Calorie restriction attenuates inflammatory responses to myocardial ischemia-reperfusion injury

B. CHANDRASEKAR, J. F. NELSON, J. T. COLSTON, AND G. L. FREEMAN. Calorie restriction attenuates inflammatory responses to myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 280: H2094–H2102, 2001.—The life-prolonging effects of calorie restriction (CR) may be due to reduced damage from cumulative oxidative stress. Our goal was to determine the long-term effects of moderate dietary CR on the myocardial response to reperfusion after a single episode of sublethal ischemia. Male Fisher 344 rats were fed either an ad libitum (AL) or CR (40% less calories) diet. At age 12 mo the animals were anesthetized and subjected to thoracotomy and a 15-min left-anterior descending coronary artery occlusion. The hearts were reperfused for various periods. GSH and GSSG levels, nuclear factor-κB (NF-κB) DNA binding activity, cytokine, and antioxidant enzyme expression were assessed in the ischemic zones. Sham-operated animals served as controls. Compared with the AL diet, chronic CR limited oxidative stress as seen by rapid recovery in GSH levels in previously ischemic myocardium. CR reduced DNA binding activity of NF-κB. The κB-responsive cytokines interleukin-1β and tumor necrosis factor-α were transiently expressed in the CR group but persisted longer in the AL group. Furthermore, expression of manganese superoxide dismutase, a key antioxidant enzyme, was significantly delayed in the AL group. Collectively these data indicate that CR significantly attenuates myocardial oxidative stress and the postischemic inflammatory response.

Reperfusion after ischemia generates elevated levels of free radicals. If an ischemic episode is brief, i.e., if it is <20 min, the resultant condition after reperfusion is termed “stunning” (3, 4); the condition is not lethal to the myocardium and recovery in function may take from hours to days. All cells resident to myocardium (cardiomyocytes, fibroblasts, endothelial cells, and smooth muscle cells) generate reactive oxygen intermediates (ROIs). The high levels of ROIs generated during reperfusion oxidize lipids, proteins, and nucleic acids. Also ROIs activate redox-sensitive transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (5, 34).

NF-κB is a multisubunit nuclear transcription factor that is present in almost all mammalian cells. It exists in an inactive form in cytoplasm due to its binding to inhibitory molecules that belong to the inhibitory κB (IκB) family. Various stimuli including ROIs activate NF-κB (5, 34). This activation results from phosphorylation and subsequent degradation of IκB in the cytoplasm. NF-κB then translocates to the nucleus and binds to promoter regions of genes containing κB-responsive elements. Previously we have shown induction of AP-1, CCAAT enhancer binding protein (C/EBP), and NF-κB in reperfused myocardium after a single episode of sublethal ischemia (10–12). Although AP-1 and C/EBP were induced in a monophasic manner, NF-κB DNA binding activity showed a biphasic expression (10).

Interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), which are cytokines with negative inotropic activity, contain κB-responsive elements in their promoter/enhancer regions and are therefore induced by activated NF-κB. In fact we have previously shown induction and upregulation of all three cytokines in postischemic myocardium (8–12), and administration of diethyldithiocarbamate (a free-radical scavenger and NF-κB inhibitor) significantly inhibited NF-κB DNA-binding activity and concomitantly the expression of IL-1β, IL-6, and TNF-α in postischemic myocardium (12), which suggests that activation of NF-κB may play a causative role in reperfusion injury.

One widely held view is that aging is associated with increased exposure to free radicals resulting in chronic oxidative stress (16, 17, 20, 29, 36, 38, 39). High levels of free-radical generation in the presence of weaker antioxidant defenses may result in peroxidation of membrane lipids and oxidation of nucleic acids (especially DNA) resulting in mutations and changes in the three-dimensional structure of proteins (13, 21, 33). Because adult rat cardiomyocytes are terminally differentiated, such changes may have profound deleterious effects on myocardial function during aging especially because the heart has high rates of oxidative metabolism. Although the heart consists of various...
free-radical scavenging systems including antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione peroxidase (GSHPx), alteration in the expression or activity of these systems may result in oxidative stress during aging. In fact several reports have demonstrated lowered antioxidant enzyme activity during aging (22) and moderate restriction of dietary calories augmented the activity similar to that of younger animals (24, 32, 36).

Chronic calorie restriction (CR) without malnutrition remains the only robust means of increasing life span and delaying the onset of a wide range of age-related diseases and physiological changes in rodents (38). However, its mode of action to retard aging remains unknown. Chronic reduction of stress exposure over the life span has been hypothesized to play an important role in the antiaging effects of CR (38). Among its wide-ranging effects, CR appears to reduce oxidative stress at steady state and to have anti-inflammatory properties (38). Also CR decreases susceptibility of various tissues in vitro to acute oxidative stress (37, 38). However, more direct tests of the hypothesis that CR decreases oxidative stress and attenuates inflammatory processes are lacking. Myocardial reduction of stress exposure over the life span has been hypothesized to play an important role in the antiaging effects of CR (38). However, its mode of action to retard aging remains unknown. Chronic reduction of stress exposure over the life span has been hypothesized to play an important role in the antiaging effects of CR (38). Among its wide-ranging effects, CR appears to reduce oxidative stress at steady state and to have anti-inflammatory properties (38). Also CR decreases susceptibility of various tissues in vitro to acute oxidative stress (37, 38). However, more direct tests of the hypothesis that CR decreases oxidative stress and attenuates inflammatory processes are lacking. Myocardial reduction of stress exposure over the life span has been hypothesized to play an important role in the antiaging effects of CR (38). However, its mode of action to retard aging remains unknown.

METHODS

Experimental animals and diets. All studies were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85–23, Revised 1996]. Male Fisher 344B rats (n = 36) were obtained at 4 wk of age from Charles River Laboratories (Kingston, NY) and housed singly in a barrier facility where a 12-h light/12-h dark cycle was followed. At 6 wk of age the rats were separated into two dietary groups (n = 18 per group). Rats in the ad libitum (AL) group were fed a standard semisynthetic diet that has been previously described in detail (18, 19), and rats in the CR group were fed 60% of the caloric intake of the AL group. Food intake of AL-fed rats was measured twice a week and the amount ingested per day was calculated. The animals were used at the age of 12 mo, which was chosen to provide a midlife assessment of the effect of chronic CR on the animals.

Induction of I/R. Preparation of animals, administration of anesthesia, and induction of I/R were performed essentially as has been previously described (8, 10–12). After 15 min of left anterior descending coronary artery ligation, the hearts were reperfused for different durations: 0.25, 0.5, 1, 2, 3, and 6 h (n = 4 rats per group). Sham-operated animals served as controls (n = 4 rats per group). After the experimental period the heart was rapidly excised and rinsed in ice-cold physiologic saline. The right ventricle and atria were trimmed away. The left ventricle was divided into ischemic and nonischemic zones and was snap-frozen in liquid N2 for further analyses. We have previously reported (8, 10) that free radical generation, NF-κB DNA binding activity, and proinflammatory cytokine expression were very similar between sham-operated animals and in nonischemic tissue adjacent to the ischemic zones. Therefore in the present investigation we used tissue from only the ischemic zones for all the analyses reported.

Tissue glutathione levels. Reduced and oxidized glutathione (GSH and GSSG, respectively) levels were measured as previously described (9) in cardiac tissue using a commercially available kit (Glutathione Assay Kit, Cayman Chemical, Ann Arbor, MI).

Electrophoretic mobility shift assay. Preparation of protein extracts and electrophoretic mobility shift assay (EMSA) were performed as has been described (8, 9, 10, 12). A double-stranded oligonucleotide (NF-κB, 5′-AGTTGAGGGGACTT- TCCAGGC-3′; Santa Cruz Biotechnology, Santa Cruz, CA) containing the decameric consensus sequence (5′-GGGACTTTCC-3′) was used as a probe. For the competition assay, the protein extract (20 μg) was preincubated with homologous unlabeled oligonucleotide for 5 min on ice and the labeled probe was then added. Absence of protein extract, competition with 100-fold molar excess unlabeled NF-κB, and mutant NF-κB oligo (5′-AGTTGAGGGGACCTTTTACCCAGGC-3′; Santa Cruz Biotechnology) served as controls.

Total RNA isolation and Northern blot analysis. Total RNA extraction, Northern blotting, autoradiography, and densitometry were performed as previously described (8, 9, 12, 31). The following probes were used: miL-1β (0.6 kb, BamHI-SmaI), hTNF-α (1.3 kb, BamHI-HindIII; American Type Culture Collection) (12), pgCAT (EcoRI, antisense, SP6), and pgSOD (HindIII, antisense T7), an oligo for GSHPx (8). h2SS rRNA (40 mer; Oncogene Science, Uniondale, NY) was used as an internal control.

Western blot analysis. Extraction of protein homogenates, Western blotting, autoradiography, and densitometry were performed as previously described (8, 9, 11, 31). Rabbit anti-IκB antibody (I-19; Santa Cruz Biotechnology) and TNF-α antibodies were purchased from Biosource International (Camarillo, CA) and were used at concentrations of 3.5 μg/ml. Goat anti-human β-actin antibodies (I-19; Santa Cruz Biotechnology) were used at a concentration of 2.5 μg/ml. In a separate set of experiments, immunoblotting was performed using the above antibodies after preincubation with their respective antigens (1 h at 37°C then 14 h at 4°C). In these studies no signals were detected for either cytokine, which demonstrates the specificity.

Statistical analysis. For parameters in which data were not observed for specific durations within treatments, each observation was scored as non-significant, then treatment means were compared at each duration. If an interaction was significant (P < 0.05 considered significant) and one-way ANOVAs with post hoc Bonferroni-adjusted Student’s t-tests (with 10× P < 0.05 considered significant) to compare durations within each treatment. For parameters in which data were not observed for specific durations within treatments, each observation was scored as zero. Because these durations had no variance, the two-way ANOVA was limited to durations having data that were not all zero for each treatment. Similar comparisons using the same critical values for significance as those for the cell types with complete data were performed. If an interaction was significant, then treatment means were compared at each
duration having observed data using unpaired Student's t-tests, and one-way ANOVAs with post hoc Bonferroni-adjusted Student's t-tests comparing nonzero mean durations within a treatment were performed. In addition to these post hoc tests, one-sample Student's t-tests were used to check whether nonzero mean durations within a treatment were significantly different from zero so that by inference such durations within a treatment were significantly different from durations having a mean and SD of zero. All statistical analyses were performed using the Statistical Analysis System (SAS Institute; Cary, NC) PROC GLM and PROC t-test.

RESULTS

GSH and GSSG levels. High levels of GSH were detected in sham-operated animals from both AL and CR groups (Fig. 1). However, during reperfusion, GSH levels significantly dropped in the ischemic zones of myocardium from the CR group at 15 min of reperfusion ($P < 0.001$) and increased gradually over time. In the AL group GSH levels fell similarly at 15 min of reperfusion ($P < 0.001$) and remained at these low levels up to 2 h of reperfusion; levels increased thereafter and did not reach those of sham-operated animals even at 6 h of reperfusion. Furthermore, GSH levels were significantly lower in the AL group compared with CR animals at 2 and 6 h of reperfusion ($P < 0.001$). In contrast to GSH levels, GSSG levels rose significantly higher at 15 min of reperfusion ($P < 0.001$), remained elevated at 30 min, and fell gradually thereafter. Similar to the levels in CR group, GSSG levels rose significantly higher at 15 min of reperfusion in the AL group. Although the levels declined gradually over time, the GSSG levels never reached those of corresponding sham-operated animals even at 6 h of reperfusion. Furthermore, compared with the CR group, GSSG levels were significantly higher in the AL group at 1 ($P < 0.001$), 2, 3, and 6 h of reperfusion ($P < 0.05$; see Fig. 1). The GSH-GSSG ratio fell at least 2.5-fold in both groups at 15 min of reperfusion and rose gradually in CR animals over time. In the AL group, even after 6 h of reperfusion the GSH-GSSG ratio was significantly lower compared with respective sham-operated controls. The ratios were also significantly lower in the AL group at 30 min of reperfusion and thereafter compared with the CR group at respective time periods, indicating persistent oxidative stress during I/R in the AL group.

NF-κB DNA binding activity. In both the AL and CR groups, NF-κB DNA binding activity (as measured by the intensity of autoradiographic bands obtained in EMSA) was detected at very low levels in sham-operated controls (Fig. 2) and in the nonischemic portion adjacent to the ischemic zone (internal control; data not shown). In the ischemic zones from both groups, increased NF-κB DNA binding activity was detected at 15 and 30 min of reperfusion and again at 3 h of reperfusion, which suggests a biphasic regulation. However, in the AL group, NF-κB levels were significantly higher at both 30 min (1.56-fold; $P < 0.002$) and 3 h of reperfusion (1.51-fold; $P < 0.001$).

Because no change was detected in NF-κB DNA binding activity in both sham-operated and nonischemic regions of the myocardium from experimental animals (internal controls), in subsequent studies we utilized myocardium from only sham-operated controls and not from internal controls.

IL-1β and TNF-α expression. The mRNA expression of cytokines is shown in Fig. 3 and that of the protein is shown in Fig. 4. Neither IL-1β nor TNF-α mRNA was detected in sham-operated controls from either group. Also, the expression was not detected in reperfused myocardium for $\leq$30 min (Fig. 3). Expression was induced at 1 h of reperfusion and remained high up to 3 h of reperfusion in both groups. Although the expression fell to basal levels at 6 h of reperfusion in the CR group, expression of IL-1β and TNF-α persisted up to 6 h of reperfusion in the AL group (Fig. 3). The protein levels of both cytokines followed a similar trend as that of the mRNA expression in both the CR and AL groups (Fig. 4): falling to baseline at 6 h of reperfusion in the CR group but remaining elevated in the AL animals. In addition to spectrophotometric determination of protein concentration, equal loading of protein homogenates was also verified by immunoblotting for β-actin, and the autoradiographic results revealed that the β-actin levels were similar in all the wells (data not shown).

Catalase, GSHPx, and Mn-SOD expression. Figure 5 shows mRNA expression of three key antioxidant enzymes. In the CR group, catalase mRNA expression was not detected in sham-operated controls or in reperfused myocardium for up to 30 min. Its levels were induced at 1 h and increased further at 2 h of reperfu-
sion then declined gradually thereafter. However, in the AL group, catalase mRNA expression was highest at 3 h of reperfusion and remained elevated even at 6 h of reperfusion. Furthermore, in the AL compared with the CR group, catalase expression was significantly higher at both 3 and 6 h of reperfusion ($P < 0.001$; see Fig. 5). However, GSHPx mRNA expression was significantly higher in the CR group only at 3 h of reperfusion ($P < 0.0001$). In contrast to catalase and GSHPx, Mn-SOD expression was detected at low levels in sham-operated animals from either group. In the CR group, Mn-SOD levels remained low for up to 30 min of reperfusion, increased significantly at 1 h ($P < 0.0001$), remained at these high levels for up to 3 h ($P < 0.0001$), and then fell to basal levels at 6 h of reperfusion. In contrast in the AL group, Mn-SOD expression remained low for up to 2 h of reperfusion; levels rose significantly higher at 3 h of reperfusion and remained at these high levels even at 6 h of reperfusion ($P < 0.0001$ vs. CR animals; see Fig. 5).

**DISCUSSION**

Results from the present study show that moderate dietary CR attenuates the posts ischemic inflammatory response in the heart. CR compared with AL feeding significantly attenuated GSSH and the GSH-GSSG ratio during reperfusion after a single episode of sublethal ischemia. Furthermore, GSH levels reached baseline values faster in the CR group. CR significantly attenuated NF-$\kappa$B DNA binding activity at 30 min and 3 h of reperfusion and attenuated the duration of $\kappa$B-responsive IL-1$\beta$ and TNF-$\alpha$ expression. Finally, although I/R induced antioxidant enzyme gene expression in both AL and CR animals,
catalase and Mn-SOD expression returned to baseline levels more rapidly in CR animals compared with AL animals. This suggests that CR attenuates the myocardial postischemic inflammatory response by reducing oxidant stress.

Although there is much correlative evidence to indicate that CR animals are in a state of reduced oxidant stress relative to AL animals, this study is among the few to directly test this hypothesis by challenging the animal with a profound oxidant stress and then measuring the outcome. Recent reports indicate that the brains of CR animals are also protected from the stress of I/R (43) as well as neurotoxic insult (6). The resolution of foot-pad edema after carrageenan injection (a common test of anti-inflammatory efficacy) is also much more rapid in CR mice than in AL mice (25). Together these results support an emerging view that CR enhances organismic and cellular mechanisms that reduce tissue damage induced by oxidant stress and other toxic insults.
Our study was conducted when rats were middle-aged and had been exposed to CR for $>10$ mo. The reduced inflammatory responses seen in the CR rats could reflect: 1) an attenuation of an age-related increase in inflammatory response normally seen in AL rats; 2) an attenuation independent of age (i.e., seen even in young rats shortly after the onset of CR); or 3) a combination of these two possibilities. Our previous studies (8) in younger AL rats show comparable changes to those seen in the middle-aged AL rats of the present study, but it is not possible to compare the results quantitatively to assess whether there is an age-related increase in inflammatory reactivity. However, it is likely that CR induces a relatively rapid change in the inflammatory response to cardiac I/R because the attenuated response to middle cerebral artery I/R and carrageenan-induced inflammation were observed within several weeks after the onset of CR (6, 25, 43).

It has been reported that during I/R, increased oxidative stress results from reduced antioxidant levels (e.g., GSH, vitamin E, and SOD) (1). In the present study, however, the levels of GSH and the GSH-GSSG ratio were similar at steady state in the AL and CR animals and the levels fell significantly after 15 min of reperfusion. Although GSH levels were restored to near steady-state levels after prolonged reperfusion in the CR animals, levels remained significantly lower in the AL animals even after 6 h of reperfusion. Furthermore the AL animals exhibited increased antioxidant enzyme ac-
tivity at later periods of reperfusion than did the CR animals. This could indicate that the need for elevated activity was foreshortened by CR, although the alternative that the ability of elevated enzyme activity in CR animals was reduced remains a possibility.

Activation of NF-κB indicates cellular stress: it plays a pivotal role in the regulation of a variety of genes involved in various immune, inflammatory, and acute phase reactions. In the present study, a biphasic activation of NF-κB was detected in the AL group at 12 mo of age, and restricting dietary calories did not affect its activation pattern. However, NF-κB levels were significantly lower in CR animals at 0.5 and 3 h of reperfusion compared with the AL group. Again, this result is consistent with a lower oxidant stimulus in the CR animals or a more efficient NF-κB mechanism. Distin-
guishing among these possibilities is an important research direction.

The mechanism(s) responsible for the observed biphasic activation of NF-κB in the heart after sublethal ischemia is unknown. The early phase (15 min) likely results from oxidant stress-induced dissociation and subsequent degradation of IκB from preformed NF-κB. The second phase of NF-κB DNA binding activity observed at 3 h of reperfusion may result from a second wave of IκB degradation and/or de novo synthesis of NF-κB subunits. In fact we have previously reported the induction of p65 gene activity and increased protein levels in postischemic myocardium in the absence of changes in p50 subunit expression (10). A further possibility involves the activation of NF-κB via a mechanism that does not require IκB degradation (7). Irrespective of the mechanism(s) involved in NF-κB activation, the present study shows that CR attenuated the magnitude but not the temporal pattern of NF-κB DNA binding activity.

Despite the lower NF-κB levels in CR animals, induction of IL-1β and TNF-α was quite similar in both groups. Although the expression returned to baseline after 6 h of reperfusion in the CR group, expression of IL-1β and TNF-α persisted for up to 6 h in the AL group, which indicates that CR inhibits the postischemic inflammatory response. Given the role of NF-κB in the response of these cytokines, the reduced amplitude of the NF-κB peaks could underlie the attenuated cytokine response. Both IL-1β and TNF-α have been shown to have negative inotropic effects in various myocardial preparations (whole heart, isolated perfused heart, papillary muscle preparation, and cultured cardiac myocytes) (14, 15, 23, 26, 30, 40). Chronic upregulation of these cytokines may therefore have profound negative effects on myocardial function. Thus it is tempting to speculate that the attenuation of the cytokine response provides an example of reduced tissue damage after insult that characterizes the CR state and may contribute to retarded aging.

In contrast to the negative inotropic effects, these cytokines also induce antioxidant enzyme gene expression. TNF-α and IL-1β either alone or in combination have been shown (35, 41, 42) to induce Mn-SOD mRNA expression with increased protein and enzyme activity without significantly altering the protein levels or enzyme activities of other antioxidant enzymes (Cu,Zn-SOD, catalase, or GSHPxs). Because of its strategic location in the mitochondria, which is a major site of ROI production, enhancement of Mn-SOD may reduce R/I injury by preventing further free-radical damage. In the present study, however, the levels and expression patterns of catalase and GSHPxs were very similar in both groups although expression of Mn-SOD differed considerably between the AL and CR groups. Although Mn-SOD expression peaked very early during reperfusion in CR animals (1 h of reperfusion), its expression was considerably delayed in the AL group, which indicates a delayed antioxidant enzyme expression in the AL animals in response to I/R. This may lead to prolonged oxidative damage in these hearts.

Our study must be interpreted in view of certain limitations. First, we did not measure biochemical parameters such as cholesterol, triglycerides, and glucose in the serum of our animals. However, CR is known to reduce serum cholesterol, triglycerides (27), and glucose (28) in male F344 rats. Although elevations of these substances may increase risk for atherosclerosis and other cardiovascular diseases, we are unaware of any direct role they may play in the myocardial response to I/R. A further limitation of the present investigation is that we did not study functional or morphological changes in these animals, which precludes establishment of a direct correlation between these parameters and the biochemical and molecular changes that we observed. A more complete understanding of these relationships warrants further investigation.

Taken together, results from the present study indicate that moderately restricting dietary calories attenuates oxidative stress and damage during cardiac I/R injury as seen by more rapid recovery of GSH levels, attenuated expression of proinflammatory cytokines, and rapid response of the free-radical scavenging system. This finding adds to earlier studies showing that CR is neuroprotective against toxic insults including I/R and has anti-inflammatory action in peripheral tissues. Thus myocardial I/R provides a new model for probing the cytoprotective mechanisms of CR.

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