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Caloric Restriction Improves Endothelial Dysfunction During Vascular Aging: Effects on Nitric Oxide Synthase Isoforms and Oxidative Stress in Rat Aorta

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Abstract

Aging is characterized by activation of inducible over endothelial nitric oxide synthase (iNOS and eNOS), impaired antioxidant activity and increased oxidative stress, which reduces nitric oxide bioavailability and causes endothelial dysfunction. Caloric restriction (CR) blunts oxidative stress. We investigated whether CR impacts endothelial dysfunction in aging and the underlying mechanisms. Aortas from young (YC, 6 months of age) and old (OC, 24 months of age) rats ad libitum fed and from old rats caloric-restricted for 3-weeks (OR, 26%) were investigated. Endothelium-dependent vasorelaxation was impaired in OC, associated with reduced eNOS and increased iNOS expression ($P < 0.05$). Aortic nitrite was similar in OC and YC, but the contribution of calcium-independent NOS to total NOS activity was increased whereas that of calcium-dependent NOS was reduced ($p \leq 0.0003$). Plasma thiobarbituric acid-reactive substances (TBARS) were elevated in OC as well as aortic nitrotyrosine ($P < 0.05$). Expression of manganese superoxide dismutase (MnSOD) and total SOD activity were impaired in OC ($P < 0.05$ vs. YC), whereas copper-zinc (CuZn) SOD expression was similar in OC and YC. CR restored endothelial dysfunction in old rats, reduced iNOS expression, total nitrite and calcium-independent NOS activity in aorta ($P < 0.05$) without changes in eNOS expression and calcium-dependent NOS activity. Sirtuin-1 expression did not differ among groups. Plasma TBARS and aortic nitrotyrosine were reduced ($P < 0.05$) in OR compared with OC. In OR CuZnSOD protein and SOD activity increased ($P < 0.05$) without changes in MnSOD expression. Short-term CR improves age-related endothelial dysfunction. Reversal of altered iNOS/eNOS ratio, reduced oxidative stress and increased SOD enzyme activity rather than enhanced NO production appear to be involved in this effect.

Keywords

Aging, Endothelium, Caloric Restriction, Nitric Oxide, Superoxide

Introduction

Aging is associated with phenotypic and functional changes in vascular structure and function, thus being a strong risk factor for cardiovascular disease (1). Endothelial dysfunction which characterizes vascular aging is strictly associated with decreased nitric oxide (NO) bioavailability (2-5), resulting in impaired vasodilation, increased plaque formation and thrombosis. Several mechanisms are involved in reduced NO availability in aging. First, endothelial nitric oxide synthase (eNOS) activity and therefore eNOS-derived NO production decline with increasing age (6-8); second, excess reactive oxygen species (ROS) produced by arteries during aging combine with NO to form peroxynitrite, a powerful oxidant, which is increased in the arterial media of aging vessels (1, 5, 9-13). However, in this process, efficient ROS scavenging by inducible NOS (iNOS)-derived NO, which is also activated during aging (1, 6, 14), may represent a counteractive response against vascular fibrosis. Several studies support this hypothesis, having shown that ROS stimulate arterial fibrosis during aging (15, 16) and that treatments able to reduce oxidative stress prevent vascular remodelling (17). Also, long-term inhibition of iNOS results in collagen deposition in the media and ROS overproduction despite reduced nitrotyrosine in aorta (16, 18, 19). Moreover, as a result of increased oxidative stress, most of the antioxidant enzyme expression and activity is altered in the aged vasculature (12, 20-22). The deficiency of both the mitochondrial free radical scavenger MnSOD and the cytosolic CuZnSOD contributes to endothelial dysfunction in experimental models of aging (23, 24).

Lifelong caloric restriction prolongs lifespan, reduces atherosclerosis in humans and improves endothelial function (25, 26). The underlying mechanisms include improved cardiovascular risk factor profile, reduced vascular oxidative stress and inflammation as reflected by lower concentrations of circulating inflammatory cytokines and decreased vascular superoxide production in both caloric-restricted humans and animals (27). However, at present very little information is available on the effects of short-term caloric restriction (3 weeks) initiated late in life on NOS isoform expression and activity, oxidative stress and on the vascular antioxidant enzymes.

To address these issues, we measured endothelium-dependent and independent vasorelaxation in aortas from young-adult and old rats fed ad-libitum or moderately caloric-restricted for three weeks. To investigate the potential mechanisms underlying CR-induced changes, we measured eNOS and iNOS expression and function, oxidative stress (in the form of plasma thiobarbituric acid-reactive substances: TBARS, and arterial expression of nitrotyrosine, a marker of protein oxidation and peroxynitrite formation), and antioxidant potential (CuZn and MnSOD expression and total SOD activity) in aorta from experimental animals. Finally, arterial sirtuin-1 (SIRT-1) expression was measured, since it is a known stimulator of eNOS expression and activity (28) and it is upregulated during long-term caloric restriction (29).

Materials and Methods

Materials

Primary antibodies were purchased from Transduction Laboratories (Franklin Lakes, NJ, USA) (anti-eNOS and anti-iNOS), Stressgen (Ann Arbor, MI, USA) (anti-MnSOD and anti-CuZnSOD), Abcam (Cambridge, MA, USA) (anti-SIRT-1), and Millipore (Billerica, MA, USA) (anti-NT mAb clone 1A6). Secondary HRP-conjugated antibodies from Jackson Laboratories (West Grove, PA, USA) (anti-mouse), Cell Signaling Technologies (Beverly, MA) (anti-rabbit) and Abcam (anti-goat). Chemiluminescence reagents for Western blot and ELISA were purchased from Pierce (Rockford, IL) and Cell Signaling Technologies. Human haemoglobin for NOS activity assay was extracted, purified and concentrated by dialysis. Immediately prior to assay it was dissolved in PBS 0.01M pH 7.2 and the large prevalence of the HbO₂ form in the solution was confirmed by absorption spectrum. Concentration was also determined spectrophotometrically by absorbance at 415 nm, using an absorption constant of 131000 M⁻¹ cm⁻¹. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Animal care and experimental procedures were approved by the local Institutional Review Committee for animal studies. Forty old (24 months of age) and twenty young (6 months of age) Fischer 344 male rats were purchased from Harlan (Indianapolis, IN, USA) and fed a standard rat chow for at least two weeks. Animals were then randomly assigned to control or caloric-restricted diets for three weeks. The control (young control, YC and old control, OC) animals were allowed free access to a standard laboratory diet for ad libitum feeding. The old, caloric-restricted group (OR) received the control diet but limited to 74% of the caloric intake of the control groups.

Throughout the experiment animals were individually housed in wire-bottom cages in a controlled environment (12 h light-dark cycle, 20-22° C, 50-60% relative humidity). Body weight and food consumption were recorded regularly. At the end of the experimental period, animals were injected an intraperitoneal overdose of sodium pentobarbital (70 mg/kg body weight). Aortas were then immediately harvested from all rats and blood was collected through cardiac puncture. From all rats in each group, thoracic aorta was divided into 6 rings, two for Western blot analysis, one for assessment of nitrite and nitrate (NO_x) production, 1 for SOD activity and the rest for vascular reactivity studies. Blood samples were centrifuged at 2000 rpm at 4°C for 15 minutes and plasma stored at -80°C until analysis.

The study was part of a larger protocol assessing the effects of caloric restriction on energy metabolism.

Determination of Blood Glucose and Insulin

Blood glucose was measured using an AccuCheck glucose monitor (Roche Diagnostic Corporation, Indianapolis, Indiana, USA). Plasma insulin concentrations were measured by radioimmunoassay using a commercially available kit (Linco, St Louis, MO, USA).

Analyses of Vascular Reactivity

Rings (4 mm long) from each thoracic aorta were used for assessing vascular reactivity. Rings were suspended in organ chambers filled with 25 ml of gassed (95% O₂ and 5% CO₂) modified Krebs-Ringer bicarbonate solution (pH 7.4, temperature 37°C; composition in mmol/l: 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, 0.026 calcium sodium EDTA, and 11.1 glucose). The rings were allowed to equilibrate for 1 hour and then stretched to the optimal point on the length-tension curve as determined by repeated exposure to 20 mmol/l KCl. The maximal contraction of each ring was determined by KCl 60 mmol/l. Submaximal contractions were obtained using a 10⁻⁶ to 10⁻⁷ mol/l concentration of phenylephrine. Acetylcholine (10⁻⁹ to 10⁻⁵ mol/l) was added cumulatively during a submaximal contraction to phenylephrine. Concentration responses to DEA NONOate (10⁻¹⁰ to 10⁻⁵ mol/l) were similarly performed.

Measurement of NO_x Concentration and of the Relative Contribution of NOS Isoforms to Total NOS Activity in the Aorta

NO content within the vessel wall was assessed by the determination of the stable end products of NO metabolism, NO₂⁻ and NO₃⁻ (NO_x) using a commercially available kit (Cayman Chemical, Ann Arbor, MI) and following the manufacturer's instructions. Briefly, endothelium-intact aortic rings were homogenized in PBS (pH 7.4) and centrifuged at 10,000 g for 20 min. Supernatant was then collected and ultracentrifuged at 100,000 g for 30 min through a 30 kDa molecular cut-off filter (Millipore). In the first step nitrate reductase was added to the filtrate to convert nitrate into nitrite. Then Griess reagent was used to convert nitrite into an azo compound. Photometric measurement of this azo compound at 548 nm determines nitrite concentration. Aortic protein content was measured by the bicinchoninic acid (BCA) assay (Pierce).

Total and isoform related NOS activity was measured in rat aorta according to Salter et al. (30). This assay is based on the conversion of oxyhemoglobin (HbO_2) to methemoglobin (MetO_2) by NO. Briefly, frozen aortic rings were pulverized on ice in lysis buffer (SDS 1% (w/v) and protease inhibitors cocktail). Insoluble cellular debris was removed by centrifuging at 10000 g for 15 min at 4°C. Total protein content was measured using the BCA assay (Pierce). 12 μg of total protein from each sample were incubated at room temperature for 10 minutes in a 96-well microplate in PBS 0.01 M pH 7.2, with the addition of both MgCl_2 (1 mM) and EGTA (1 mM) or of CaCl_2 (250 μM), in order to distinguish NO production from calcium independent isoform (iNOS) from the total NOS activity. In some experiments L-NMMA (100 μM), a non selective NOS inhibitor, was added to ensure NOS dependence of the HbO_2 to MetHb conversion. An assay buffer (final concentration: HbO_2 0.15 μM , L-Arginine 20 μM , NADPH 100 μM) was then added and HbO_2 to MetO_2 conversion was monitored for 15 minutes by measuring the difference in absorbance between 421 and 401 nm. Calcium dependent (constitutive) NOS isoforms contribution in NO synthesis was calculated by subtraction of the calcium independent production rate from the total NO release of each sample.

Aortic Protein Expression and SOD Activity

Protein levels of eNOS, iNOS, MnSOD, CuZnSOD and SIRT-1 and were analyzed by Western blot analysis. Briefly, aortic segments were isolated and immediately stored in liquid nitrogen. For eNOS, iNOS and MnSOD quantifications the frozen segments were pulverized and resuspended in lysis buffer (50 mmol/l Tris HCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 0.1% SDS, 0.1% deoxycholate, 1% Igepal, 2 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ aprotinin, 1 mmol/l PMSF, 1 $\mu\text{g}/\text{ml}$ pepstatin). Aortic debris was homogenized on ice, then centrifuged at 14000 rpm for 10 minutes to remove the insoluble pellet, and protein concentration was determined by the BCA method. For SIRT-1 and CuZnSOD the same homogenate described for NOS activity was used. After separation by SDS/PAGE, the resolved proteins were transferred to 0.2 μm nitrocellulose membrane using a

semi-dry electrophoretic transfer system (Bio-Rad). Equal protein load was confirmed by Ponceau staining. Blots were blocked and incubated with anti-eNOS (1:250), anti-iNOS (1:4000), anti-MnSOD (1:5000), anti-CuZnSOD (1:1500) and anti-SIRT-1 (1:1000) antibodies overnight at 4 °C. After extensive washing, appropriate horseradish peroxidase-linked secondary antibodies were added. Chemiluminescence detection was performed by exposure of x-ray films to membranes after incubation with ECL reagents. After densitometric scan (GS-700, BioRad) films were analyzed by appropriate image analysis software (Molecular Analyst, BioRad; ImageJ, NIH).

For the SOD activity assay the aortas were carefully cleaned of fat and connective tissue and were then cut into pieces in ice-cold lysis buffer. The minced tissue-buffer mixture was homogenized with 50 passes of a ground glass-on-glass homogenizing system and centrifuged at 4°C at 4,000 rpm for 15 min to remove cellular debris. The supernatant was collected for SOD activity measurement by the reduction of cytochrome C method (31). Briefly, xanthine/xanthine oxidase was used to generate O_2^- which was detected by the reduction of cytochrome C. Spectrophotometrical measurement of the rate of reduction of cytochrome C in the presence of increasing amounts of SOD protein was performed. Total SOD activity was determined from the amount of inhibition of cytochrome C reduction. Protein content of the samples was assayed by the BCA assay. All of the assays were performed at 25°C.

Oxidative stress

Lipid peroxidation in plasma was determined by measurement of TBARS, using a commercially available kit (Oxitek, ZeptoMetrix Co., Buffalo NY, USA) with slight modifications. Briefly, after pre-treatment of plasma samples with SDS 5%, a reaction buffer containing thiobarbituric acid 36.8 mM was added and samples were heated at 96°C for 60 min and then centrifuged at 1000 g for 15 min to remove precipitates. To eliminate reading interference by haemoglobin and its derivatives, as by Yagi (32), one volume of 1-butanol was added followed by centrifugation at 10000 g for 5'. 200µl of the butanol phase were then transferred to a 96-well

microplate and sample fluorescence (excitation: 535 nm, emission: 595 nm) was read in a microplate fluorimeter (Infinite F200, Tecan Group Ltd., Männedorf, CH). Malondialdehyde concentration was calculated by standard curve interpolation and expressed as $\mu\text{mol/l}$ plasma. Inter-assay coefficient of variation was 3.56%.

Nitration of protein tyrosine in aortic tissue samples was measured by chemiluminescence enhanced indirect ELISA, basing on the method described by Naseem et al. (33) with modifications. The same aortic homogenate described for NOS assay was used. Samples appropriately diluted in coating buffer (buffer 0.05M pH 9.6) were coated on a white 96-well plate (Luminunc, Nunc, Roskilde, Denmark) for 2h at 37°C. Nonspecific binding sites were then blocked with blocking buffer (Ovalbumin 0.5% (w/v), Tween-20 0.05% (v/v), PBS 0.01M pH 7.4) for 2h at 37°C. Primary detection antibody (1:2000) in blocking buffer (Anti-NT mAb clone 1A6) was added and incubated at 37°C. Following repeated washing of unbound antibody (PBS 0.01M pH7.4, Tween-20 0.05% (v/w)), the plate was incubated with secondary HRP conjugated antibody (1:1500) in blocking buffer for 1h at room temperature. After washing, chemiluminescence substrate LumiGLO (Cell Signaling) was added and luminescence read at 10 minutes in a microplate luminometer (Synergy2, BioTek). Measurements were obtained by interpolation from a standard curve of serial dilutions of nitrosylated bovine serum albumin (BSA^{NT}) and expressed in pmol BSA^{NT} equivalents/mg protein. The assay showed a level of detection of 2.25 nmol/l BSA^{NT} equivalents and linearity in the range of 4.5-150 nmol/l ($r^2=0.992$) BSA^{NT} equivalents. Impact of SDS on the assay was assessed. Results showed that SDS in the concentration used in the samples did not interfere with detection linearity. Specificity of the assay was tested by coating some samples in the presence of sodium hydrosulfite 50 mM, which reduces nitrotyrosine to aminotyrosine, and resulted in >90% reduction of the sample 3-NT detection. Overall intra-assay coefficient of variation was 6.0%

Statistical Analysis

Data are presented as mean \pm SE. Statistical analysis was performed by ANOVA to detect significant differences in multiple comparisons. An unpaired Student's *t* test was used to detect significant differences when two groups were compared. Post hoc analysis was performed, where appropriate, by using a *t* test with Bonferroni's adjustment. A value of $P < 0.05$ was considered to be statistically significant.

Results

Body Weight and Metabolic Profile

After three weeks of diet, OR rats weighed significantly ($P < 0.05$) less than YC and OC (Table 1). Plasma insulin was significantly ($P < 0.05$) lower in old caloric-restricted rats, while there was no difference in blood glucose levels among the three groups (Table 1).

Vascular Reactivity

Endothelium-dependent vasorelaxation to acetylcholine was markedly ($P \leq 0.02$) reduced in old versus young control rats, but was improved ($P < 0.05$) by caloric restriction (Fig 1A). This effect selectively involved the endothelium, since endothelium-independent vasorelaxation to DEA-NONOate was similar in the three groups (Fig. 1B). Contractions in response to phenylephrine also did not differ among groups.

NOS Isoform Expression and NO Production

Old control rats showed profound alterations in NOS isoform expression (Fig 2). eNOS expression was markedly ($P<0.05$) reduced while that of iNOS was four-fold higher ($P<0.05$) in OC compared with YC (Fig 2B and A). CR modified NOS isoform expression by selectively and substantially ($P<0.05$) reducing iNOS protein to levels comparable to YC animals (Fig. 2A). This effect was not associated with changes in the expression of eNOS (Fig 2B).

Since expression levels of NOS enzymes regulate vascular NO_x content, the latter was measured in homogenates from aortic rings. Similar levels of NO_x were found in aortas from young and old control rats (Fig 3A). In contrast, aortic NO_x was reduced ($P<0.05$) in OR compared to both YC and OC, consistent with reduced iNOS expression in this group (Fig 3A). Analysis of the relative contributions of calcium-independent and calcium-dependent NOS to total NOS activity showed a marked ($P=0.0001$) increase of calcium-independent NOS activity in OC vs. YC, which decreased ($P=0.0001$) following caloric restriction (Fig. 3B). Calcium-dependent NOS activity was reduced in OC ($P=0.0003$), whereas it did not change after CR (Fig. 3B).

Oxidative Stress and Antioxidant Enzymes

To determine whether changes in endothelial function are associated with changes in oxidative stress and antioxidant enzymes, we measured plasma levels of TBARS, aortic nitrotyrosine concentration, a marker of peroxynitrite formation, the activity of SOD and the expression of both CuZnSOD, the predominant isoform of SOD in vessels and of mitochondrial MnSOD. Plasma TBARS, a marker of lipid peroxidation, were 120% higher in OC vs. YC ($P<0.05$) and decreased by 31% in OR vs. OC ($P<0.05$, Fig 4A). Aortic nitrotyrosine, a cellular marker of peroxynitrite formation, was 60% higher in OC ($P<0.01$), but reduced ($P<0.01$) in OR vs. OC (Fig 4B). Western blot analysis revealed an age-dependent reduction ($P<0.01$) in MnSOD expression that was associated with decreased ($P<0.05$) total SOD activity (Figs. 5A and C) in OC. CuZnSOD levels were decreased in OC vs. YC although not significantly (Fig. 5B). CR was associated with increased CuZnSOD expression ($P<0.05$) and higher, although not completely normalized SOD

activity ($P < 0.01$, Figs. 5B and C). This effect was independent from changes in MnSOD expression (Fig 5A), suggesting enzyme activation possibly related to post-translational modifications following CR and/or prominent effect of CR on other SOD isoforms (namely CuZnSOD).

SIRT-1 Expression

Protein expression of SIRT-1 was not significantly different in aorta of YC, OC and OR (Fig 2C). This finding demonstrates that 3-weeks moderate caloric restriction, as in this study, does not activate vascular SIRT-1.

Discussion

The results of this study demonstrate that caloric restriction prevents many functional changes associated with vascular aging, namely: a) it normalizes iNOS expression in aged arteries (b) it consensually reduces NOx species production by selectively suppressing calcium-independent iNOS activity; c) it decreases oxidative stress and peroxynitrite formation d) it upregulates CuZnSOD expression increasing also SOD activity. Finally, although decreased eNOS expression and activity were not reversed by CR, the overall effect on vascular function was an improvement of endothelial dysfunction in old rats. Taken together, these findings suggest that short-term caloric restriction initiated late in life reduces oxidative stress and potentiates antioxidant defenses in the aorta of old rats. These results provide novel and potentially important insights on the protective effects of caloric restriction on endothelial function during aging.

In this study, aging resulted in endothelial dysfunction to acetylcholine as previously demonstrated (1-4), which was reversed by caloric restriction. This effect has been reported recently also by Ungvari et al (27). Improved vasorelaxation was not due to a difference in smooth muscle cell function, because we observed similar vasorelaxation in aortas from young, old control and old-caloric restricted rats when we used the endothelium-independent agent DEA-NONOate. Several

factors associated with caloric-restricted diets, such as reduced oxidative stress, blunted vascular inflammation and improved cardiovascular risk factor profile may participate to prevent endothelial dysfunction during vascular aging.

Since NO is the key regulator of endothelial function, we measured vascular NO production in the form of nitrates and nitrites (NO_x) and the expression and the activity of calcium independent and dependent NOS, as a measure of iNOS and eNOS activity, to determine the relative contribution of each isoform to total NO production. In old animals, aortic NO_x was similar compared to young rats; however the expression of NOS isoforms and the relative contribution of each isoform to total NO activity was substantially different in the two groups. In old rats iNOS expression was markedly increased, whereas eNOS expression was reduced; accordingly iNOS contribution to total NOS activity was higher in this group, suggesting a major role of iNOS in total NO production. These data are in line with previous studies showing upregulation of iNOS in aged arteries (1, 6, 14). Caloric restriction normalized iNOS protein expression and activity in old rats without restoring eNOS protein levels and activity; consistently, total NO_x production decreased.

With regards to eNOS expression in aging, conflicting data have been reported, showing reduced (1), unchanged (8) or increased (6, 12, 34) eNOS protein levels during aging. These discrepancies may possibly be attributed to animal species, strain, age, experimental model and vascular bed-dependent differences. In this study we found reduced eNOS protein expression in aortas from old animals, which, unlike iNOS, was not restored to normal levels by caloric restriction. Therefore, it appears from our data that changes in eNOS expression and activity are not responsible either for preserved NO availability or for improved vasorelaxation in old caloric restricted animals. One of the prominent mechanisms by which caloric restriction increases eNOS is by activating the deacetylase sirtuin 1 (SIRT-1) (28). Although previous studies have shown that lifelong caloric restriction stimulates eNOS expression and activity in aorta via SIRT-1 (29), it is currently unknown whether the same effect can be achieved by 3 week caloric restriction. The results of this study demonstrate that SIRT-1 protein expression was not significantly different

among young, old and old-caloric restricted animals. Considered together, these data provide a mechanism for the lack of effect of caloric restriction on eNOS protein expression and activity in aortas of old animals.

Rather the results of this study suggest that in old rats other mechanisms were implicated in improved endothelial dependent vasodilation following CR than increased NO production. This idea is supported by the finding of reduced NOx in old calorically restricted rats despite improved endothelial function. One hypothesis is that short-term CR may reduce ROS generation. Oxidative stress represents an important contributor to the pathogenesis of endothelial dysfunction associated with aging (5, 9-11), as demonstrated by the finding that treatment with antioxidants reverses aging-associated endothelial dysfunction (35). Our results are in agreement with previous studies (5, 9-10, 21, 27), showing increased oxidative stress in the aging vasculature, as evidenced by increased plasma TBARS, aortic nitrotyrosine accumulation and decreased MnSOD expression and SOD activity as a counteractive mechanism to reduce oxidative stress. During vascular aging, ROS overproduction and iNOS overexpression, resulting in peroxynitrite accumulation have been linked to arterial fibrosis (15-19). In this setting, therapeutic strategies to reduce oxidative stress may prevent arterial remodelling and endothelial dysfunction. Lifelong caloric restriction has been shown to reduce oxidative stress (27) and in parallel to decrease age-related vascular fibrosis (17). In the present study, short-term caloric restriction initiated late in life reduced oxidative stress and iNOS activation; as a result, nitrotyrosine deposition, which reflects peroxynitrite formation, was markedly lower in aorta from old calorically-restricted rats. Although our data do not allow to determine if reduced oxidative stress or iNOS downregulation, simultaneously present following caloric restriction is primarily responsible for improved endothelial function, these findings provide a rationale for the antisclerotic effect of caloric restriction (17, 18) during vascular aging and extend the observations of reduced oxidative stress and decreased iNOS activation from long-term to short-term caloric restriction (29).

Changes in the levels of antioxidant enzymes have also been described during aging (1, 19, 21, 34, 36, 37). Age-related MnSOD nitration is responsible for reduced enzyme activity in this setting (12). In aged aorta deficiency of both MnSOD and CuZnSOD causes endothelial dysfunction and oxidative stress (23, 24). Since caloric restriction selectively reduces ROS production, we measured the expression of MnSOD and of CuZnSOD, which is the predominant isoform in vessels along with SOD activity in rat aorta. The results show that the expression of MnSOD and SOD activity were significantly reduced in vessels from old control compared to those from young animals, whereas CuZnSOD expression was less affected by aging. These changes are consistent with the increased oxidative stress in the old group of rats, as an inverse relationship between oxidative stress and antioxidant capacity has been demonstrated in aortas from old animals (18). Caloric restriction did not restore MnSOD expression to normal levels, however CuZnSOD expression and SOD activity significantly increased in aortas from old rats compared to the ad-libitum fed group of old animals. Whether increased SOD activity is due to increased CuZnSOD activity or to reduced nitration of MnSOD associated with caloric restriction cannot be predicted on the basis of these data.

Insulin sensitivity is an important determinant of endothelial function. In this study, circulating glucose was similar in the three groups, but insulin levels were lower in the calorically-restricted old rats, suggesting improved insulin sensitivity, in line with previous findings in primates and humans (38). It is possible that improved insulin sensitivity contributed to reversal of endothelial dysfunction in OR. However, SIRT-1 which is implicated in both improved insulin sensitivity and eNOS activation during caloric restriction was similar in the three groups. In contrast iNOS has been strongly linked with insulin resistance and inhibition of iNOS reduces insulin levels (39) and restores the response to insulin vascular effects in diabetic animals (40). Thus, iNOS downregulation might contribute to improved insulin sensitivity and endothelial function in old rats during CR.

In conclusion, our data demonstrate that short-term caloric restriction results in decreased iNOS expression and oxidative stress (directly, via reduced lipid peroxidation and peroxynitrite formation and indirectly via upregulation of SOD activity and CuZnSOD expression) in old rat aorta. These changes are associated with improvement of age-associated endothelial dysfunction. Increased eNOS-derived NO as a result of enhanced eNOS expression and function does not seem to be involved in this effect, as SIRT-1 expression is not increased in aorta following 3 week CR. Thus, CR might contribute to reduce cardiovascular disease in aging by attenuating oxidative-stress induced endothelial dysfunction and vascular remodeling.

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Fig. 1. A: Concentration response curves to acetylcholine in young (YC), old (OC), and old caloric-restricted rats. Vascular reactivity studies were performed on aortic segments from each group.

* $P \leq 0.02$ or less vs. YC. $^{\S}P < 0.05$ vs. OC. $n=20$. Contractions to phenylephrine were similar in the two experimental groups. B: C: Concentration response curves to DEA-NONOate in young control (YC), old control (OC) and old caloric-restricted (OR) rats. Contractions to phenylephrine were not significantly different in the three groups. $n=20$. $-\log M =$ negative logarithm of molar concentration.

Figure 1

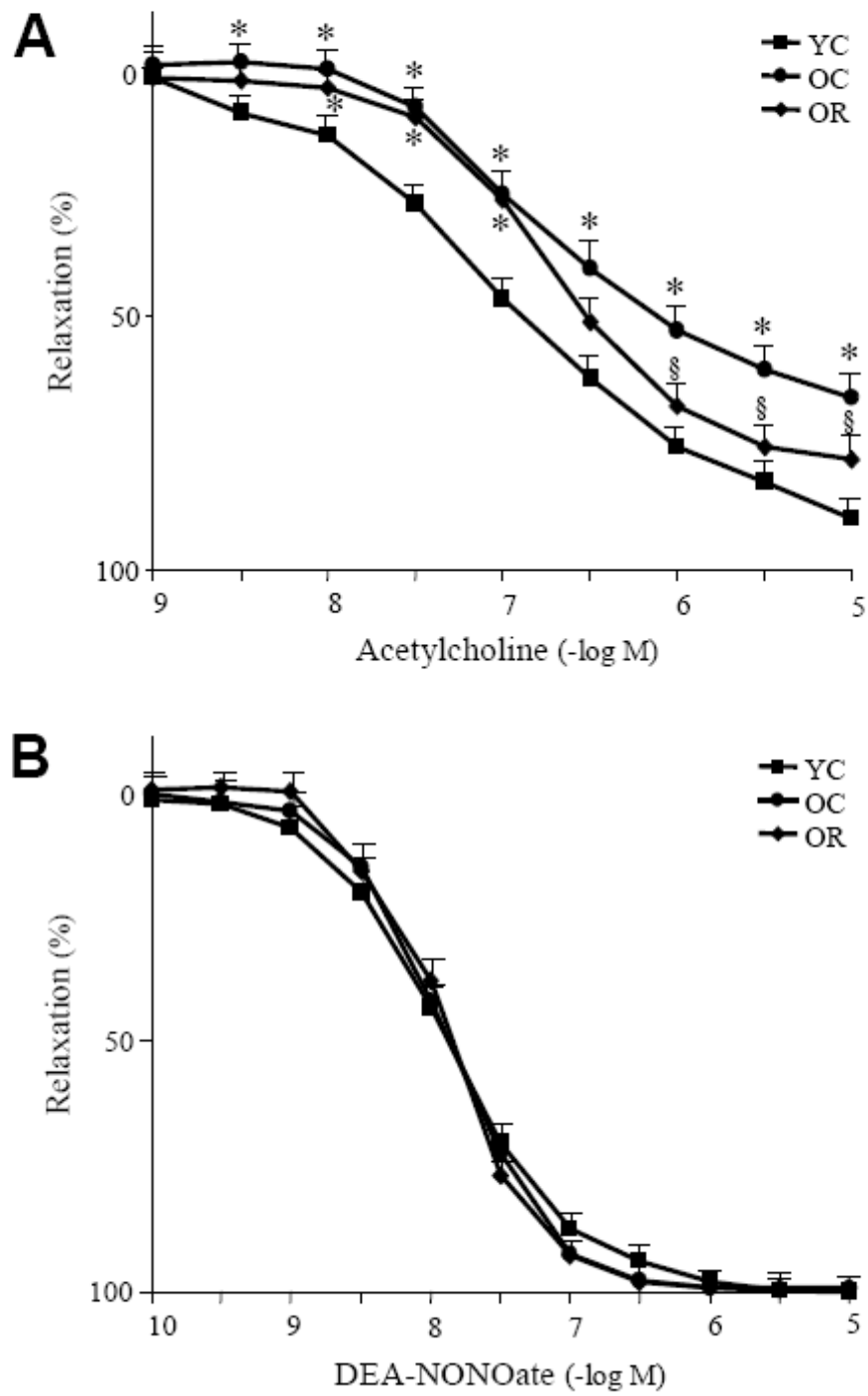


Fig. 2. A: Top: Expression of iNOS protein (130 kDa) in aorta from young (YC, lanes 1-2), old (OC, lanes 3-4) and old caloric-restricted rats (OR, lanes 5-6) as detected by Western blot analysis.

Bottom: Densitometric analysis of iNOS Western blotting. Data represent mean value of 8 animals from each group. * $P < 0.05$ or less vs. YC and OR. a.u.: arbitrary units.

B: Top: Expression of eNOS protein (140 kDa) in aorta from young (YC, lanes 1-2), old (OC, lanes 3-4) and old caloric-restricted rats (OR, lanes 5-6) as detected by Western blot analysis. Bottom:

Densitometric analysis of eNOS Western blotting. Data represent mean value of 8 animals from each group. eNOS expression was 200% higher in aorta from YC vs. OC and OR. * $P \leq 0.05$. a.u.: arbitrary units.

C: SIRT-1 protein expression in aorta of young, (YC, lane 1), old (OC, lane 2) and old caloric-restricted (OR, lane 3) rats. Top panel shows representative blot, bottom panel: densitometry arbitrary units (a.u.). $n=8$.

Figure 2

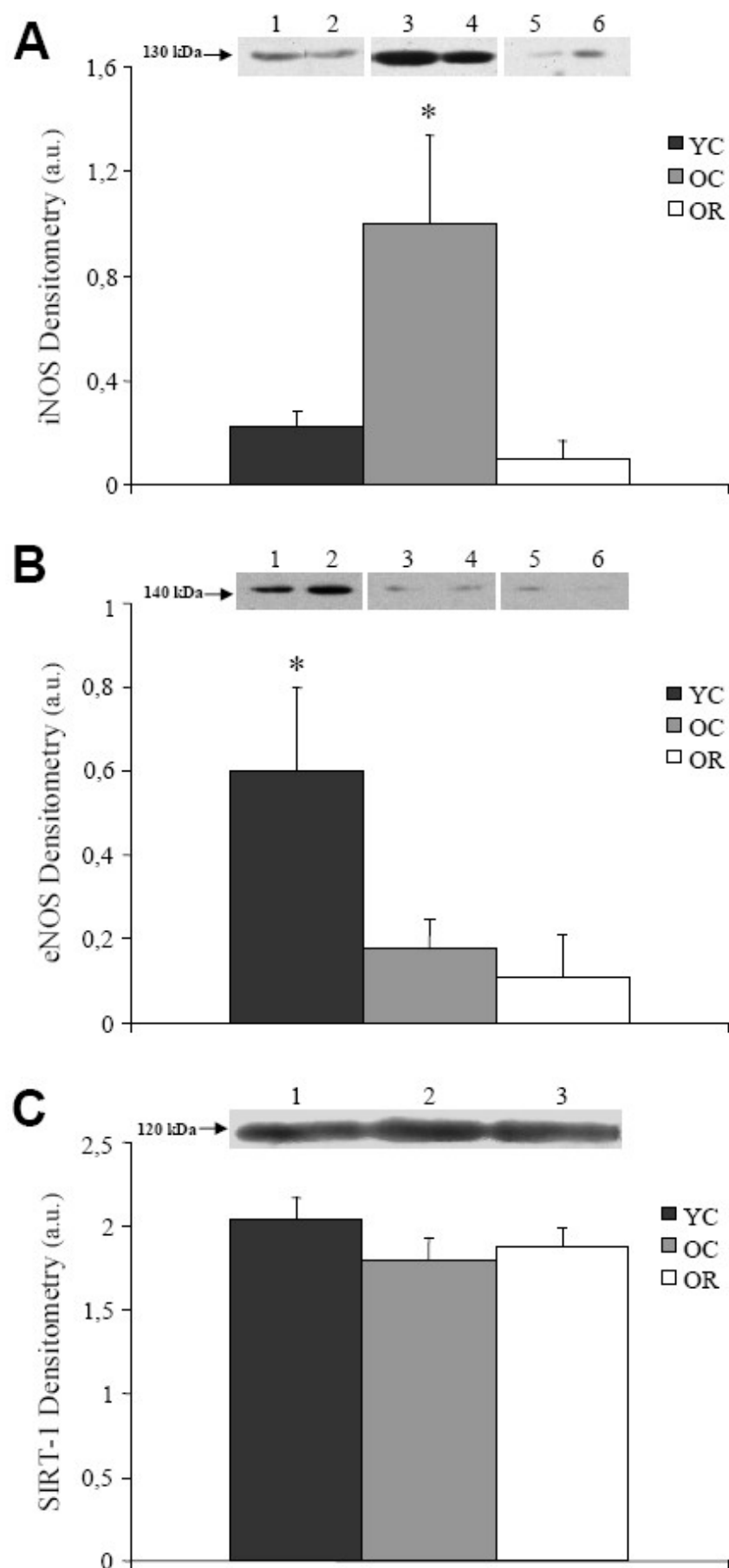


Fig. 3. A: Total nitrite and nitrate ($\text{NO}_2^- + \text{NO}_3^-$) in aorta from experimental animals. Data are expressed as μMol per mg of protein. $n=10$. $*P<0.05$ or less old caloric-restricted rats (OR) vs. young control (YC) and old control (OC). B: Contribution of NOS isoforms to total NOS activity. Results are expressed as % of each group total NOS activity assumed as 100%. $n=7$. $*P=0.0001$ calcium-independent NOS activity in OC vs. YC and OR; $^sP=0.0003$ calcium-dependent NOS activity in OC vs. YC and OR.

Figure 3

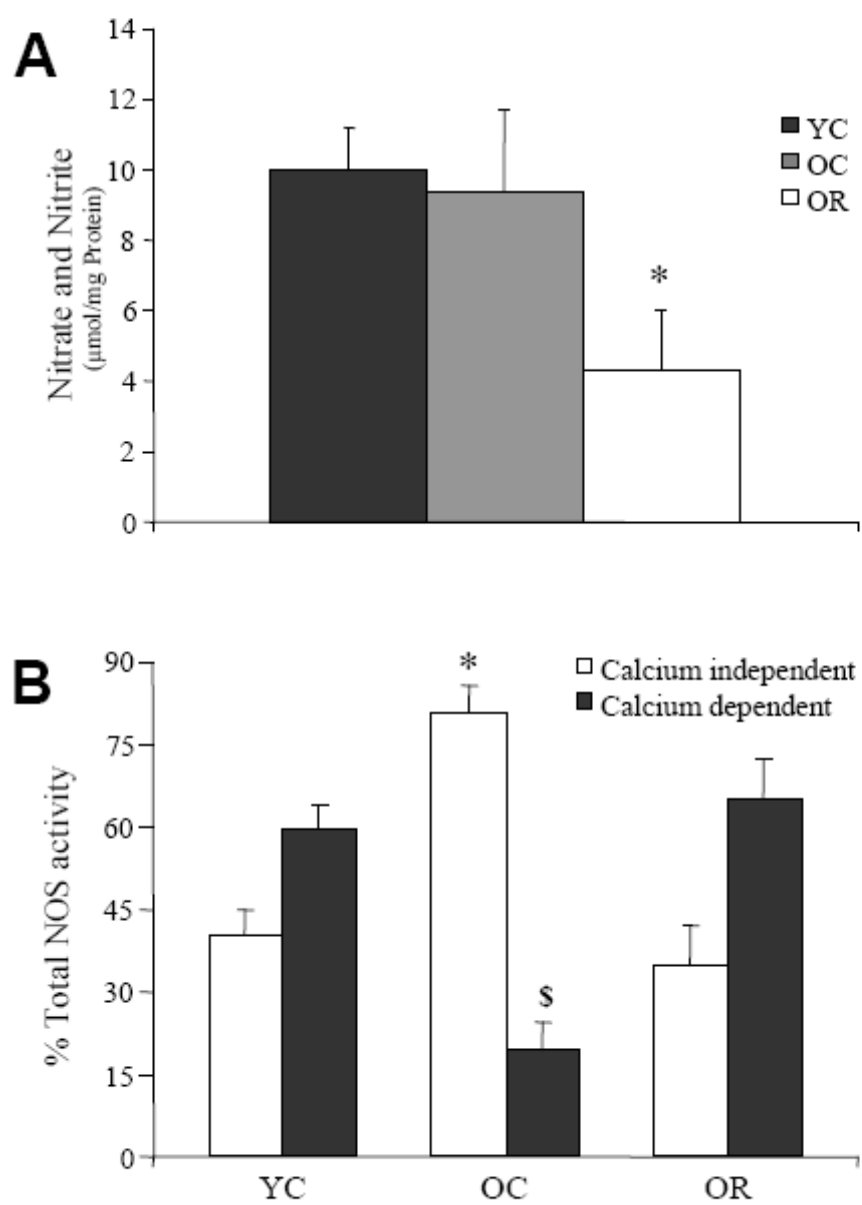


Fig 4. A: Plasma thiobarbituric acid-reactive substances (TBARS) in young (YC), old (OC) and old caloric-restricted rats (OR). * $P < 0.05$ YC vs. OC and OR; $^{\$}P < 0.05$ OR vs. OC. $n = 9$.

B: Nitrotyrosine content in aorta of young (YC), old (OC) and old caloric-restricted rats (OR) assessed by ELISA. Results were confirmed by Western blot analysis (data not shown).; $n = 9$; ** $P < 0.01$ OC vs. YC and OR. BSA^{NT} = nitrosylated bovine serum albumin.

Figure 4

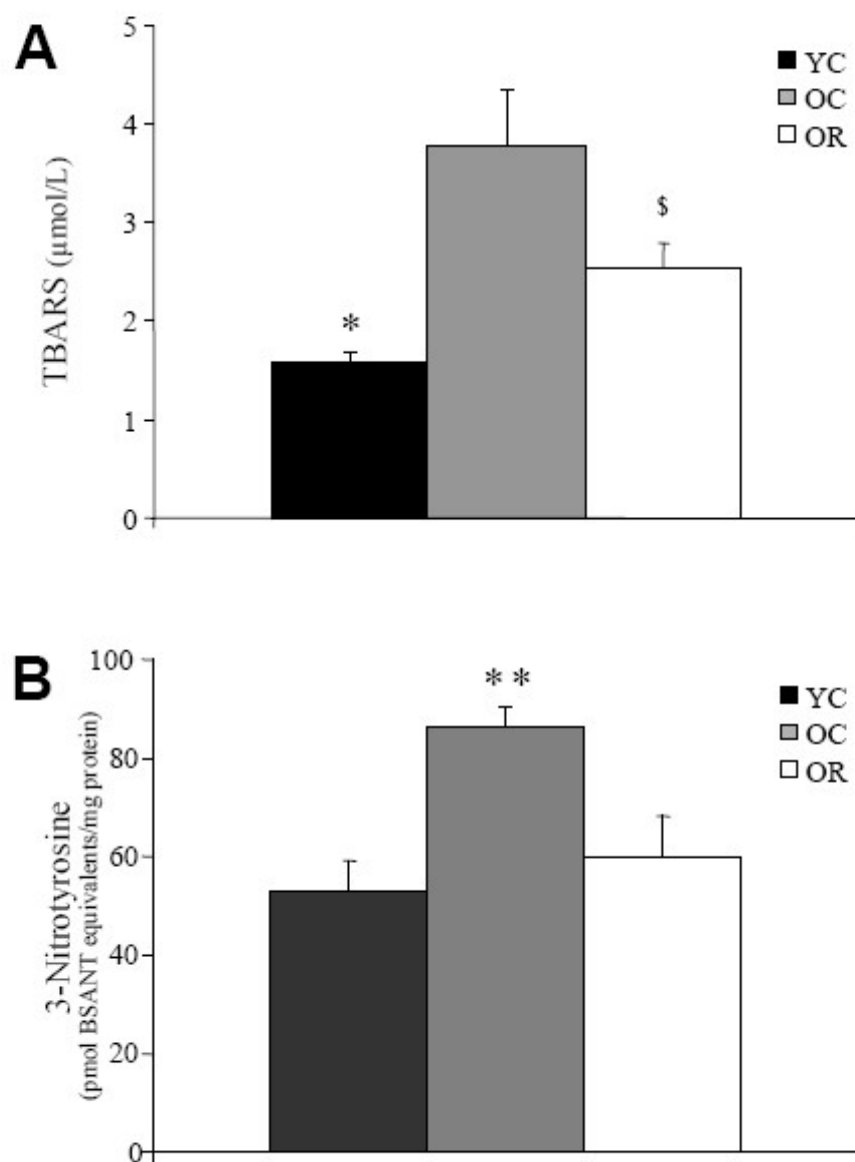


Fig 5. A: Top: MnSOD expression (25 kDa) in aortas from young (YC, lanes 1-3), old (OC, lanes 4-6) and old caloric-restricted rats (OR, lanes 7-9) as detected by Western blot analysis. Bottom: densitometric analysis of MnSOD Western blotting. * $P \leq 0.01$ YC vs. OC and OR. B: CuZnSOD expression (19 kDa) in aorta from YC, OC and OR. Top: representative Western blot; bottom: densitometric analysis. $^{\S}P < 0.05$ OR vs. OC. Data represent mean of 8 aortic rings from each experimental group. a.u.: arbitrary units. C: SOD activity in aortas from young, (YC) old (OC) and old caloric-restricted rats (OR). $n=10$. au.: arbitrary units.; * $P < 0.05$ YC vs. OC and OR. $^{\S}P < 0.01$ OR vs. OC.

Figure 5

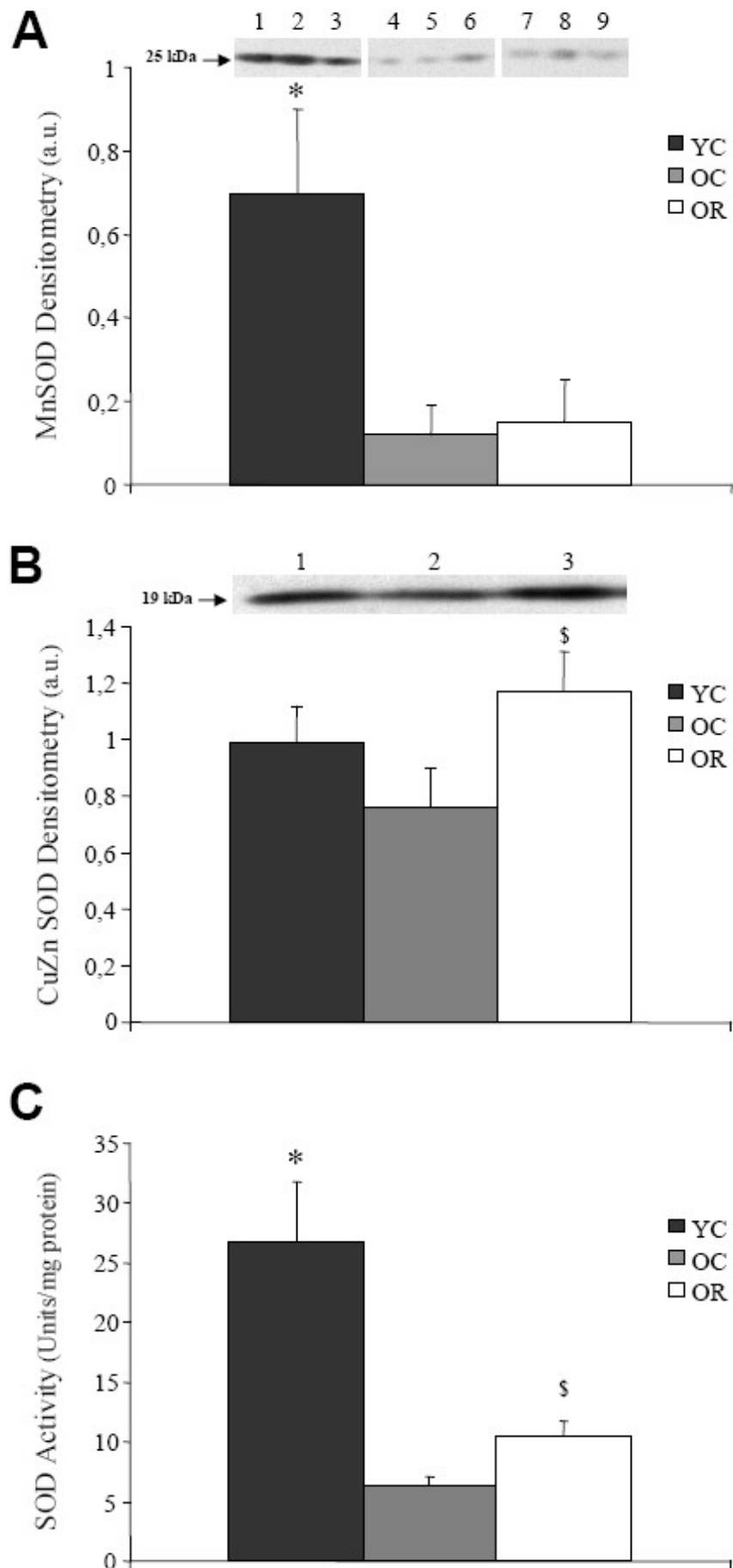


Table 1. Characteristics of the three experimental groups of animals after 3 weeks of treatment.

YC= Young Control; OC=Old Control; OR= Old Caloric-Restricted rats. *P<0.05.

	Young Control	Old Control	Old Caloric-Restricted
Final Body Weight (g)	398±6	404±5	368±5*
Blood Glucose (mg/dl)	139±4	131±5	132±5
Insulin (ng/ml)	6.7±1.3	5.7±0.9	3.4±0.3*
