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Dietary Fat and Aging Modulate Apoptotic Signaling in Liver of Calorie-Restricted Mice

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Imbalance between proliferation and cell death accounts for several age-linked diseases. Aging, calorie restriction (CR), and fat source are all factors that may influence apoptotic signaling in liver, an organ that plays a central metabolic role in the organism. Here, we have studied the combined effect of these factors on a number of apoptosis regulators and effectors. For this purpose, animals were fed diets containing different fat sources (lard, soybean oil, or fish oil) under CR for 6 or 18 months. An age-linked increase in the mitochondrial apoptotic pathway was detected with CR, including a decrease in Bcl-2/Bax ratio, an enhanced release of cytochrome *c* to the cytosol and higher caspase-9 activity. However, these changes were not fully transmitted to the effectors apoptosis-inducing factor and caspase-3. CR (which abated aging-related inflammatory responses) and dietary fat altered the activities of caspases-8, -9, and -3. Apoptotic index (DNA fragmentation) and mean nuclear area were increased in aged animals with the exception of calorie-restricted mice fed a lard-based fat source. These results suggest possible protective changes in hepatic homeostasis with aging in the calorie-restricted lard group.

Key Words: Apoptosis—Calorie restriction—Dietary fat—Liver.

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AGE-RELATED diseases, including neurodegenerative disorders and cancer, for which aging is considered the most important risk factor (1), are the clinical expression of the age-linked loss of function in tissues. The mechanisms underlying such loss of tissue function are not yet fully explained. The free radical theory of aging suggests that reactive oxygen species, resulting from either enzymatic or nonenzymatic processes, cause cumulative oxidative damage in biomolecules (2). Reactive oxygen species may cause DNA oxidation and induce apoptosis. Imbalance between proapoptotic and antiapoptotic proteins belonging to the Bcl-2 family determines cellular fate. Mitochondrial permeabilization, which allows cytochrome *c* to enter the cytosol, triggers a caspases cascade. Activation of final effectors is followed by DNA fragmentation and organelle dismantling (3). Apoptosis can also be initiated by extracellular ligands (4,5).

A correct balance between proliferation and death is required to regulate homeostasis, and the role of apoptosis appears to be tissue specific. Although excessive cell death can lead to dysfunction in postmitotic organs (6), defective apoptosis prevents removal of damaged cells in proliferative organs, potentially resulting in neoplastic, autoimmune, or viral disease (7). A variety of apoptosis-avoiding strategies have been identified in tumors (8,9), and resistance to apoptosis has been highlighted as one of the basic attributes of neoplastic cells (10).

The liver plays a central role in energy metabolism, glycogen storage, and detoxification. Hepatocytes have a high proliferative potential, despite their low division rate under normal conditions (11). It has been reported that apoptotic cell death in the liver increases with age both in rats (12) and mice (13), although the underlying mechanisms remain controversial. Despite this, a decline in the apoptotic response to genotoxic stress has also been observed in liver from aged rats (14), indicating that multiple factors may influence hepatic apoptotic signaling during aging. The liver is highly exposed and particularly sensitive to environmental factors, including infections, alcohol, and diet (15).

Calorie restriction (CR) without malnutrition extends mean and maximum life span in rodents and is known to delay the onset of age-related diseases (16). Although the underlying mechanisms have not been fully elucidated, a decrease in oxidative stress likely contributes to CR effects (17,18). Additionally, CR delays the incidence of hepatocellular carcinoma in mice (19) and increases levels of spontaneous apoptosis in preneoplastic foci and hepatoma-prone strains (13,20,21), which however do not correlate with an increase in caspase activity, suggesting the implication of alternative mechanisms (22).

CR lowers long-chain polyunsaturated fatty acid content in mitochondrial membranes (23). Saturated fatty acids are less prone to oxidation, and fatty acid unsaturation level negatively correlates with maximum life span in mammals (24). Hence, alterations in tissue phospholipid fatty acid composition have been suggested as a mechanism of CR action (25). Moreover, changes in membrane composition may not only exert a passive role as a target for reactive oxygen species but also modulate important membrane processes such as proton leak (26–28). Dietary fat source alterations also modulate apoptotic signaling. We previously demonstrated that a diet containing olive oil prevented in Wistar rats the age-dependent decrease of hepatic caspase-3, -8, and -9 that was otherwise observed with diets containing sunflower oil (29). Additionally, a CR diet containing fish oil decreased proapoptotic signaling in both mitochondrial and plasma membranes from skeletal muscle of young mice (30).

The aim of this work was to determine the extent to which CR, fat source variations, and aging interact to modulate liver apoptotic signaling in mice.

Methods

Animals, Diets, and Liver Samples

Male C57BL/6J mice (Charles River Laboratories, Spain) were fed a commercial rodent chow diet (Harlan Teklad #7012, Madison, WI) until they were 3 months old and then randomly assigned into four dietary groups fed a modified AIN-93G semipurified diet containing 20.3% protein, 63.9% carbohydrate, and 15.8% fat (% total kcal/d). Control group was fed 95% of a predetermined ad-libitum intake (12.5 kcal) to prevent obesity, whereas CR dietary groups were fed 40% less calories. All diets were identical except for dietary lipid source, which was soybean oil (high in n-6 polyunsaturated fatty acids, Super Store Industries, Lathrop, CA) for the Control-Soy fed mice and one of the CR groups. The two remaining CR groups were fed diets containing fish oil (high in n-3 polyunsaturated fatty acids: 18% eicosapentaenoic acid, 12% docosahexaenoic acid, Jedwards International, Inc., Quincy, MA) or lard (high in saturated and monounsaturated fatty acids, ConAgra Foods, Omaha, NE). To insure adequate linoleic acid levels, the CR-fish diet also contained soybean oil (14% of total fat content). Fatty acid compositions of all diets have been previously reported (28). After a dietary intervention period of 6 or 18 months, animals were sacrificed by cervical dislocation and liver samples were stored frozen at −80°C for later analysis. The age of animals at the end of intervention period was 9 or 21 months. All experimental procedures and animal handling were in accordance with the Pablo de Olavide University Ethical Committee rules and the 86/609/EEC Directive. Liver mitochondrial and cytosolic fractions were obtained by homogenization and differential centrifugation and then stored at −80°C (Supplementary Methods).

Caspase Activities

Proteolytic activity of caspases-8, -9, and -3 was determined using fluorogenic 7-amino-4-methylcoumarin-conjugated substrate peptides. Noncaspase proteolytic activity was substrated by developing parallel assays in the presence of the corresponding inhibitory peptides (Supplementary Methods). Activities were then expressed as arbitrary units/mg protein.

Quantification of DNA Fragmentation (Apoptotic Index)

DNA fragmentation was measured by ELISA quantification of cytosolic mono- and oligonucleosomes (Roche Diagnostics, Mannheim, Germany). Absorbance was determined at 405 nm using a Flex Station 3 (Molecular Devices, Sunnyvale, CA) and data were presented as arbitrary optical density units/mg cytosolic protein.

Polyacrylamide Gel Electrophoresis and Western Blot Immunodetection

Samples of about 50 μg protein were denatured, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5% acrylamide), and transferred to nitrocellulose membranes. Bcl-2 polypeptide was measured in whole homogenates, whereas Bax was measured both in homogenates and in mitochondria-enriched fractions. Homogenate Bcl-2 and mitochondrial Bax data were used to calculate Bcl-2/Bax ratio. X-linked inhibitor of apoptosis protein (XIAP) was measured in whole homogenates. Apoptosis-inducing factor (AIF) and cytochrome *c* levels were measured in mitochondria-enriched fractions and in cytosols. Actin staining was used to reveal equal protein loading in electrophoretic separations of whole homogenates and cytosols. Immunostaining was carried out with corresponding primary antibodies (Santa Cruz Biotechnology or Sigma, Supplementary Table 1). Horseradish peroxidase–conjugated secondary antibodies (Sigma and BD Biosciences Pharmingen) were used to reveal binding sites by enhanced chemiluminescence (ECL-Plus, GE Healthcare Life Sciences). Quantification of Ponceau S-stained lanes was used for normalization of protein load (31). Changes

produced by CR per se were studied by comparing Control-Soy and CR-Soy groups, whereas the effects of dietary fat under CR were studied by comparing CR-Lard, CR-Soy, and CR-Fish groups. These two classes of studies were performed in separate electrophoresis gels and blots, which were optimized for each case (Supplementary Methods).

Light Microscopy

Pieces of liver tissue were aldehyde fixed and embedded in EMbed 812 resin following standard protocols (Supplementary Methods). Semithick (0.5–1 μm width) sections were stained with toluidine blue. Micrographs were taken with a Leica DME light microscope using a 40× objective. Because the increase of nuclear size is a well-known marker of aging liver (32), we carried out a planimetric study of nuclear size using the ImageJ software (NIH). About 1,000 nuclei from five animals per diet were measured.

Statistical Analysis

Values are means ± *SEM*. D'Agostino–Pearson tests were performed to determine normality. The effect of CR was assessed by Student's *t* test (CR-Soy group vs Control-Soy group). In case data did not pass the normality test, the Mann–Whitney test was followed. The effect of dietary fat under CR was assessed by one-way analysis of variance followed by a post hoc analysis (Tukey's test for multiple comparisons) to assess for significant differences among groups. Post hoc analysis of linear trend was also performed to investigate putative alterations of tested parameters among CR diets ordered as CR-Lard → CR-Soy → CR-Fish, which resulted in a progressive increase of the n-6/n-3 ratio in phospholipid highly unsaturated fatty acids (28). In case data did not pass the normality test, the Kruskal–Wallis test was followed. Means were considered statistically different with $p < .05$. All statistical analyses were performed using Graphpad Prism 5.03 (Graphpad Software Inc., San Diego, CA).

Results

Bcl-2, Bax, and Bcl-2/Bax Ratio

Aging produced a significant decrease of Bcl-2 levels in liver homogenates from Control-Soy and CR-Soy groups (Figure 1A, Supplementary Figure 1A). Bax was not altered significantly with age when measured in homogenates (Figure 1C, Supplementary Figure 1A), but it was increased significantly in old animals from CR-Soy group when measured in mitochondria-enriched fractions (Figure 1E, Supplementary Figure 1E). Whereas age-related changes of Bcl-2 levels were not affected by CR (Figure 1A), reduction of calories did produce a decrease of Bax levels in liver homogenates that was statistically significant for young mice (Figure 1C). The aging-related decrease in Bcl-2 levels we observed in the CR-Soy group was not reproduced in the CR-Lard and CR-Fish groups (Figure 1B, Supplementary Figure 1B). We did not observe significant differences when comparing the three CR dietary groups within a given age, except for Bcl-2 levels in old animals, which were significantly higher in the CR-Lard compared with CR-Soy group (Figure 1B). Interestingly, changing fat source under CR produced a different pattern of Bax levels alterations with age in liver homogenates (Figure 1D): The lack of age-related changes we previously observed for the CR-Soy group was not maintained in the CR-Fish mice, in which a significant increase of Bax levels was observed for old animals. Bax also tended to increase with age in the CR-Lard group, although results did not reach statistical significance ($p = .09$). Nevertheless, when measured in mitochondria-enriched fractions, it was clearly observed that aging produced a significant increase of mitochondria-associated Bax in all CR groups (Figure 1F, Supplementary Figure 1F).

Aging produced a significant decrease of Bcl-2/Bax ratio in membranes, both in the Control-Soy and CR-Soy groups. Conversely, CR produced a significant increase of Bcl-2/Bax ratio in young and old animals (Figure 1G), and this effect was maintained in all CR groups (Figure 1H). When studying the effect of dietary fat under CR conditions, we found that Bcl2/Bax ratio was lower in CR-Soy in comparison with CR-Lard and CR-Fish groups (Figure 1H).

Cytochrome c *and AIF Release and Accumulation in the Cytosol*

Aging produced a dramatic increase of cytosolic cytochrome *c* in Control-Soy and CR-Soy groups, but no differences were found between the two dietary groups (Figure 2A, Supplementary Figure 1C). This age-related change was also observed in CR-Lard and CR-Fish groups (Figure 2B, Supplementary Figure 1D), being particularly evident in CR-Lard group (10-fold increase). Furthermore, levels of cytosolic cytochrome *c* were significantly elevated in the old CR-Lard group compared with the old CR-Fish group, but no significant differences were observed between the remaining groups (Figure 2B).

AIF release and accumulation to the cytosol did not change with age in the Control-Soy group and was even significantly decreased in old animals of the CR-Soy group (Figure 2C, Supplementary Figure 1C) with a similar trend being observed in the CR-Fish group, although differences were not statistically significant. No age-related changes in AIF release and accumulation to the cytosol were observed in the CR-Lard group. Finally, levels of AIF in the liver cytosolic fractions were significantly higher in CR-Lard than in the CR-Soy or CR-Fish groups in old but not in young mice (Figure 2D, Supplementary Figure 1D).

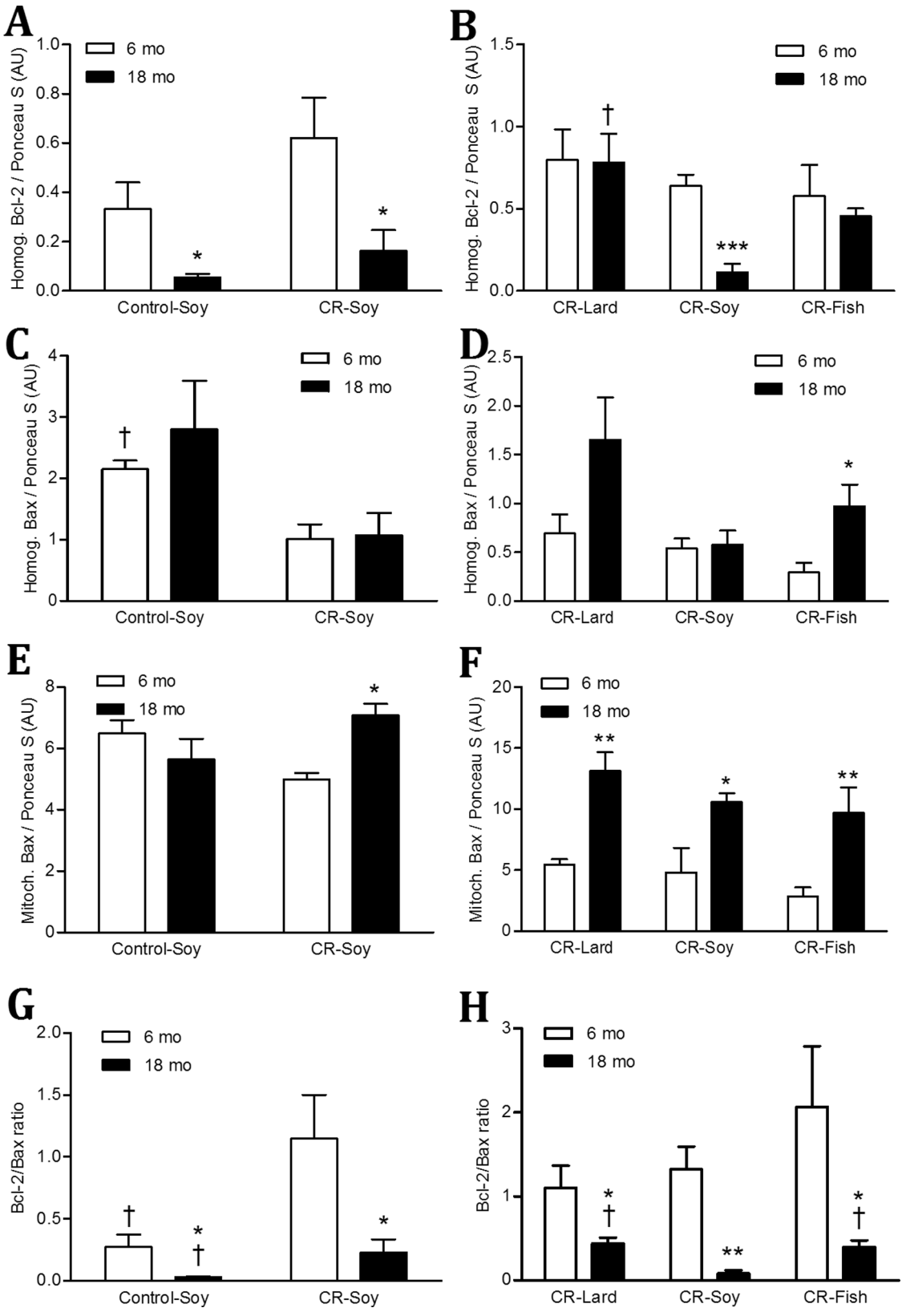

Figure 1. Bcl-2 and Bax levels in liver total homogenates (Panels **A**–**D**), Bax levels in mitochondria-enriched fractions (Panels **E** and **F**), and membrane Bcl-2/Bax ratio (Panels **G** and **H**) after 6 or 18 months of dietary intervention (the age of animals at the end of intervention period was 9 and 21 months, respectively). Data are mean ± *SEM*; $n = 4$ per group. Significant differences between 6 and 18 months for the same diet are denoted with asterisks (*$p < .05$, **$p < .01$, ***$p < .001$); †$p < .05$ vs CR-Soy for the same age. AU = arbitrary units; CR = calorie restriction.

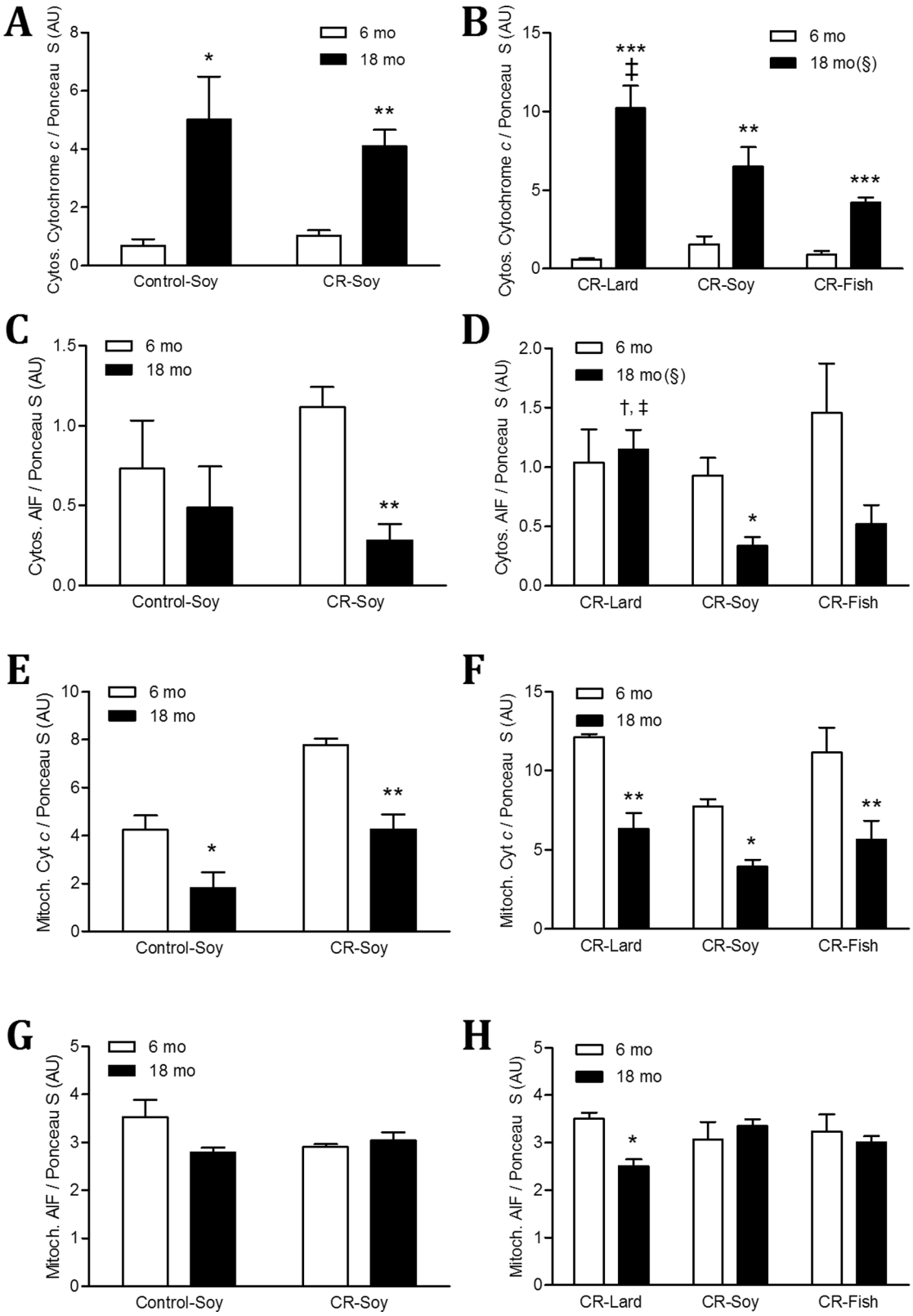

Figure 2. Levels of cytochrome *c* and AIF in cytosolic and in mitochondria-enriched fractions. (Panels **A** and **B**) Cytosolic cytochrome *c*. (Panels **C** and **D**) Cytosolic AIF. (Panels **E** and **F**) mitochondrial cytochrome *c*. (Panels **G** and **H**) mitochondrial AIF. Animals were subjected to 6 or 18 months of dietary intervention (the age of animals at the end of intervention period was 9 and 21 months, respectively). Data are mean ± *SEM*; $n = 3$–4 per group. Significant differences between 6 and 18 months for the same diet are denoted with asterisks (*$p < .05$, **$p < .01$, ***$p < .001$); $^{\dagger}p < .05$ vs CR-Soy for the same age group; $^{\ddagger}p < .05$ vs CR-Fish for the same age group; $^{\S}p < .05$ for a linear trend within the three CR diets in the same age group. AIF = apoptosis-inducing factor; AU = arbitrary units; CR = calorie restriction.

Interestingly, changes of cytochrome *c* levels with aging in mitochondria-enriched fractions were opposite to those in cytosols, with a significant decrease being observed in old animals from all dietary groups (Figure 2E and F, Supplementary Figure 1E and F). On the contrary, no significant alterations with age were found in the case of AIF, excepting for a decrease in the CR-Lard group (Figure 2G and H, Supplementary Figure 1E and F).

Caspases Activities

In contrast with the decrease of Bcl-2/Bax ratio and the release and accumulation of proapoptotic cytochrome *c* to the cytosol with aging in the Control-Soy group, the activities of caspases related to the mitochondrial pathway did not increase in old animals. On the contrary, caspase-9 activity tended to decrease and the effector caspase-3 was significantly decreased with aging in this group (Figure 3B and C). Caspase-8 was also decreased in old animals (Figure 3A), which adds support for a general decrease of hepatic caspases activities with age in Control-Soy group.

CR resulted in significant decreases in hepatic activities of caspases-8, -9, and -3 in young mice, but no significant alterations by CR were observed in old animals (Figure 3A–C). Interestingly, when studying the effect of aging in the CR-Soy group, we found that the age-dependent change in the activities of the two regulatory caspases we previously found in the Control-Soy group was reversed in the CR-Soy group, where activities of both caspase-8 and caspase-9 increased significantly with age (Figure 3A and B). However, results obtained for the effector, caspase-3, in the CR-Soy group were in contrast to those obtained for the regulatory caspases-8 and -9 because the activity of caspase-3 also decreased with aging in CR mice (Figure 3C).

The patterns obtained as a function of dietary fat were very similar for caspase-8 and caspase-9. Activities increased significantly with age in the three dietary groups and a slight yet significant trend among the three diets ordered as CR-Lard → CR-Soy → CR-Fish was observed, with maximal caspase-8 activity found in the CR-Lard group and minimal activity found in CR-Fish. This linear trend was statistically significant for both young and old animals in the case of caspase-9, although in the case of caspase-8 statistical significance was only maintained for young animals. Additionally, when considering the activities of young animals, the activities of caspase-8 and -9 in the CR-Fish group were significantly decreased compared with both the CR-Lard and CR-Soy groups (Figure 3A and B). Finally, the aging-related decrease of caspase-3 in the CR-Soy group was not maintained in CR-Lard and CR-Fish groups (Figure 3C).

XIAP Levels

XIAP was dramatically increased in aged animals from the Control-Soy group (Figure 4A, Supplementary Figure 1A). Although an increase in XIAP levels with aging was also found in the CR-Soy group, the extent of this increase was considerably attenuated by CR in old animals. As a result, XIAP was significantly lower in the old CR-Soy than in the old Control-Soy group (Figure 4A). Aging-related changes in animals fed under CR with different fat source were similar for all the three CR groups, although the aging-related increase did not reach statistical significance in the case of the CR-Fish group (Figure 4B, Supplementary Figure 1B).

Chromatin Fragmentation

Chromatin fragmentation was dramatically increased with aging in the Control-Soy group and this increase was also observed in the CR-Soy group. Interestingly, when comparing the three CR diets, we observed that aging-related increase of mono- and oligonucleosomes was maintained in the CR-Fish group, but completely abolished in the CR-Lard group (Figure 5A).

Hepatic Histology and Mean Nuclear Area of Hepatocytes

Hepatic tissue did not show evident signs of damage in young mice fed any of the experimental diets. The only conspicuous modification that was associated to one experimental diet was the occurrence of abundant intracellular lipid inclusions in hepatocytes of the Control-Soy group (Supplementary Figure 2A). These inclusions were not present in any of the three CR groups (Supplementary Figure 2B–D).

Microscopic structure of liver tissue was altered by diet in old animals. Hepatocytes did not show large lipid inclusions. Remarkably, multiple inflammation foci containing abundant leukocytes, which were found mainly in close proximity with blood vessels, were observed in the old Control-Soy group (Supplementary Figure 3A). The occurrence of inflammation foci was abated in all CR groups (Supplementary Figure 3B–D). Nuclear size increased significantly with aging in the Control-Soy group, and this change was attenuated by CR both in young and in old animals. Of note, as previously observed for chromatin fragmentation, the increase of nuclear size with aging was also observed in CR-Fish but was completely abolished in the CR-Lard group (Figure 5B; see also Supplementary Figures 2 and 3).

Discussion

An increase of apoptotic rate with aging has been documented in liver and other tissues (12,13,33). However, a decrease in the hepatic apoptotic response to genotoxic stress has been also reported (34). The decrease of Bcl-2/Bax ratio and cytochrome *c* content in mitochondria, concomitant with the increase of cytosolic cytochrome *c* and chromatin fragmentation with aging under most of our

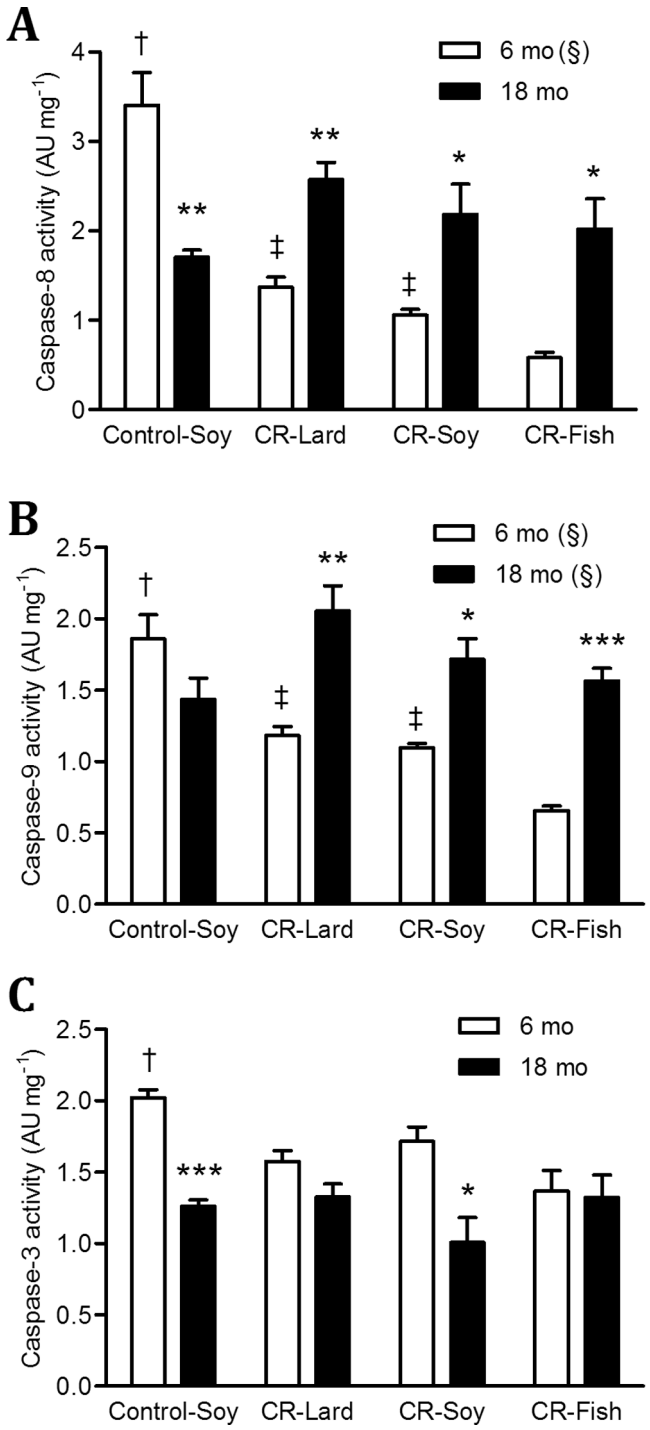

Figure 3. Activity of caspase-8 (Panel **A**), caspase-9 (Panel **B**), and caspase-3 (Panel **C**) in cytosolic fractions of liver after 6 or 18 months of dietary intervention (the age of animals at the end of intervention period was 9 and 21 months, respectively). Data are mean ± *SEM*; $n = 4$ per group. Significant differences between 6 and 18 months for the same diet are denoted with asterisks (*$p < .05$, **$p < .01$, ***$p < .001$); $^{\dagger}p < .05$ vs CR-Soy for the same age group; $^{\ddagger}p < .05$ vs CR-Fish for the same age group; $^{\S}p < .05$ for a linear trend within the three CR diets in the same age group. AU = arbitrary units; CR = calorie restriction.

experimental conditions, support this age-related increase in apoptosis. However, the influence of CR and dietary fat source in apoptotic signaling depicts a complex pattern in which variations of upstream regulators are not always effectively transmitted to final effectors.

Previous studies reported no significant changes of Bcl-2, Bax, or Bcl-xL expression levels with aging or CR in rat liver (35), and another article reported that Mcl-1 expression level increased with age but was not altered by CR in mouse liver (36). Our results indicate a strong regulation

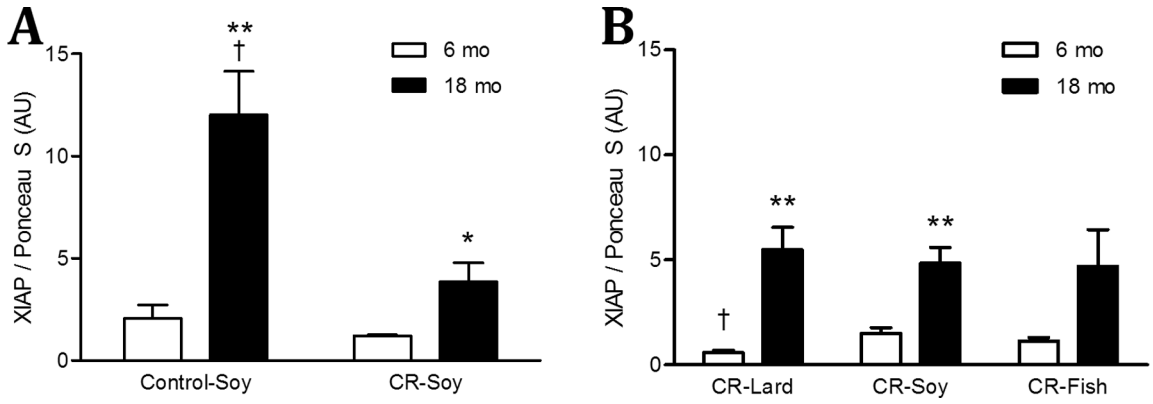

Figure 4. XIAP levels in total homogenates of liver after 6 and 18 months of dietary interventions (the age of animals at the end of intervention period was 9 and 21 months, respectively). Data are mean ± *SEM*; $n = 4$ per group. Significant differences between 6 and 18 months for the same diet are denoted with asterisks (*$p <$ .05, **$p <$ .01); $^{\dagger}p < .05$ vs CR-Soy for the same age group. AU = arbitrary units; CR = calorie restriction; XIAP = X-linked inhibitor of apoptosis protein.

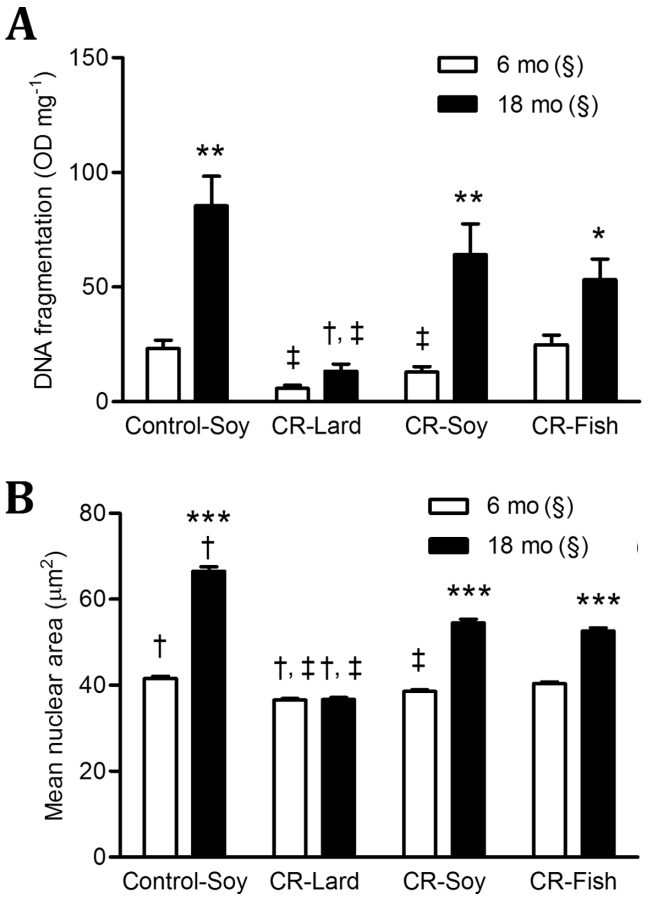

Figure 5. (Panel **A**) Presence of chromatin fragments in liver cytosolic fractions after 6 or 18 months of dietary intervention (the age of animals at the end of intervention period was 9 and 21 months, respectively). (Panel **B**) Mean nuclear area of hepatocytes after 6 or 18 months of dietary intervention. Data are mean ± *SEM*; $n = 4$ per group. Significant differences between 6 and 18 months for the same diet are denoted with asterisks (*$p < .05$, **$p < .01$, ***$p < .001$); $^{\dagger}p < .05$ vs CR-Soy for the same age group; $^{\ddagger}p < .05$ vs CR-Fish for the same age group; $^{\S}p < .05$ for a linear trend within the three CR diets in the same age group. CR = calorie restriction.

of Bcl-2 and Bax protein levels, resulting in a general age-dependent decrease of Bcl-2/Bax ratio, regardless of energy intake and dietary fat. Interestingly, the underlying mechanisms appear to be dependent on dietary fat source, because only mice fed soybean oil–containing diets exhibited an age-related decrease of Bcl-2 levels, whereas mitochondrial Bax levels increased with age in all CR groups. The ability of dietary fat or specific fatty acids to control apoptotic signaling through the regulation of Bcl-2 family proteins abundance has been studied previously. A short-term

treatment with docosahexaenoic acid decreased Bcl-2 and increased Bax protein levels in colon cancer cell lines (37), whereas olive oil (high in n-9 monounsaturated fatty acids) had the same effect in aged rat liver when compared with a sunflower oil–based diet (29). These studies may indicate regulation at a posttranscriptional level, although further research is needed.

Zhang and colleagues (38) reported a decrease of cytosolic cytochrome *c* levels in old rats, allegedly due to its rapid degradation once released to the cytosol, which is in contrast with our observations. The dramatic age-dependent increase of hepatic cytochrome *c* release and accumulation to the cytosol we show here is however in accordance with another report documenting a 10-fold increase of cytosolic cytochrome *c* in old rats (39), which is also consistent with the significant decrease of Bcl-2/Bax ratio in old mice.

Several studies have described an age-linked increase of caspase activities in liver and other mitotic organs (38,40–43). However, we detected lower hepatic caspases-8 and -3 activities, and caspase-9 activity also tended to decrease, in aged mice from the Control-Soy group, which is apparently in contrast with these previous reports and with our own observations about upstream and downstream components of the apoptotic signaling pathway. However, a decrease in hepatic caspases activities with aging in Control-Soy group is indeed in agreement with our previous results obtained from Wistar rats fed diets containing sunflower oil as the predominant fat source. Interestingly, this age-dependent decrease was abolished when animals were fed a diet containing olive oil (29). Because sunflower and soybean oils share a very similar fatty acid composition characterized by a high content in n-6 polyunsaturated fatty acids (44), these observations suggest an influence of the edible oils on regulation of caspase activities with aging.

An increase in inflammatory responses as a result of the consumption of n-6 fatty acid–enriched diets has been widely described (45,46), and the use of corn oil as the predominant fat source causes activation of the nuclear factor-κB in hepatic Kupffer cells (47). Inflammatory responses mediated by nuclear factor-κB include the upregulation of prosurvival proteins, such as XIAP (48), which potently inhibits effector caspases (-3 and -7) and regulatory caspase-9 (4,49,50). Expression of c-Flip, a caspase-8 inhibiting protein, is also enhanced by nuclear factor-κB (51). Thus, proinflammatory conditions elicited by n-6 fatty acids–enriched diets may account for the dramatic increase in XIAP expression we detect in old animals from the Control-Soy group and also for the decrease in caspase activities. The uncoupling between the mitochondrial pathway (decrease of Bcl-2/Bax ratio and release and accumulation of cytochrome *c* toward the cytosol) and caspases activities is a likely consequence of this mechanism.

Despite representing a prosurvival expression profile, the blockage of apoptotic pathways can result in the induction of necrotic cell death as a result of inflammation-related cell damage. Inflammatory processes in the liver are often associated with necrosis, which cannot be properly distinguished from apoptosis when determining the extent of chromatin fragmentation in tissue samples. Histological liver sections from old animals of the Control-Soy group showed leukocyte infiltration, which is consistent with age-dependent chronic hepatic inflammation in this group. It is thus likely that the high values of chromatin degradation in liver from Control-Soy old mice represent a combined estimation of apoptosis and necrosis.

When we consider the three groups fed under CR, alterations of Bcl-2/Bax ratio, cytochrome *c* release and accumulation, and caspase-8 activity are consistent with enhanced hepatic proapoptotic signaling with advanced age. CR is known to prevent age-linked chronic inflammation, reducing tumor necrosis factor-α circulating levels and nuclear factor-κB expression and activation (52,53). Of note, hepatic XIAP levels were significantly lower in mice fed under CR for 18 months when compared with their Control-Soy counterparts. An increase in caspases-8 and -9 activities with aging was detected in animals from the CR-Soy group, whereas mice from the Control-Soy group exhibited the inverse trend. In accordance, hepatic leukocyte infiltration in old mice was abated in the three CR groups. Besides a general age-related increase of caspase-8 and -9 activities, subtle changes due to dietary fat source were also observed in the CR groups because activities of regulatory caspases were higher in CR-Lard group and lowest in CR-Fish group, both in young and old animals.

Our data indicate an age-induced increase of chromatin fragmentation. Interestingly, the CR-Lard diet abolished this increase, although this does not fit with alterations of the studied effector proteins. In fact, caspase-3 activity was enhanced and the upstream caspase-9 activity remained unaltered with aging in this group. Cytosolic levels of AIF tended to decrease with age in all the CR diets with the exception of CR-Lard. AIF release occurs downstream of cytochrome *c* release and has been suggested to be a caspase-dependent process (54). Consequently, the divergent patterns of cytosolic AIF and cytochrome *c* release and accumulation could be mediated by mechanisms involving mitochondrial outer membrane sequential permeabilization. DNA fragmentation can also be mediated by external agents, namely Kupffer and stellate cells, which engulf the remaining apoptotic bodies after hepatocyte apoptosis (55).

Interestingly, age- and diet-related alterations in hepatocyte mean nuclear area were in accordance with DNA fragmentation data. Hepatocytes from old animals fed CR diets, with the exception of CR-Lard, exhibited an increase in nuclear size compared with their young counterparts. Nuclear size is directly related to polyploidy, a common feature among hepatocytes, which increases with aging (56). High ploidy levels have been previously associated to cell senescence and increased apoptosis (57,58), although the physiological significance of this process is

not fully understood (56). Oxidative stress contributes to polyploidization in the adult liver (59,60). The possibility exists that saturated and monounsaturated fatty acids contained in the CR-Lard diet may have influenced the ploidy levels through generation of a less prooxidant environment with aging.

Regardless of the mechanism underlying the apparent lack of enhanced apoptosis in old CR-Lard mice, its biological significance needs to be carefully considered. Unrestrained proliferative stimuli may generate neoplastic disease in tissues lacking an adequate regulation of programmed cell death. Nevertheless, excessive apoptosis has been recently proven to be a source of hepatocarcinogenesis in mice with organ-specific deletions of genes codifying antiapoptotic Bcl-2-related proteins, and development of hepatocellular carcinoma was prevented in *Mcl-1*$^{-/-}$ *Bak*$^{-/-}$ double knockout strains (61,62). Similarly, a relation between hepatocellular carcinoma and polyploidization is currently under debate, although polyploidy and carcinogenesis have been previously linked in other tissues (56).

Although the lack of an age-dependent increase of nuclear size would be consistent with improved liver maintenance with aging, further studies will be required to elucidate whether lower levels of hepatic apoptotic DNA fragmentation in old mice from the CR-Lard group are actually related with protective changes in hepatic homeostasis. Pathophysiological data, together with life-span studies, are expected to enhance our understanding about how different fat sources affect hepatocytes function and population dynamics.

Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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