme proteases (Cain et al., 2002; Ravagnan et al., 2002; Van Loo et al., 2002; Wang, 2001). However, it has also been suggested that Bcl-2 may play a role in regulating apoptosis independently from apoptosomal formation (Marsden et al., 2002) and apoptosis can occur in a caspase-independent fashion, for example through the actions of apoptosis-inducing factor (AIF) (Cande et al., 2002; Cregan et al., 2002; Joza et al., 2001) or endonuclease G (Li et al., 2001).

In spite of the substantial body of knowledge gathered regarding long-term CR, the effects of short-term CR (less than 2-month duration) with respect to alterations in radical production, the attenuation of oxidative stress (Gredilla et al., 2001a) and on apoptosis are less well documented. Unlike long-term CR, no enhancement in apoptosis rate was observed in rats after 6 weeks of CR (Higami et al., 2000). The following study was designed to examine the effects of short-term CR on hepatic apoptosis in Fischer 344 rats after 8 weeks of CR (40% reduction compared to ad libitum controls). We hypothesized that short-term CR would enhance the amount of hepatic apoptosis, through an increase in the levels of various caspases, specifically caspases-3, -7, -9 and -12. In addition, we examined whether this hypothesized increase in apoptosis following short-term CR could be, in part, due to a decrease in the level/s of two potential inhibitors of apoptosis, namely the X-chromosome-linked inhibitor of apoptosis protein (XIAP; an endogenous inhibitor specifically of caspase-3 and -7) and heat shock protein 70 (HSP70), which is thought to inhibit or neutralise both the apoptotic protease activating factor 1 (APAF-1) and AIF: (Beere et al., 2000; Cande et al., 2002; Ravagnan et al., 2001). In this study, we observed a significant increase in the level of hepatic apoptosis (DNA fragmentation) during short-term CR, but this was not associated with any increase in caspase levels or with any decrease in two inhibitors of apoptosis.

2. Experimental procedures

2.1. Animals

Male ad libitum and caloric restricted Fischer 344 rats were purchased from the National Institute of Aging (Indianapolis, IN, USA) at 4 months of age. CR was started at 3.5 months of age (10% restriction), increased to 25% restriction at 3.75 months and then maintained from 4 months onwards at 40% restriction (of ad libitum control levels) until the termination of the experiment at 6 months of age. All animals were individually housed at The University of Florida Animal Care Services (Gainesville, FL, USA), under a photoperiod of 12L:12D and an ambient temperature of 18–20 °C from 4 months of age onwards. At 6 months of age, two individuals per day (one ad libitum and one caloric restricted) were weighed, anaesthetized using an intraperitoneal injection of pentobarbital sodium solution (Abbot Laboratories, IL, USA; 5 mg/100 g body weight) and then the liver was rapidly dissected out. The cytosolic fraction was separated using differential centrifugation during which tissues were homogenized (on ice) in 1:10 wt/vol of an ice-cold buffer (220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 2 mM Hepes (pH 7.4) and 0.1% fatty-acid free BSA) using a Potter–Elvehjem glass–glass homogeniser. The liver homogenates were centrifuged (Eppendorf, 5810R, Brinkmann Instruments Inc., NY, USA) at 600 g (4 °C) for 10 min, the resulting supernatant centrifuged for 18,000*g* (4 °C) for 10 min, collected and stored at −80 °C. This experiment received local institutional animal care and animal use committee approval.

2.2. Induced cell death levels

The amount of hepatic cytoplasmic histone-associated-DNA fragmentation (mono- and oligo-nucleosomes) was quantified in the cytosolic fractions using a Cell Death Detection ELISA (Roche Diagnostics, GmbH, Germany). Endogenous endonucleases activated during apoptosis cleave double stranded DNA in the linker region between nucleosomes to produce mono- and oligo-nucleosomes of 180 base pairs (or multiples) and these were used to determine cell death. Samples were run in triplicate and the means expressed as arbitrary OD units normalized to milligram cytosolic protein, with protein values determined throughout using the Bradford method.

2.3. Caspase-3, -7, -9, -12, XIAP and HSP70 levels

Western analysis was carried out on liver cytosolic fractions separated using 4–20% PAGEr® Gold precast Tris–glycine gels (BioWittaker Molecular Applications, Rockland, ME, USA) under denaturing conditions, transferred on nitrocellulose membranes (0.2 μm, Trans-Blot® Transfer Medium, Bio-Rad Laboratories, CA, USA) and blocked overnight using a 5% milk solution. Samples from both groups were always loaded on the same gel, to avoid any potential inter-gel variation, standardized to one another using protein content and we probed each gel for actin to verify this. Membranes were incubated and rocked for 90 min at room temperature (RT) in 5% blocking solution plus either, polyclonal primary antibody: Caspase-3 (1:1000, NeoMarkers, CA, USA), caspase-7 (1:1000, NeoMarkers, CA, USA), caspase-12 (1:1000, Oncogene Research Products, MA, USA), XIAP (1:200, Medical and Biological Laboratories

Co., Japan) or HSP70 (1:1000, Stressgen Biotechnologies, Canada). Membranes were subsequently washed 4 times (0.5% Tween PBS), the secondary antibody, of either anti-rabbit or anti-mouse IgG horseradish peroxidase-linked whole secondary antibody (Amersham Biosciences, UK Ltd, UK), added then membranes were incubated and rocked as before. Membranes were then washed 4 times (0.5% Tween PBS), exposed to photographic film (Hyperfilm™ ECI™, Amersham Pharmacia Biotech, UK) and analysed using the NIH APPLE-J program. All 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'. Cytosolic caspase-3 activity was measured using a fluorometric assay kit (Promega-Corporation, Madison, WI USA) following the manufactures protocol. The substrate Ac-DEVD-AMC is cleaved proteolytically by caspase-3 and fluorescence was determined using a Spectra Max Fluorescent Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at excitation: 485 nm and, emission: 538 nm for AMC. Values were expressed as arbitrary units of OD per milligram protein.

2.4. 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 normally distributed were log-transformed. Statistical analyses were carried out using MINITAB 13 statistical package (Minitab Inc., State College, PA, USA). Statistical significance was considered where $p < 0.05$. All data is reported as mean ± SEM.

3. Results

3.1. Body mass and liver mass

Short-term CR resulted in a significant lower body mass ($t = 16.71$, $p < 0.001$) and liver mass ($t = 12.45$, $p < 0.001$), of 30% and 46%, respectively, compared to the ad libitum group (Table 1). This reduction in liver mass during short-term CR was proportional to the decreases observed in body mass, as when employing a general linear model with body mass as covariate, no significant differences were observed between groups in liver mass ($F = 0.830, p = 0.374$).

Table 1
Body mass and liver mass in ad libitun (ad lib) and short-term caloric restricted (CR) rats.

	Ad lib (g)	CR (g)	% decline in mass
Body mass	390.5 ± 5.2	272.5 ± 4.7*	30.2
Liver mass	13.25 ± 0.37	7.22 ± 0.31*	45.5

Mean ± SEM body mass and liver mass in ad libitum (ad lib) and short-term caloric restricted (CR) rats. $N = 10$ AD and $N = 8$ CR. * Denotes significant difference to ad libitum group. % Decline in mass compared to ad libitum group.

3.2. Cell death levels

The amount of cytoplasmic histone-associated-DNA fragments (mono- and oligo-nucleosomes) resulting from the cleavage of double stranded DNA during apoptosis was quantified in the liver of the ad libitum and short-term caloric restricted rats. In this study, we thought that any change in the level of apoptosis and DNA fragmentation observed, as a consequence of CR, would be minimal and therefore difficult to detect. We chose the ELISA technique due to its greater sensitive in quantifying DNA fragmentation when compared to other methods such as TUNEL. A significant difference was observed between the groups (Fig. 1), with significantly higher levels of DNA fragmentation, i.e. apoptosis, being observed in the short-term caloric restricted group ($t = 2.49$, $p = 0.028$).

3.3. Caspase-3, -7 and -9 levels

The levels of caspase-3, -7 and -9 were measured by Western blotting. However, the levels of both procaspase-3 (Fig. 2A) and caspase-3 (Fig. 2B) were significantly lower in the caloric restricted compared to the ad libitum group ($t = 3.01$, $p = 0.015$; $t = 4.27$, $p = 0.002$, respectively). Similarly, both procaspase-7 (Fig. 2C; $t = 2.78$, $p = 0.018$) and caspase-7 levels (Fig. 2D; $t = 2.823$, $p = 0.017$) were also significantly lower in the short-term caloric restricted group compared to the control levels. However, despite the levels of both procaspase-9 (Fig. 3A, $t = 1.18$, $p = 0.264$)

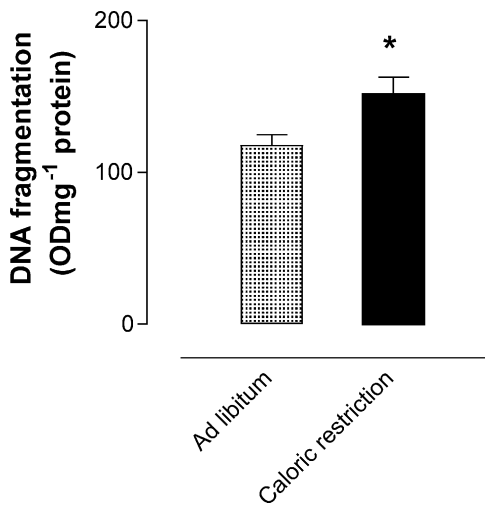

Fig. 1. Quantified levels of hepatic cytoplasmic histone-associated-DNA fragments (mono- and oligo-nucleosomes). Short-term CR resulted in a significantly higher level of DNA fragmentation in the liver compared to the ad libitum control group ($p = 0.028$). $N = 10$ *AD* and $N = 8$ *CR*.

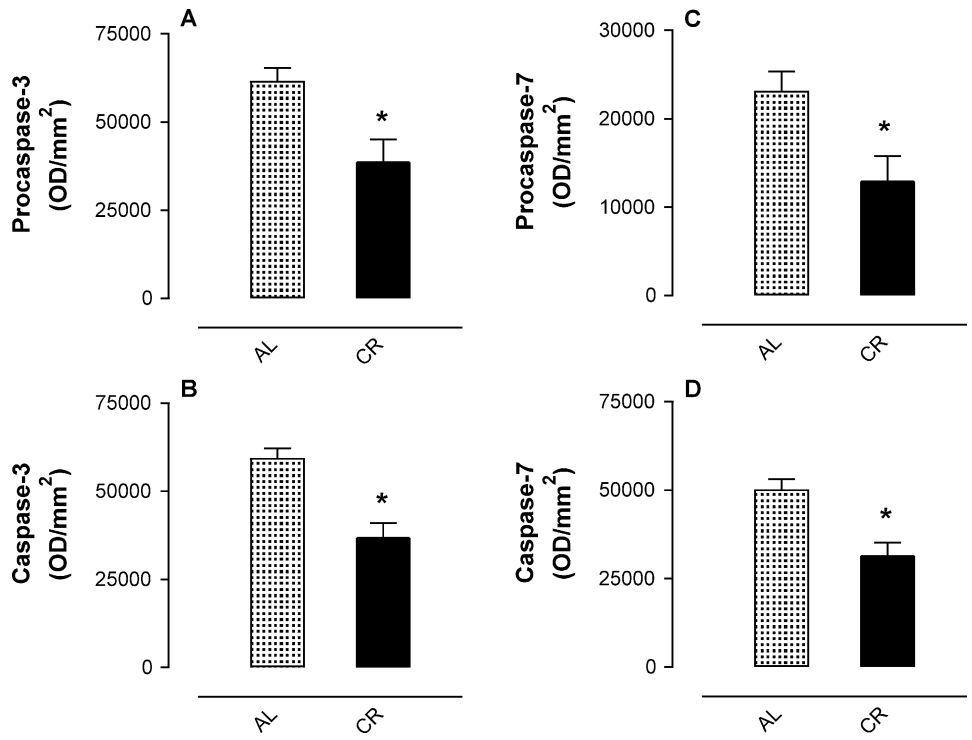

Fig. 2. (A) Procaspase-3, (B) caspase-3, (C) procaspase-7 and (D) caspase-7 levels (OD/mm^2) in liver cytosol of ad libitum and short-term caloric restricted rats. The levels of both procaspase-3 and caspase-3 were significantly lower in short-term caloric restricted rats compared to ad libitum controls ($p = 0.015$ and $p = 0.002$, respectively. Both procaspase-7 levels ($p = 0.018$) and caspase-7 levels (p = 0.017) were significantly lower in the short-term caloric restricted groups compared to ad libitum controls ($p = 0.018$). $N = 7$ in each group.

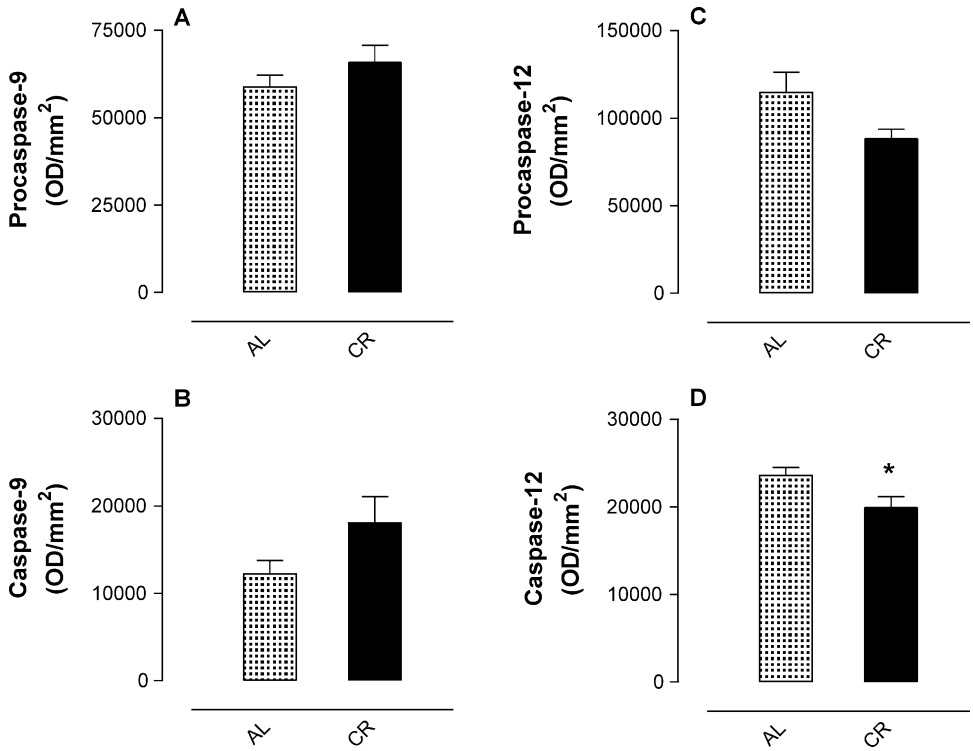

Fig. 3. The levels (OD/mm^2) of (A) procaspase-9, (B) caspase-9, (C) procaspase-12 and (D) caspase-12 in liver cytosol of ad libitum and short-term caloric restricted rats. There was no significant difference between the short-term caloric restricted and the ad libitum groups in either procaspase-9 levels ($p = 0.264$) or in caspase-9 levels ($p = 0.125$). While no significant difference was observed between the ad libitum and short-term caloric restricted groups in procaspase-12 levels ($p = 0.082$), a significant difference was observed between groups in caspase-12 levels, with the CR group having a significantly lower level ($p = 0.041$). $N = 7$ in each group.

Table 2
Inhibitor of apoptosis XIAP and HSP70 in the livers of ad libitum (ad lib) and short-term caloric restricted (CR) rats.

	Ad lib	CR	p value
XIAP	106859 ± 3360	99789 ± 3106	0.151
HSP70	42282 ± 4588	32705 ± 2302	0.099

Mean ± SEM levels (OD/mm^2) of the inhibitor of apoptosis XIAP and HSP70 in the livers of ad libitum (ad lib) and short-term caloric restricted (CR) rats. $N = 7$ in each group.

and caspase-9 (Fig. 3B, $t = 1.71$, $p = 0.125$) being elevated in the CR compared to the ad libitum group, these differences were not statistically significant. There was no significant difference observed between the AD and CR rats when determining caspase-3 activity using a fluorometric assay (3224 ± 245.8 and 3272 ± 166.8 arbitrary units of OD per milligram protein, respectively, $n = 9$ in each group). However, any lack of significance in caspase activity, compared to caspase levels in this study, may be partly explained by the potential insensitivity of these fluorometric assays.

3.4. Caspase-12 levels

There was no significant difference in the levels of procaspase-12 (Fig. 3C), which is activated under endoplasmic reticulum stress, between experimental groups, although it did approach statistical significance ($t = 2.08$, $p = 0.082$). However, the levels of caspase-12 (Fig. 3D) were significantly reduced in the CR rats compared to the ad libitum controls ($t = 2.32$, $p = 0.041$).

3.5. XIAP and HSP70 levels

The hepatic levels of both XIAP (Table 2; $t = 1.55$, $p = 0.151$), an inhibitory protein of apoptosis particularly caspase-3 and -7, and HSP70 ($t = 1.87$, $\times$ $p = 0.099$), a potential inhibitor of both APAF-1 and AIF, did not differ between groups, although the levels of both were lower in the short-term caloric restricted groups (Table 2).

4. Discussion

Long-term CR extends lifespan (McCarter, 1995; Sohal and Weindruch, 1996), probably via decreased radical production and resultant oxidative stress (Barja, 2002; Gredilla et al., 2001b; Lass et al., 1998; Leeuwenburgh et al., 1997; Lopez-Torres et al., 2002), and is associated with increased hepatic apoptosis and a decreased frequency and severity of various cancers (James and Muskhelishvili, 1994; James et al., 1998; Wachsman, 1996). However, the effect of short-term CR on radical production and particularly on apoptosis are less clear, appearing both time- and tissue-dependent (Gredilla et al., 2001a,b; 2002; Higami et al., 2000). In the following study, we observed that short-term CR resulted in a significant reduction in body mass and liver mass and, as seen during long-term CR protocols, a significantly higher amount of hepatic apoptosis (indicated by DNA fragmentation) compared to ad libitum controls. However, this increase in hepatic apoptosis was not associated with any increase in various caspase levels activated under either mitochondrial stress (caspases-3, -7 or -9) or endoplasmic reticulum mediated stress (caspase-12). Indeed, the levels of both the procaspase and the proteolytic caspase in all cases, except with caspase-9, were significantly reduced in the CR rats, despite the higher levels of DNA fragmentation observed in these animals. It is certainly feasible that the enhanced rate of apoptosis during short-term CR may have been initiated via a caspase-independent manner (Joza et al., 2001; Li et al., 2001). The translocation of AIF from the mitochondria to the nucleus (sensitive to various pro-apoptotic factors), has been shown to induce caspase-independent DNA fragmentation (Cregan et al., 2002; Joza et al., 2001; Rideout and Stefanis, 2001; Van Loo et al., 2002), possibly without chromatin condensation or nuclear body formation (Cregan et al., 2002). It has been suggested that AIF can be antagonized and blocked through the actions of HSP70 (Ravagnan et al., 2002) and while not significant ($p = 0.099$), there was a trend towards lower hepatic HSP70 levels in the short-term CR rats (23% reduction compared to ad libitum controls). Furthermore, it does appear that molecular chaperones are found in proportion to the energy intake of rodents (Dhahbi et al., 2001a; Spindler et al., 1990).

This potential induction of apoptosis through a caspase-independent pathway may confer protection to particular cell types in the liver, as suggested to occur in neuronal cells after various models of stroke Rideout and Stefanis, 2001. The liver is central to metabolism and is particularly sensitive to changes in diet (Lopez-Torres et al., 2002). A ‘selective’ and coordinated cell death, rather than ‘wholesale’ cell death initiated via the caspase cascade, may be essential in maintaining liver function during CR, a period of reduced and scarce energy availability coupled with a significant reduction in liver mass. The reduction in liver mass of 45.5% with CR is likely to occur very rapidly, probably over a couple of weeks, therefore, it is possible that selective cell death in the liver takes place during CR to prevent any further cell loss, thus leading to removal of unwanted and/or damaged cells only. In future studies, it would be interesting to examine the role of HSP27, as recent evidence suggests that this chaperone may play a major role during in preventing apoptosis during cellular stress. HSP27 has been shown to inactivate various caspases through its ability to sequester cytochrome-c and procaspase-3 and also through its ability to cause redox modulation in cells (Concannon et al., 2001; Concannon et al., 2003). In addition, CR is associated with elevated autophagic proteolysis (Cavallini et al., 2001), protein catabolism, gluconeogenesis and turnover and

replacement of extra hepatic proteins (Dhahbi et al., 2001b). Therefore, CR must involve significant changes in hepatic homeostasis and it is certainly not established whether particular cell types or structures in the liver are selectively removed or maintained during short-term CR.

In summary, we observed that short-term CR (2 months duration) in Fischer 344 rats resulted in an enhanced rate of apoptosis, as indicated by an increase in cytoplasmic histone-associated-DNA fragments (mono- and oligo-nucleosomes), compared to ad libitum controls. However, this enhanced apoptosis rate was actually associated with a decrease in the levels of various members of the caspase-dependent apoptotic cascade. A further examination of the role of ROS in initiating both caspase-dependent and -independent apoptosis is required, as the greater amount of hepatic apoptosis during short-term CR was actually associated with a period of significantly reduced hepatic ROS production (Gredilla et al., 2001a). Further investigations are required to examine whether there is an enhancement of factors, such as AIF or endonuclease G, which are involved in caspase-independent pathways of cell death (Wang, 2001) and in addition whether apoptosis actually plays a significant role in the hepatic atrophy associated with CR. Future studies also need to determine if caspase activation is visible when employing immunohistochemistry on specifically prepared liver tissue from CR and control animals. Finally, the role of the proteasome during CR must be addressed, as it appears crucial to the degradation of various cytosolic proteins, including those involved in cell proliferation and cell death (Glockzin et al., 1999; Grune, 2000; Merker et al., 2001) and has been suggested to be central to the regulation of both caspase-dependent and -independent apoptosis (Wu et al., 1999).

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