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# Hepatic Oxidative Stress During Aging: Effects of 8% Long-Term Calorie Restriction and Lifelong Exercise

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## ABSTRACT

**Hepatic aging may involve alterations in redox status, resulting in enhanced oxidant production and changes in specific signaling pathways that lead to a pro-inflammatory response. The authors investigated whether mild calorie restriction and long-term voluntary exercise could attenuate these changes. Four groups of male Fischer 344 rats were compared: young (6 mo), old (24 mo), old calorie restricted (8% CR, 24 mo) and old CR with daily voluntary wheel running (Exercise; 8% CR, 24 mo). Levels of endogenous reactive oxygen species (ROS), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^{-}$) were significantly higher in the old *ad libitum* fed group compared to the young group. Sulfhydryl (-SH) content was significantly reduced and glutathione (GSH) content tended to be lower in the old animals. Old rats had significantly increased nuclear presence of NF-κB and in connection, increased levels of regulatory cytosolic phosphorylated I-κBα and decreased dephosphorylated I-κBα, suggesting an increased inflammatory response. Interestingly, a significant increase in liver RNA oxidation (8-oxo-7,8-dihydroguanosine) in the old *ad libitum* fed rats was detected and DNA oxidation (8-oxo-7,8-dihydro-2′-deoxyguanosine) tended to be increased. The age-associated increase in oxidative stress and upregulation of pro-inflammatory proteins was attenuated in the livers from both the CR and the exercise + CR groups.** *Antioxid. Redox Signal.* 8, 529–538.

## INTRODUCTION

THE AGING PROCESS results in a gradual and progressive structural deterioration of biomolecules and cellular compartments (9, 35, 39, 44, 45) and is associated with many pathological conditions, including cardiovascular disease (36, 38), stroke (54), and Alzheimer's disease (12), in addition to disorders causing functional decline such as osteoporosis (41), sarcopenia (6, 9), and liver dysfunction (58). The aged liver shows functional reductions in blood flow, metabolite clearance, and tissue injury repair by regeneration. Moreover, there is a decline in liver antioxidant and detoxifying enzymes with age and an increase in inflammatory signaling, which may cause oxidative damage to proteins and DNA. Reduced enzyme activity may also negatively impact the metabolic clearance of drugs, which could have major implications for drug dosing in the elderly (60).

The mechanisms underlying the aging process have not been completely understood but may partly involve inflammatory processes and oxidative damage (4, 8). Reduced calorie intake and physical exercise have been identified as possible strategies to decrease the incidence of age-related diseases and delay the aging process (22, 52). Lifelong exercise is known to extend mean lifespan in animals while calorie restriction (CR) can increase both mean and maximum lifespan (22–24). However, the mechanisms by which these interventions work remain unclear. Animal studies have shown that both CR and exercise reduce oxidative damage

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and may increase antioxidant enzyme activities, such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), as well as glutathione (GSH) content in various tissues (5, 11, 59). Moreover, it has been suggested that CR and exercise attenuate induction of inflammatory genes (8, 13, 47), which may be associated with both intra- and extracellular oxidative stress.

Indeed, various *in vivo* and *in vitro* studies have shown that reactive oxidants can damage macromolecules and also play a role in redox-sensitive signal transduction (10, 48). Both the mitochondria and immune cells can produce oxidants and play crucial roles in determining the degree of cellular oxidative stress. In addition to being sources of oxidants, mitochondria are also targets of ROS damage, and mitochondrial DNA (mtDNA) is especially susceptible to oxidant-induced damage (33). Furthermore, the cytosolic transcription factor nuclear factor κB (NF-κB), which regulates inducible nitric oxide synthase (iNOS) (56), cyclooxygenase-2 (COX-2) (31), vascular cell adhesion molecule-1 (VCAM-1) (34), intercellular adhesion molecule-1 (ICAM-1) (25), and pro-inflammatory cytokine production in various tissues, is redox-sensitive and can be activated through an altered redox environment (49).

Oxidative stress is often quantified by assessing carbonyl formation in certain amino acid side residues (53) and by measuring the formation of the guanine oxidation product 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), originated from DNA precursors (20). Several studies have indicated that cells and tissues from old animals have increased nuclear DNA oxidation (3). However, it was recently reported that total RNA, being mainly cytoplasmic, was considerably more oxidized than total DNA (19, 51) in cell cultures exposed to $H_2O_2$. Therefore, RNA oxidation has been an underrepresented field in nucleic acid damage and aging research. Hence, both RNA and DNA oxidation markers were investigated in this study with a recently developed method with simultaneous measurement of the oxidized RNA product 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxodGuo by high-performance liquid chromatography coupled to electrochemical detection (HPLC-EC-UV).

We investigated the effects of long-term CR (8%) and lifelong voluntary exercise (with 8% CR) on hepatic oxidative stress. We were interested in determining whether mild (8%) CR, being more realistic to impose in a human situation than the commonly used 30%–40% CR (23, 45), would show significant effects. Redox status was assessed by quantifying glutathione (GSH) and total sulfhydryl (-SH) content as well as activation of the redox-sensitive transcription factor NF-κB. Hence, the aim of this study was to determine whether increased oxidant production and altered redox status leads to chronic inflammatory responses with age and whether lifelong calorie restriction and long-term exercise could reverse these age-related changes.

## MATERIALS AND METHODS

### *Animals*

Male Fischer 344 rats were purchased from Harlan (Indianapolis, IN) at 10–11 weeks of age and were housed at the University of Florida's Animal Care Services facilities (Gainesville, FL) until sacrifice at 6 (young) or 24 (old) months of age. Rats were assigned to one of four groups: (i.) young sedentary *ad libitum* fed (Young; $n = 12$), (ii.) old sedentary *ad libitum* fed (Old; $n = 19$), (iii.) old lifelong 8% calorie restricted (CR; $n = 20$) and (iv.) old lifelong 8% calorie restricted with lifelong daily voluntary wheel running (Exercise + CR; $n = 20$). Rats fed an *ad libitum* diet tend to decrease their running activity abruptly, but slight food restriction (8%–10%) has been shown to prevent this decline (23, 24). Food intake for the 8% CR and 8% CR exercised groups of rats was therefore restricted by 8% below the *ad libitum* food intake of a separate group of sedentary, age-matched, male Fischer 344 rats, which were housed in the same facilities. Throughout the duration of the study, food intake of these two groups was adjusted accordingly each week (based on *ad libitum* food intake from the previous week). All animals were singly housed in a temperature (20° ± 2.5°C) and light-controlled (12:12 h light-dark cycle) environment with unrestricted access to water. All sedentary rats were housed in standard rodent cages supplied by the University of Florida's Animal Care Services. Rats in the wheel running group were housed in cages equipped with Nalgene Activity Wheels (1.081 meters circumference) obtained from Fisher Scientific (Pittsburgh, PA) and had free access to the wheels (27). Each wheel was equipped with a magnetic switch and a counter with liquid crystal display (LCD) that recorded the number of wheel revolutions. The number of revolutions was recorded for each animal daily. Body weights of all rats were recorded weekly. Animals were euthanized with isoflurane (administered via inhalation using a precision vaporizer at 5%), sacrificed by heart puncture, and the livers removed, rinsed in phosphate-buffered saline and immediately frozen in liquid nitrogen (−196°C). All experimental procedures were approved by the University of Florida's Institute on Animal Care and Use Committee.

### *Western blot analysis*

The nuclear extracts from rat liver were prepared as described by Han *et al.* (15) with slight modification. Liver (100 mg) was homogenized in 1 ml of ice-cold hypotonic buffer A (10 m*M* HEPES, pH 7.8; 10 m*M* KCl; 2 m*M* $MgCl_2$; 1 m*M* dithiotheritol (DTT); 0.1 m*M* EDTA; 0.1 m*M* phenylmethylsulfonylfluoride (PMSF)). After 25 min incubation on ice, the nucleoprotein complexes were collected by centrifugation at 500 *g* for 10 min. The following supernatant was collected and centrifuged at 1000 *g* for 30 min to obtain the postmitochondrial fraction, and the supernatant was collected and stored at −80°C. The nuclei were washed once in buffer A containing 0.2% NP-40, centrifuged, resuspended in 250 µl of buffer B (50 m*M* HEPES, pH 7.8, 50 m*M* KCl, 300 m*M* NaCl, 0.1 m*M* EDTA, 1 m*M* DTT, 0.1 m*M* PMSF, and 20% glycerol), and centrifuged for 5 min at 14,800 *g*. The supernatant containing nuclear protein was collected and stored at −80°C after determination of protein concentrations.

Western blotting was carried out as described previously (14). Nuclear extracts (20 µg) and cytosolic extracts (50 µg) were denatured under reducing conditions by heating to 95°C in Laemmli buffer and subjected to gel electrophoresis in

12% SDS-polyacrylamide at room temperature. Separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes by electrophoresis. Transfer efficiency and verification of equal protein loading were evaluated by staining with Ponceau S (Sigma Chemical Co., St. Louis, MO). Membranes were blocked in Tris buffered saline with Tween 20 (TBST: 150 m*M* NaCl, 0.05% Tween 20, 50 m*M* Tris-HCl; pH 7.4) containing 5% nonfat dry milk. Transferred proteins were incubated in TBST buffer with the specific primary antibodies rabbit anti-p65, rabbit anti-p50, rabbit anti-IκBα (all from Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:500), and mouse anti-phospho-IκBα (diluted 1:5000, Santa Cruz Biotechnology) overnight at 4°C. After thorough washing procedures with TBST, membranes were exposed to secondary antibodies (Santa Cruz Biotechnology), and detected with enhanced chemiluminescence reagents (ECL Kit, Amersham Biosciences, Buckinghamshire, UK). Prestained SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA, USA) were used to identify proteins. Protein expression was determined after scanning the exposed films using the Image J processing program (NIH, Bethesda, MD, USA).

### *Preparation of cytosolic extracts for fluorometric analyses*

One gram of liver was homogenized on ice in 5 ml of homogenate buffer (20 m*M* glycerophosphate, 20 m*M* NaF, 2 m*M* sodium orthovanadate, 1 m*M* EDTA, 0.5 m*M* phenylmethylsulfonyl fluoride, 1 *M* pepstatin, 80 mg/L trypsin inhibitor, and 100 m*M* Tris-HCl; pH 7.4) and centrifuged at 900 *g* at 4°C for 15 min. The supernatant was collected and then centrifuged at 12,000 *g* at 4°C for 15 min. The postmitochondrial supernatant fraction was analyzed immediately (47).

### *Overall ROS generation*

To determine overall liver ROS formation, including lipid hydroperoxides, $O_2^{\bullet -}$, $H_2O_2$, $HO^{\bullet}$, $^1O_2$, and $ONOO^-$, a fluorometric assay was used (2). This assay measures the oxidative conversion of nonfluorescent 2′,7′-dichlorofluorescein-diacetate ($H_2$DCF-DA) to the highly fluorescent 2′,7′-dichlorofluoorescein (DCF) (7). $H_2$DCF-DA was dissolved in absolute ethanol at 12.5 m*M* and kept at −70°C in the dark. Before experiments, $H_2$DCF-DA was diluted in 50 m*M* phosphate buffer (pH 7.4) to 125 μ*M* and then added to the liver homogenates in a 96-well plate to achieve a final concentration of 25 μ*M*. The plates were incubated at 37°C for 30 min and the fluorescence was determined at two time points (0 and 30 min) using a microplate fluorescence reader (excitation 485 nm/emission 530 nm; GENios, TECAN AG, Männedorf, Switzerland). Readings were calibrated against a standard curve of DCF ranging from 0 to 200 n*M*.

### *Assessment of $NO^{\bullet}$ generation*

For detection of $NO^{\bullet}$, 1 mg of 4,5-diaminofluorescein (DAF-2) was dissolved in 0.55 ml dimethyl sulfoxide and diluted 1:400 in 50 m*M* phosphate buffer, pH 7.4. Liver homogenates were incubated in 50 m*M* phosphate buffer at room temperature under shaking for 5 min in a 96-well plate. Then, DAF-2 was added to the final concentration 25 μ*M* and the fluorescence determined at five time points (2-min intervals between 0 and 10 min) using a microplate fluorescence reader (excitation 495 nm/emission 515 nm) (42).

### *Measurement of $ONOO^-$ levels*

$ONOO^-$ was measured by monitoring the oxidation of DHR 123 using the method of Kooy *et al.* (32) with slight modification. A stock solution of 5 m*M* DHR 123 in dimethylformamide was purged with nitrogen and stored at −20°C. A solution of 5 μ*M* DHR 123 was placed on ice in the dark immediately prior to the study. A buffer with 90 m*M* sodium chloride, 50 m*M* sodium phosphate (pH 7.4), and 5 m*M* potassium chloride was purged with nitrogen and placed on ice before use. Just before use, 100 μ*M* diethylenetriaminepentaacetic acid (DTPA) was added. $ONOO^-$ was measured by oxidation of DHR 123 on a microplate fluorescence reader GENios (TECAN AG) with excitation and emission wavelengths of 485 nm and 530 nm, respectively, at room temperature. Authentic $ONOO^-$ rapidly oxidizes DHR 123 and the oxidation product's fluorescent intensity is stable over time. It should be noted that use of DHR 123 and DAF-2 for detection of $ONOO^-$ and $NO^{\bullet}$ may not be absolutely specific (inhibitors of $ONOO^-/NO^{\bullet}$ sources were not used).

### *Total SH and GSH levels*

To measure total sulfhydryl (-SH) levels, 0.2 *M* Tris buffer (pH 8.2; 250 μl), 0.01 *M* DTNB (5,5′-dithiobis-2-nitrobenzoic acid; 25 μl), and methanol (1 ml) were added to 25 μl of liver cytosol with incubation for 15 min at room temperature. After centrifugation at 4000 *g* for 20 min, the absorbance of the supernatant was determined at 412 nm ($\epsilon$ = 13 $mM^{-1}cm^{-1}$) (50). For detection of GSH levels, concentrated metaphosphoric acid was mixed into homogenized tissues to reach 25% v/v. Following centrifugation (12,000 *g*, 10 min), the supernatants were taken for assay and 1 m*M* EDTA-containing phosphate buffer (50 m*M*, pH 7.4) was added, followed by the addition of 100 μg/ml *o*-phthalaldehyde. After 20 min at room temperature, fluorescence was measured (excitation 360 nm/emission 485 nm) (18).

### *Measurement of RNA and DNA oxidation*

The cold (0°C) 3 *M* guanidine isothiocyanate (GTC) method for analysis of RNA and DNA oxidation in tissues such as liver (and cells) described by Hofer *et al.* (21) was used. Briefly, after homogenization in GTC in the presence of the metal chelator deferoxamine mesylate (DFOM; Sigma), proteins and lipids were removed using organic solvents. After salt/isopropanol precipitation of nucleic acid at −80°C and washing in 70% ethanol, nucleic acids were dissolved in 30 μ*M* DFOM and hydrolyzed using 4 U Nuclease $P_1$ (MP Biomedicals, Irvine, CA) and 5 U alkaline phosphatase (Sigma) in 30 m*M* sodium acetate, 20 μ*M* $ZnCl_2$, pH 5.3 at 50°C for 60 min. After filtration to remove enzymes, nucleosides were separated using HPLC-EC-UV and analyzed for Guo (RNA) and dGuo (DNA) by UV, and 8-oxoGuo (RNA) and 8-oxodGuo (DNA) electrochemically using a Coulochem detector from ESA Inc. (Chelmsford, MA) (21). HPLC peaks

were quantified against daily made calibration curves of standards from Sigma and Calbiochem (San Diego, CA).

### *Statistical analysis*

Statistical analysis was performed using Prism 4 (GraphPad Software Inc., San Diego, CA). The significance level was set at $p < 0.05$ using Student's *t* test.

## RESULTS

Calorie restriction by 8% showed a significant reduction in average body weights compared to the *ad libitum* fed group, beginning at 5 months of age (Table 1). The exercising (with 8% CR) animals had the lowest body weights, already apparent at 3 months of age (Table 1). The maximum body weight in all groups was achieved at 17 months of age. Moreover, there was no significant difference in body weight at 23 months of age between the old and old CR animals. For the exercise + CR group, the running activity was the highest between 4 and10 mo (and peaked at 6 mo) after which running activity stabilized until very old age (Table 1). The CR and exercise + CR groups average lifespan tended to be longer than the *ad libitum* fed group when comparing Kaplan-Meier survival curves using the log rank test (data not shown). The remaining old ($n = 9$), CR ($n = 12$), and exercised + CR ($n = 12$) animals were sacrificed at 24 months of age.

Hepatic ROS generation was increased by 52% in the old *ad libitum* fed rats compared to the young group (Fig. 1A). Lifelong CR decreased the ROS levels by 12.8% and exercise + CR by 20.4% ($p < 0.01$) compared to the old *ad libitum* fed group. Furthermore, a significant increase in $NO^{\bullet}$ (39%) was detected in old animals compared with young rats (Fig. 1B). Although the change was not significant, both CR and exercise + CR decreased the levels of $NO^{\bullet}$ by 7.8% and 17.8%, respectively, compared to the old *ad libitum* fed group (Fig. 1B). $ONOO^-$ levels were significantly increased by 88% in livers from old *ad libitum* fed rats compared to young (Fig. 1C). Levels of $ONOO^-$ were significantly lowered with CR by 22.5% ($p < 0.05$) and with exercise + CR by 24.5% ($p < 0.01$) compared to the old *ad libitum* fed group (Fig. 1C).

Thiols possess antioxidant function and play a critical role in maintaining cellular redox status. The total liver thiol (-SH) levels from old *ad libitum* fed rats were significantly decreased by 20.6% compared to young (Fig. 2A). Lifelong CR increased the total thiol levels by 12.3% ($p < 0.05$), but no significant changes were found in the exercise + CR group compared to the old *ad libitum* fed group (Fig. 2A). Levels of reduced glutathione (GSH) followed a similar pattern to total thiols, but no significant differences were found (Fig. 2B).

Western blot analysis to assess NF-κB activation in liver was performed by analyzing the content of the NF-κB subunits p65 and p50 in isolated nuclei. As confirmation of acti-

Table 1. Body Weights, Survival, and Running Distance for All Groups During the 24-Month Study

| *Age activity (months)* | *Young g* | *Old g* | *8% CR Old g* | *Exercise; 8% CR Old g* | *Running m/day* |
|---|---|---|---|---|---|
| 2 | — | 261.0 ± 1.6 (19) | 257.6 ± 2.3 (20) | 253.2 ± 5.2 (20) | — |
| 3 | — | 278.5 ± 1.8 (19) | 278.7 ± 2.2 (20) | 259.3 ± 3.8***(20) | 666 ± 160 |
| 4 | 301.3 ± 8.2* (12) | 320.5 ± 2.2 (19) | 323.2 ± 2.8 (20) | 301.5 ± 3.1***(20) | 1267 ± 304 |
| 5 | 340.5 ± 9.7 (12) | 356.0 ± 3.1 (19) | 345.1 ± 3.6* (20) | 313.9 ± 4.9***(20) | 2314 ± 457 |
| 6 | 364.2 ± 10.5 (12) | 376.9 ± 3.6 (19) | 353.0 ± 3.8***(20) | 312.8 ± 6.7***(20) | 2462 ± 435 |
| 7 | — | 394.0 ± 4.3 (19) | 366.7 ± 4.1***(20) | 324.0 ± 6.4***(20) | 1769 ± 286 |
| 8 | — | 407.2 ± 4.7 (19) | 371.9 ± 4.6***(20) | 335.4 ± 6.0***(20) | 1408 ± 251 |
| 9 | — | 413.1 ± 4.6 (19) | 378.5 ± 4.8***(20) | 345.9 ± 5.4***(20) | 1385 ± 268 |
| 10 | — | 416.8 ± 4.6 (19) | 389.8 ± 4.3***(20) | 362.3 ± 4.8***(20) | 1210 ± 247 |
| 11 | — | 412.7 ± 4.7 (19) | 385.2 ± 4.6***(20) | 360.6 ± 4.2***(20) | 1132 ± 227 |
| 12 | — | 424.0 ± 4.8 (19) | 392.0 ± 4.4***(20) | 363.1 ± 4.7***(19) | 1110 ± 233 |
| 13 | — | 423.4 ± 5.3 (19) | 389.1 ± 4.2***(20) | 355.3 ± 5.1***(19) | 1121 ± 223 |
| 14 | — | 431.7 ± 5.7 (19) | 392.7 ± 3.5***(19) | 355.9 ± 5.5***(19) | 1188 ± 250 |
| 15 | — | 445.8 ± 5.5 (19) | 414.9 ± 3.8***(19) | 371.9 ± 5.1***(19) | 1438 ± 325 |
| 16 | — | 450.1 ± 5.0 (19) | 420.3 ± 3.9***(19) | 377.8 ± 5.5***(19) | 1104 ± 244 |
| 17 | — | 456.3 ± 5.4 (19) | 427.7 ± 3.8***(19) | 379.2 ± 5.2***(19) | 1014 ± 248 |
| 18 | — | 447.1 ± 6.0 (17) | 420.6 ± 4.1***(18) | 370.4 ± 6.0***(18) | 1046 ± 285 |
| 19 | — | 442.6 ± 5.9 (15) | 418.7 ± 4.5**(17) | 366.1 ± 6.7***(17) | 919 ± 246 |
| 20 | — | 435.2 ± 6.0 (14) | 412.6 ± 4.4**(16) | 365.3 ± 5.0***(17) | 904 ± 223 |
| 21 | — | 428.3 ± 6.3 (13) | 403.9 ± 4.2**(16) | 359.2 ± 5.1***(17) | 926 ± 227 |
| 22 | — | 420.3 ± 7.6 (12) | 397.5 ± 4.0**(16) | 351.5 ± 5.7***(15) | 993 ± 246 |
| 23 | — | 401.6 ± 12.8 (11) | 384.1 ± 6.1 (13) | 344.2 ± 5.7***(15) | 997 ± 262 |
| 24 | — | 395.6 ± 17.9 (9) | 377.1 ± 5.4 (12) | 337.1 ± 5.5**(12) | 943 ± 175 |

The number of animals in each group is shown within parentheses. Body weights are expressed as mean ± SEM. $p < 0.05$ (*); 0.01 (**); 0.001 (***) versus old rats.

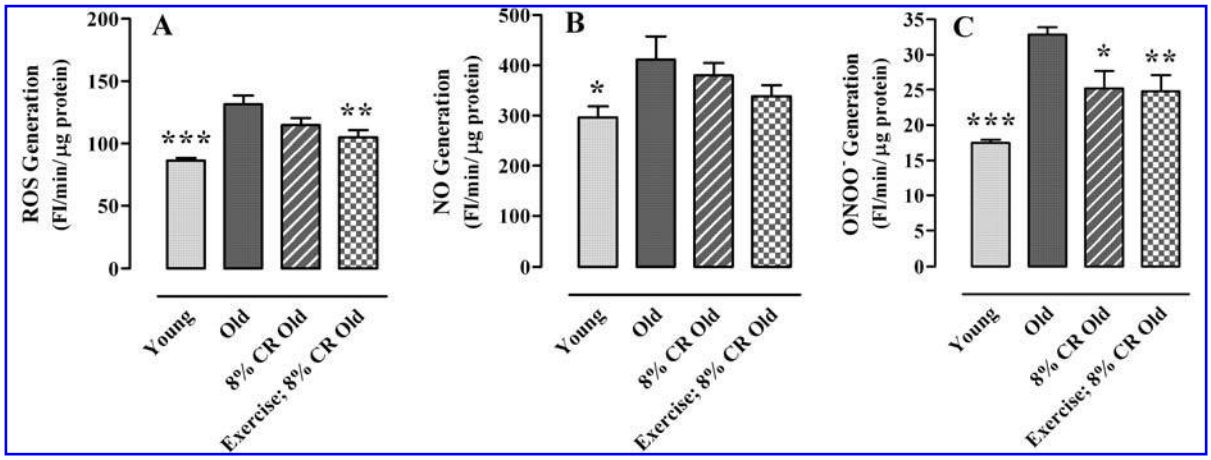


**FIG. 1. Hepatic oxidant formation.** Male Fischer 344 rats were grouped as: young (6-mo-old, *ad libitum* fed, sedentary), old (24-mo-old; *ad libitum* fed, sedentary), 8% CR old (24-mo-old; 8% caloric restriction, sedentary) and Exercise; 8% CR old (24-mo-old; 8% caloric restriction, voluntary wheel runners). Analysis of: (**A**) reactive oxidant species (ROS), (**B**) nitric oxide ($NO^{\bullet}$), (**C**) peroxynitrite ($ONOO^{-}$) formation using fluorometric assays (see Materials and Methods). Data are expressed as mean ± SEM ($n = 8$). $p < 0.05$ (*); 0.01 (**); 0.001 (***) versus old rats.

vation, cytosolic levels of regulating phosphorylated I-κBα (active form) and dephosphorylated I-κBα (inactive) were measured. We detected a significant increase in NF-κB nuclear content with age (Figs. 3A and 3B), together with increased levels of phosphorylated I-κBα (Fig. 3C) and decreased levels of dephosphorylated I-κBα (Fig. 3D). Hence, all parameters show a strong indication of NF-κB activation in hepatic tissues of old rats. Interestingly, lifelong CR was able to attenuate the age-associated increase in phosphorylated I-κBα (Fig. 3C) and reverse the age-associated decrease of levels of dephosphorylated I-κBα. Also, exercise + CR was able to attenuate the age-associated increase in phosphorylated I-κBα (Fig. 3C). Moreover, the age-associated increases in NF-κB nuclear content (p65 and p50) tended to be decreased ($p < 0.1$) with both CR and exercise + CR (Figs. 3A and 3B).

With age, a significant increase in liver RNA oxidation was observed (Fig. 4A). In contrast, the levels of hepatic DNA oxidation were not significantly increased (Fig. 4B). CR significantly lowered RNA oxidation, whereas exercise + CR was not significantly different from the old *ad libitum* fed group. RNA oxidation levels were: 1.78 ± 0.25 (young), 4.58 ± 0.75 (old), 2.63 ± 0.44 (CR), and 3.63 ± 0.47 8-oxoGuo/$10^6$ Guo (exercise + CR), respectively. Yields of liver RNA from all groups were similar (6.8–7.1 μg/mg), but yields of DNA from the young livers (1.325 ± 0.02 μg/mg) were significantly lower ($p < 0.01$) than from the old animals (1.99 ± 0.20 μg/mg), although extraction procedures of all groups was performed in parallel. DNA yields from all old animal groups were similar (1.93–2.01 μg/mg).

## DISCUSSION

The liver is a critical organ for metabolic detoxification, biological waste clearance, and immune homeostasis, mediating both innate and adaptive immunity. As the central organ of extrathymic T cell development (43), the liver could play an important role in the age-related shift to T helper 2 (Th2) response that results in production of pro-inflammatory cy-

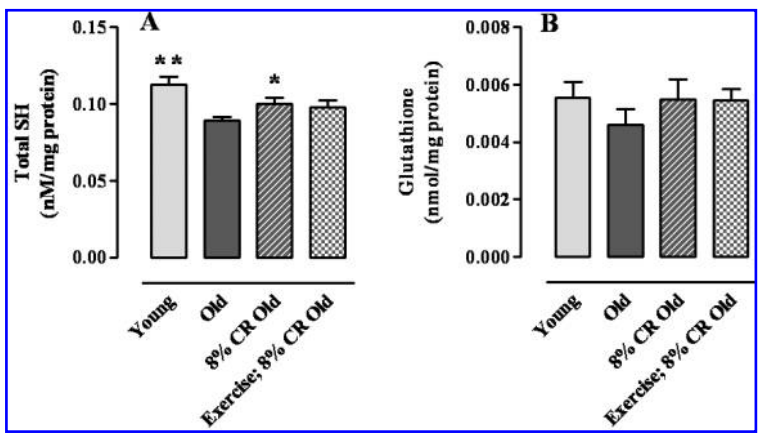


**FIG. 2. Liver total thiol and reduced glutathione levels.** (**A**) Total sulfhydryl (SH) groups and (**B**) total glutathione (GSH) content were measured to investigate liver redox status with age and the effect from lifelong CR and exercise + CR. Data are expressed as mean ± SEM ($n = 8$). $p < 0.05$ (*); 0.01 (**) versus old rats.

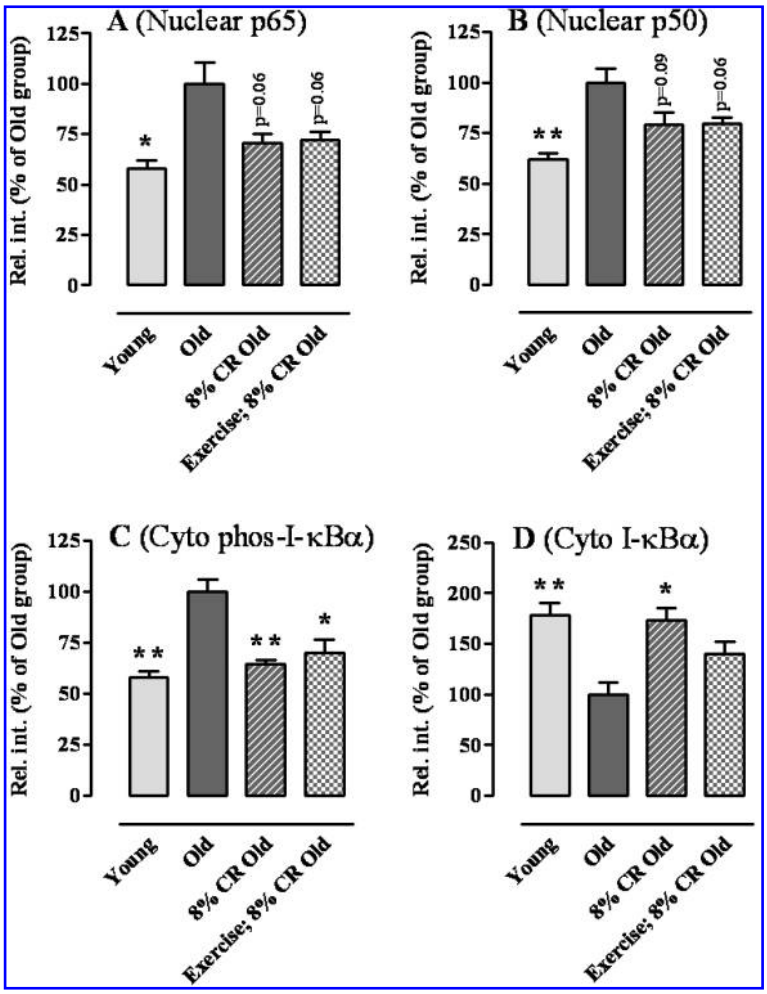


**FIG. 3. Transcription factor NF-κB activation in liver.** Western immunoblot analysis was performed to investigate NF-κB activity in the cytosol and nuclei. Both (**A**) p65 and (**B**) p50 were measured to compare NF-κB activity in isolated nuclei as well as (**C**) cytosolic dephosphorylated I-κBα and (**D**) dephosphorylated I-κBα. Densitometry derived data are expressed as mean ± SEM (triplicate). $p < 0.05$ (*); 0.01 (**); 0.001 (***) versus the old rat group which has been normalized to 100%.

tokines, and may also contribute to local and systemic inflammatory oxidative stress during the aging process. Age-associated changes in liver oxidant generation, redox status, and a pro-inflammatory signaling (NF-κB) pathway were studied in young and old rats, as well as in rats that were mildly calorie restricted and in rats that were subjected to long-term voluntary exercise plus mild calorie restriction. We were able to detect significantly increased levels of total oxidants, $NO^{\bullet}$ and $ONOO^{-}$, a decreased sulfhydryl content, increased RNA oxidation, and increased NF-κB activation in

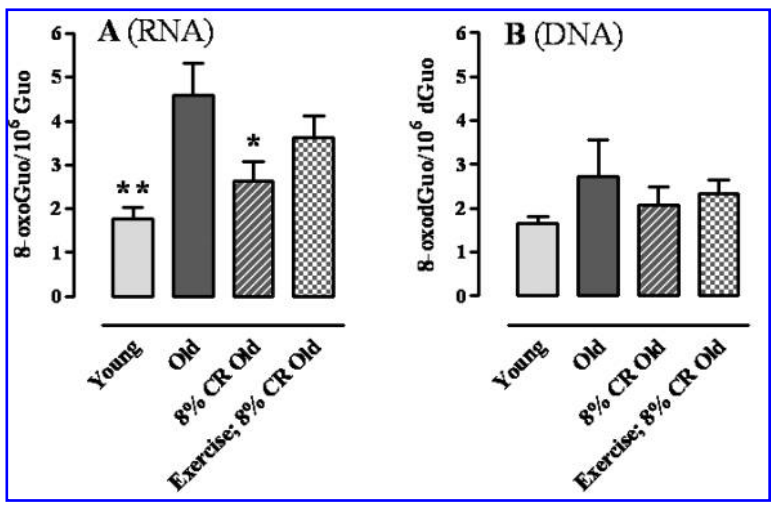


**FIG. 4. Nucleic acid oxidation in liver.** Levels of oxidative damage in nucleic acids were quantified by (**A**) 8-oxoGuo (RNA), and (**B**) 8-oxodGuo (DNA) using HPLC-EC-UV. Levels are normalized to $10^6$ (d)Guo and expressed as mean ± SEM ($n = 8–10$). $p < 0.05$ (*); 0.01 (**) versus old rats.

the old *ad libitum* fed rats compared to the young *ad libitum* fed group. In striking contrast, most of the age-associated increases in oxidative stress and pro-inflammatory signaling activation parameters were attenuated with CR and wheel running exercise + CR. Moreover, this study is the first to demonstrate RNA oxidation as a possible biomarker of aging. We found that there is an increased RNA oxidation in the aged liver and that 8-oxoGuo could be a more suitable sensor for screening nucleic acid damage with age and oxidative stress than the traditional oxidative marker, 8-oxodGuo (DNA). RNA oxidation was lowered with CR and exercise + CR. Hence, our data strongly supports both the Inflammation and Oxidative Stress Theories of Aging (8, 52).

The increase in $ONOO^-$ levels in aged livers likely stems from increased production of $NO^\bullet$ and $O_2^{\bullet -}$ known to react by a diffusion limited rate to produce $ONOO^-$ (26). The increased activation of NF-κB could have induced iNOS activity, resulting in enhanced $NO^\bullet$ production, while simultaneous increases in $O_2^{\bullet -}$ could stem from the mitochondrial respiratory chain and/or immune cells. Whereas $O_2^{\bullet -}$ and $NO^\bullet$ are relatively unreactive oxidants, their products $ONOO^-$ and $H_2O_2$ (the latter combined with the presence of redox active metals) can become highly reactive. $ONOO^-$ can dissociate into the strongly oxidizing short-lived intermediates $HO^\bullet$ (hydroxyl radical) and $NO_2^\bullet$ (nitrogen dioxide) (40), and is well known to oxidize thiols (46). $H_2O_2$ acts as a strong oxidant in the presence of reduced transition metals ($Fe^{2+}$, $Cu^+$, etc.) (19). Increases in $ONOO^-$ and $H_2O_2$ could explain the reduction in total sulfhydryl (-SH) groups (Fig. 2A) as well as the increase in RNA oxidation (Fig. 4) observed in this study. Bejma *et al.* reported similar increased intracellular oxidant production in aged rat livers (4), and Kakarla *et al.* reported similar lower hepatic GSH levels in aging and induced antioxidant system with exercise (28). However, differences in levels of thiols could also be due to, and counteracted by, differences in rates of thiol group synthesis (37).

The baseline oxidation level of RNA (1.78 ± 0.25 8-oxoGuo/$10^6$ Guo) and DNA (1.66 ± 0.16 8-oxodGuo/$10^6$ dGuo) were similar in the livers from the young animals. However, with age, RNA oxidation was significantly increased, whereas DNA oxidation merely tended to increase. Kaneko *et al.* reported late life-time onset of oxidative nuclear DNA damage in rat liver and postulated that CR could delay the onset of 8-oxodGuo accumulation (30). In agreement with our data, their study showed no significant increase in liver 8-oxodGuo content with age until 24 months and no decreasing effect of CR in the same Fischer 344 rats. However, at 27 months 8-oxodGuo begins to accumulate and CR significantly ameliorates the DNA oxidation (30). DNA is protected by histones and located inside the nucleus whereas RNA is mainly cytoplasmic, which may explain the differences in oxidation levels with age. In addition, some studies suggest that metals preferentially bind to RNA, which may render it more prone to oxidative insult (57). Also, it is possible that the DNA repair system is more efficient than for RNA. In fact, various DNA repair systems have been well described in the literature, whereas little is known regarding RNA repair. The increased RNA oxidation observed with age could cause erroneous translation and the construction of less functional hepatic enzymes. A recent structural study has found that oxidized RNA is specifically recognized by the mammalian Y box-binding protein 1 (YB-1) (16), suggesting that structural changes to the RNA molecules can be recognized and erroneous translation possibly prevented.

Helenius *et al.* have shown an increased activity of the redox sensitive transcription factor NF-κB from various tissues of old rats (17). Enhanced NF-κB activation could be a consequence of increased levels of inflammatory molecules, including cytokines (IL-1α, IL-6, TNF-α, or CRP), an increased level of ROS produced by tissue-invading macrophages and neutrophils, or from increased mitochondrial superoxide ($O_2^{\bullet -}$) production. Chung *et al.* have postulated that NF-κB plays a pivotal role in accumulating oxidative stress via chronic molecular inflammatory response and have shown that CR attenuates NF-κB activity in old rats (8). Furthermore, recent data from Radak's group clearly shows a decreased activation of liver NF-κB following 8 weeks of treadmill training in adult (18 mo) and old (28 mo) rats (47). Our present study confirms the enhanced activation of NF-κB with age and shows similar beneficial effects of both long-term CR and lifelong exercise + CR. We found a significant decrease in phosphorylated I-κBα (active form) as well as a decrease in nuclear p50/p65 with CR and exercise + CR. The increase in ROS levels observed in the old animals may alter the activity of kinases in the NF-κB signaling pathway, while CR and exercise + CR were able to attenuate the age-associated increase of p50/p65. Indeed, Storz *et al.* suggests a possible model of the NF-κB activation pathway that is associated with oxidative stress and tyrosine phosphorylation of protein kinases D (55). Interestingly, recent data shows that overexpression of NF-κB can induce CRP production, which is mainly produced in the liver and is a strong inflammatory marker (1). We recently showed that CR and exercise + CR attenuate the age-related increase in plasma CRP level (29), supporting a possible link between a decrease in oxidative stress, attenuated NF-kB activity in liver cells, and lowered CRP levels.

## CONCLUSION

The present study shows strong evidence of increased hepatic oxidative stress (increased oxidant production, thiol depletion, and RNA oxidation) and activated redox-sensitive transcription factor NF-κB with age. In striking contrast, these changes were ameliorated by slight CR (8%) and lifelong exercise (with 8% CR). No consistent additional beneficial effects were observed from exercise (with 8% CR) compared to 8% CR alone. This is in agreement with Hollozsy *et al.* who documented improved survival, but not an extension of maximum lifespan with exercise (with 8% CR) (24). However, they did show an increase in maximal lifespan with moderate CR (~30%) or moderate CR (~30%) with exercise (22–24). We postulate that cellular oxidative stress accelerates a cellular redox imbalance due to stimulating the NF-κB signaling pathway, which could lead to increased levels of pro-oxidant and pro-inflammatory gene expression (Fig. 5).

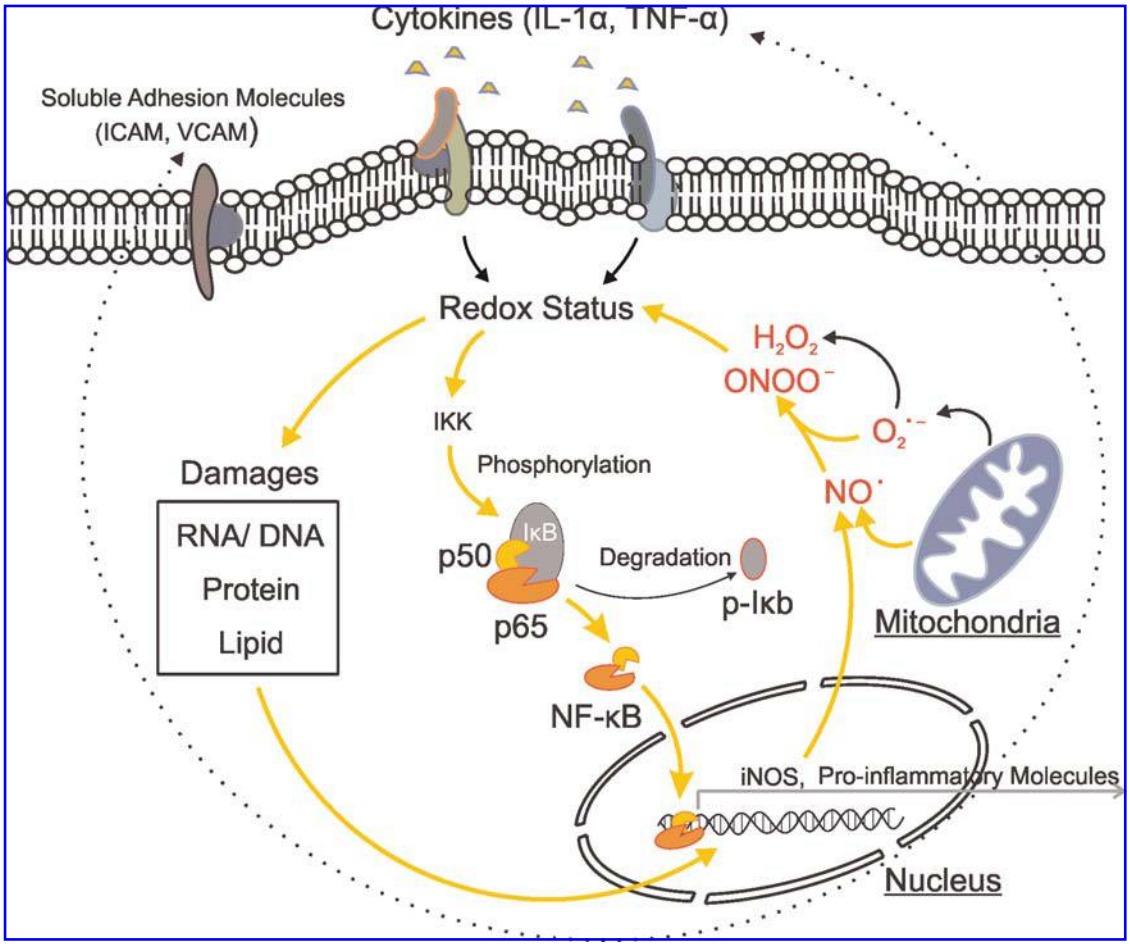


**FIG. 5. Postulated role of the redox-sensitive NF-κB in signaling oxidative stress.** With age, an increase in oxidant production and a decrease in antioxidants (such as thiols) may be causal to a decline in cellular redox state and promote a shift towards oxidative stress by triggering NF-κB activation and the upregulation of inflammatory and pro-oxidant enzymes (8), leading to the completion of a vicious cycle associated with the aging process.

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## ABBREVIATIONS

CR, caloric restriction; CRP, C-reactive protein; DAF-2, 4,5-diaminofluorescein; DCF, 2′,7′-dichlorofluorescein; dGuo, 2′-deoxyguanosine; GSH, glutathione; Guo, guanosine; $H_2$DCF-DA, 2′,7′-dichlorofluorescein-diacetate; HPLC-EC-UV, high-performance liquid chromatography coupled to electrochemical detection; NF-κB, nuclear factor kappa B; NO•, nitric oxide; $O_2^{\bullet -}$, superoxide anion; $ONOO^-$, peroxynitrite; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; ROS, reactive oxygen species.

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