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Caloric restriction induces H_2O_2 formation as a trigger of AMPK-eNOS-NO pathway in obese rats: Role for CAMKII

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ABSTRACT

Caloric restriction (CR) improves endothelial function through the upregulation of adenosine monophosphate-activated protein kinase (AMPK) and endothelial nitric oxide synthase (eNOS). Moreover, hydrogen peroxide (H_2O_2) is upregulated in yeast subjected to CR. Our aim was to assess if mild short-term CR increases vascular H_2O_2 formation as a link with AMPK and eNOS activation.

Twelve-week old Zucker obese (*fa/fa*) and control Zucker *lean* male rats were fed a standard chow either *ad libitum* (AL, n = 10) or with a 20% CR (CR, n = 10) for two weeks. CR significantly improved relaxation to ACh in *fa/fa* rats because of an enhanced endogenous production of H_2O_2 in aortic rings (H_2O_2 levels $_{fa/faAL} = 0.5 \pm 0.05$ nmol/mg *vs.* H_2O_2 levels $_{fa/faCR} = 0.76 \pm 0.07$ nmol/mg protein; $p < 0.05$). Expression of mitochondrial superoxide dismutase (Mn-SOD) and total SOD activity were increased in aorta from *fa/fa* animals after CR. In cultured aortic endothelial cells, serum deprivation or 2-deoxy-D-glucose induced a significant increase in: i) superoxide anion and H_2O_2 levels, ii) p-AMPK/AMPK and p-eNOS/eNOS expression and iii) nitric oxide levels. This effect was reduced by catalase and strongly inhibited by Ca^{2+}/calmodulin-dependent kinase II *(CamkII)* silencing.

In conclusion, we propose that mild short-term CR might be a trigger of mechanisms aimed at protecting the vascular wall by the increase of H_2O_2, which then activates AMPK and nitric oxide release, thus improving endothelium-dependent relaxation. In addition, we demonstrate that CAMKII plays a key role in mediating CR-induced AMPK activation through H_2O_2 increase.

1. Introduction

Caloric restriction (CR) is a promising approach in the prevention and/or reduction of cardiovascular disorders associated with obesity [1]. CR, defined as a reduction in *ad libitum* (AL) energy intake by 10–50% without malnutrition, is recognized to induce protective effects on longevity [2], metabolic disorders [1], and carcinogenesis [3] in a variety of species. Body weight reduction through CR in both overweight or obese rodents and humans reports numerous beneficial effects, including an enhancement of adiponectin serum levels [4], an improvement in serum lipid profile, insulin sensitivity and glucose homeostasis [1,5–11].

CR has also benefits on cardiovascular function by reducing blood pressure [10], atherosclerosis [12], and cardiac remodeling [13,14]. Underlying mechanisms for these effects include the improvement of endothelial function [10], upregulation of endothelial nitric oxide synthase (eNOS) [1,8,15,16], adenosine monophosphate-activated protein kinase (AMPK) [1,10,17] and Sirtuin 1 (Sirt1) [1,8,11] expression and activity in blood vessels. We have previously shown that a moderate and short-term CR in young Zucker *fa/fa* rats has cardiovascular benefits by both modulating endothelial function through AMPK-PI3K-Akt-eNOS activation and reducing risk factors, such as

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Abbreviations

2-DG	2-deoxy-D-glucose
AL	*ad libitum*
AMPK	adenosine monophosphate-activated protein kinase
AMTZ	3-aminotriazole
AT	adipose tissue
BAEC	bovine aortic endothelial cells
BW	body weight
CAMKII	Ca^{2+}/calmodulin-dependent kinase II
Comp C	Compound C
CR	caloric restriction
DAF-2 DA	4,5-diaminofluorescein diacetate
DAPI	4′,6-diamino-2-phenylindole
DCF	diacetate 2′,7′-dichlorofluorescein
DHE	dihydroethidium
Emax	maximum response
eNOS	endothelial nitric oxide synthase
H_2O_2	hydrogen peroxide
$LDLr^{-/}$	low-density lipoprotein receptor knockout mice
NO	nitric oxide
NOX	NADPH oxidase
O_2^-	superoxide anion
Phe	phenylephrine
Ach	acetylcholine

blood pressure, cardiac hypertrophy and plasma triglyceride levels [14]. CR also improves vascular function through a decrease in vascular oxidative stress as shown in type II diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats [18] or in models of aging [8].

Reactive oxygen species (ROS) play a critical role as second messengers by regulating physiological processes, including cell proliferation and immune response that ensure normal function of sub-cellular bioenergy systems [19–21]. One of the more stable ROS is hydrogen peroxide (H_2O_2) [19]. Special attention has been paid to H_2O_2 at the vascular level since it has been postulated as an endothelium-derived hyperpolarizing vasodilator in vascular smooth muscle [22,23]. H_2O_2 is formed by dismutation of superoxide anion ($O_2^{\cdot-}$) by the various superoxide dismutases (SOD) [24]. Although toxic effects might arise at non-physiological high levels of H_2O_2 [21,25], it is essential for normal cell function and signaling [19]. At the vascular wall, both endothelial and smooth muscle cells produce H_2O_2 [26,27], which is able to elicit relaxant effects in vessels through several endothelium-dependent and endothelium-independent mechanisms [28]. At endothelial level, H_2O_2 induces eNOS expression [29,30] and increases nitric oxide (NO)-induced relaxation of aortic rings [31,32].

Recent studies suggest an increase in H_2O_2 as the mechanism by which non-pharmacological approaches, such as aerobic exercise training, protects against endothelial dysfunction in mice [33]. Along the same line, it has been shown that CR delays aging and extends chronological lifespan in *Saccharomyces cerevisiae* by inducing an elevation of H_2O_2 levels [34]. This effect is mediated by an increase in Mn-SOD expression, the main scavenger of $O_2^{\cdot-}$ within the cell [24], thus inhibiting $O_2^{\cdot-}$ accumulation-mediated deleterious actions [34]. Since mechanisms related to CR are highly conserved in the evolutionary scale [35] and obesity is a cause of accelerated and premature vascular aging [36], we hypothesize that a mild and short-term CR induces H_2O_2 formation in blood vessels contributing to the beneficial effects of this dietary approach on endothelial function. We therefore tested whether a two-week 20% CR improves endothelial function in an early-stage model of obesity (young Zucker *fa/fa* rats) through the increase in vascular H_2O_2 production and clarified the involvement of the AMPK-eNOS pathway [10]. In addition, we aimed at elucidating the role of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) on H_2O_2 mediated effects, since it is an upstream kinase involved in AMPK activation [37] and might be activated by oxidants like H_2O_2 [38].

2. Materials and methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

2.1. Animals and dietary treatments

Eight-week old male Zucker lean (*lean*) and Zucker fatty *fa/fa* (*fa/fa*) rats were housed under controlled dark-light cycles (12h/12h from 8:00 a.m. to 8:00 p.m.) and temperature (22 °C) conditions with standard food and water *ad libitum*. Animals were housed individually for body weight (BW) and food intake monitoring for four weeks. Then, they were randomly divided into two groups and assigned either to *ad libitum* (AL, n = 10) or caloric restricted diet (CR; 80% of AL n = 10) for two additional weeks. Adjustment of 20% CR was done individually based on the previous food intake values [10]. Animals were checked daily to verify consumption of the fixed food allotment. On the last day, rats were weighed and euthanized by decapitation. Then, thoracic aortas were isolated and used for vascular function, western blotting and enzymatic activity determinations. This study was carried out in accordance with the recommendations of the Spanish Animal Care and Use Committee according to the EC Directive 86/609/EEC for animal experiments and was approved by the Ethical Committee of Universidad San Pablo-CEU (CEBA-CEU USP. Reference: PCD 050-1 V3/12) and Universidad Complutense de Madrid, Spain (reference: PROEX 413/15).

2.2. Vascular reactivity in the thoracic aorta

Vascular function was performed in the thoracic aorta as previously described [39]. Aortas were placed in Krebs-Henseleit solution (KH, 115 mM NaCl, 4.6 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.01 mM EDTA and 5.5 mM glucose), cleaned of blood and perivascular fat and cut in 2 mm-long rings, which were suspended on two intraluminal parallel wires and introduced in an organ bath containing KH at 37 °C and pH 7.4 [40]. Rings were given an optimal resting tension of 1.5 g, which was readjusted every 15 min during a 60 min equilibration period. Isometric tension was recorded in a Power Lab system (ADInstruments, Oxford, UK). Before starting the experiment, rings were contracted with 75 mM KCl to assess their contractility. Relaxation curves to acetylcholine (Ach, 10^{-9}-10^{-4} mol/L) were performed in segments pre-contracted with phenylephrine (Phe, 10^{-7} mol/L) (n = 10 rings, 5 animals) in absence or presence of catalase (1000 U/mL), which catalyzes H_2O_2 degradation. Catalase was incubated for 25 min before Phe addition. Contractions are expressed as the percentage of the contraction elicited by KCl 75 mM and relaxations are expressed as the percentage of a previous contraction to Phe. Emax values correspond to the maximal response to Ach.

2.3. Basal H_2O_2 levels measurement and catalase activity determination

Basal H_2O_2 levels and catalase activity determination in aortas from *lean* and *fa/fa* animals were assessed by Amplex Red Hydrogen Peroxide and Amplex Red Catalase Assay Kits (Invitrogen, USA), respectively. H_2O_2 levels were expressed as nmol/mg total protein content. Catalase activity was expressed as U/mg total protein content.

2.4. Western blot analysis

Western blot experiments were performed in aortas and bovine aortic endothelial cells (BAEC) as previously described [41]. Aortic and cell-lysates were loaded in Laemli buffer [50 mM Tris (pH 6.8), 10% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, and 2 mg/ml blue bromophenol] and size-separated in 10%–15% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using a transblot apparatus (Bio-Rad, Hercules, CA). For immunoblotting, membranes were blocked with 5% nonfat dried milk for 1 h. Primary antibodies against p-AMPKα (Thr172) and AMPKα (1:1000 dilution; Cell Signaling Technology, Massachusetts, USA), p-eNOS (Ser1177) (1:800 dilution; Cell Signaling Technology), eNOS (1:800 dilution; BD Transduction Laboratories, Lexington, UK), Mn-superoxide dismutase (Mn-SOD) (1:1000 dilution; Santa Cruz Biotechnology, Germany), $p47^{phox}$ and $p22^{phox}$ (1:500 dilution; Santa Cruz Biotechnology), xanthine oxidase (1:1000 dilution, Santa Cruz Biotechnology) and catalase (1:2000 dilution, Sigma-Aldrich, Spain) were applied overnight at 4 °C. After washing, appropriate secondary antibodies (anti-rabbit or anti-mouse IgG-peroxidase conjugated) were applied for 1 h at a 1:5000 dilution. Blots were washed, incubated in enhanced chemiluminescence reagents (ECL Prime, Amersham Bioscence, UK) and bands were detected by ChemiDoc XRS + Imaging System (Bio-Rad, California, USA). To prove equal loadings of samples, blots were re-incubated with β-actin antibody (1:5000 dilution; Sigma-Aldrich, Missouri, USA). Blots were quantified using Image Lab 3.0 software (Bio-Rad, USA). Values for p-AMPKα and p-eNOS were normalized with AMPKα and eNOS, respectively.

2.5. NADPH oxidase (NOX) and total superoxide dismutase (SOD) activities

Aortic NOX and SOD activities were determined as previously described [42]. NOX activity was measured by the lucigenin-enhanced chemiluminescence method. Samples were placed in potassium phosphate buffer containing 5 μM lucigenin. Chemiluminescence was then recorded every 30 s during 5 min (Sirius Luminometer, Berthold Detection Systems, Germany). Then, 100 μM NADPH was added and luminescence was recorded at the same conditions. After subtracting medium chemiluminescence value, NOX activity was calculated by the difference between the values obtained before and after addition of NADPH.

SOD activity was determined following the method described by Kuthan et al. [43]. Aortas were homogenized in 50 mM tris buffer containing 5 mM EDTA and centrifuged for 10 min at 10.000 rpm. Supernatants were mixed with working buffer at 37 °C (0.05 M, pH 7.0 potassium phosphate buffer and 0.1 mM EDTA), 0.5 mM xanthine solution and 0.1 M cytochrome c solution. Reaction was started by addition of 0.5 U/mL xanthine oxidase. The reduction of cytochrome c was followed recording the increase in the absorbance at 546 nm over 1 min in a Beckman DU 640 spectrophotomer. Results were interpolated in a calibration curve using commercial SOD as standard and expressed as specific activity (U SOD/mg protein).

2.6. Cell culture experiments and confocal microscopy

Bovine aortic endothelial cells (BAECs) were obtained from European collection, (ECACC; Sigma-Aldrich). BAECs were cultured in DMEM with 10% FBS, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL), maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and used between passages 3 and 6. For H_2O_2, $O_2^{-\cdot}$, or NO determination, BAECs were plated on glass cover slips. CR was mimicked by performing two experimental protocols: i) by serum deprivation for 16–20 h (-FBS) in medium supplemented with 0.1% BSA or ii) by addition of 2-deoxy-D-glucose (2-DG) for 10 min. Thereafter, the cells were incubated with specific fluorescent dyes for 30 min at 37 °C in humid atmosphere and 5% CO_2 in darkness. H_2O_2 and NO production were determined by using diacetate 2′,7′-dichlorofluorescein (DCF, 10^{-5} M; Molecular Probes, USA) and 4,5-diaminofluorescein diacetate (DAF-2 DA, 10^{-5} M; Sigma-Aldrich, USA), respectively. Dihidroethidium (DHE, Sigma-Aldrich, USA) was used for $O_2^{-\cdot}$ Assessment. Cells were fixed in 4% paraformaldehyde and nuclei were stained by incubation either with 4′,6-diamino-2-phenylindole (DAPI, 1 μg/ml; Molecular Probes, USA) or Hoechst 33342 (1:1.000; Invitrogen) for 15 min at room temperature in darkness. Cover slips with cells were mounted (FluoroGuard antifade®, Bio-Rad, USA) and examined under the confocal microscope. Images were captured with a LEICA SP5 confocal microscope (Leica Microsystems, Germany) using excitation 488 nm/emission 530 nm line to visualize DCF-DA, 488 nm/515 nm (DAF-2DA), 488 nm/590–620 nm (DHE) and 405 nm/410–475 nm (DAPI/Hoescht33342).

2.7. Calcium calmodulin dependent kinase II (CAMKII) gene silencing

BAECs were transfected with Dicer-Substrate siRNAs (DsiRNA) duplexes targeting bovine *CamkII*, previously designed under optimal characteristics as described by Elbashir et al. [44]. DsiRNA were designed by “DsiRNA design tool” (Integrated DNA technologies, IA). Two different duplexes were designed to increase the knockdown effectiveness (Duplex 1: 5′-GUACUUACACUACCAGAAGAUCATC-3′ and 5′-GAUGAUCUUCUGGUAGUGUAAGUACUC-3'; Duplex 2: 5′-AUGGUG UUUGAACUGGUCAACCAAG-3′ and 5′-CUUGGUUGACCAGUUCAAAC ACCAUGU-3′). Before siRNA transfection, BAECs were seeded at a density of 70.000 cells/well in 24-well plates in DMEM medium with FBS 10%. All transfections were performed using DsiRNA according to the manufacturer's recommendation. 24 h before transfection, cells were washed and incubated with DMEM without serum and antibiotics. For each well, siRNAs (50 nM) were diluted in 50 μL Opti-MEM (Life Technologies, CA), incubated 15 min at 25 °C and then added to a solution containing 3 μL lipofectamine RNAiMAX (Life Technologies, CA) diluted in 50 μL Opti-MEM. After 15 additional min at 25 °C, 100 μL of the mixture was added drop-wise to wells containing 400 μL Opti-MEM. After 6 h of transfection, medium was replaced by DMEM supplemented with FBS 2%. NO production was analyzed in both control and CAMKII silent cells, 48 h after transfection. As previously described, CR was mimicked by either serum deprivation for 16–20 h (-FBS) or by addition of 2-DG for 10 min. In a set of cells treated with 2-DG, the catalase inhibitor 3- aminotriazole (AMTZ, 10^{-2} M) was also used as a positive control. Positive controls for transfection were performed by using Block-iT Alexa Fluor Red (Life Technologies, CA). A Silencer Select Negative Control (4390843, Life Technologies, CA) was used. All samples were run in pentaplicate. Transfection efficacy was evaluated by qRT-PCR.

2.8. Quantitative real-time PCR

Total RNA was extracted using the Tri-Reagent protocol (Sigma,

MO). cDNA was then synthesized from 1 μg total RNA by using a high-capacity cDNA RT kit (Applied Biosystems, CA). Quantitative RT-PCR was performed by using designed primer pairs (Integrated DNA Technologies, IA) for *CamkII* (forward 5′-ACTGTACTGCTTTGTGTTT GGC-3'; reverse 3′-CCTGGCTCTTGATCTTACTGTGT-5′). A total of 10 ng of cDNA were used out and all samples were run in duplicate. Optimal annealing temperature was checked and set at 60 °C. SsoAdvanced Universal SYBR Green Supermix (Biorad, CA) was used for amplification according to the manufacturer's protocols in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA). Fold-expression values were normalized by the housekeeping gene *Actb* (forward 5′-CCCTCTGAACCCTAAGGCCAACCG-3'; reverse 3′- GTGGTGGTGAAGCT GTAGCCACGC-5′) and *Rn18s* (forward 5′- ACTCAACACGGGAAACC TCA-3'; reverse 3′- AATCGCTCCACCAACTAAGA-5′). The ΔΔC(T) method was used to determine relative expression levels. The expression of the control group was set as 1. All samples were run in duplicate.

2.9. Data analyses

All values are given as mean ± S.E.M. and *n* denotes the number of animals used in each experiment or independent values for *in vitro* experiments. The assignment of animals to different groups was randomized, with similar BW in both groups. Statistical significance was analysed by using either two-way ANOVA for cumulative-curve comparison, one-way ANOVA (for comparison of more than two groups) or Student's *t*-test for comparison of two groups (GraphPad Prism 5.0 Software, California, USA). A value of $p < 0.05$ was considered statistically significant.

3. Results

As already shown in previous studies of our group, CR significantly reduced BW gain in both lean and fa/fa rats [10].

3.1. CR increases endothelium-dependent relaxation by increasing endogenous H_2O_2 production in aorta of fa/fa animals

To assess the effect of CR on endothelial function, rings were pre-contracted with Phe (10^{-7} M) to assess concentration-dependent relaxations to ACh (10^{-9} to 10^{-4} M). There was no difference in the response to Phe 10^{-7} M between groups (*lean* AL = 87.7 ± 3.25%; *lean* CR = 88.35 ± 3.15%; *fa/fa* AL = 90.58 ± 2.37%; *fa/fa*

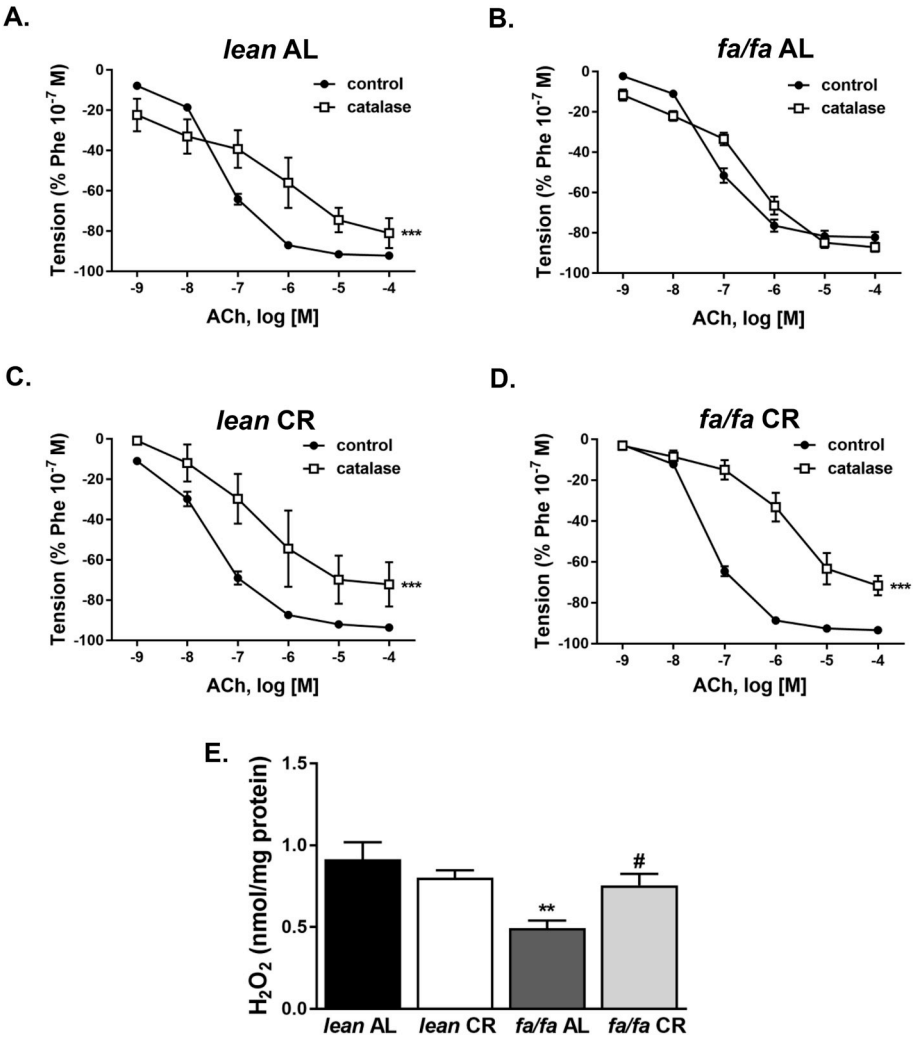


Fig. 1. Mild CR increases aortic relaxation and enhances endogenous H_2O_2 production in aorta of *fa/fa* animals. (A, D) Cumulative concentration-response curves to acetylcholine (ACh, 10^{-9}-10^{-4} M) in absence/presence of catalase (1000 U/ml). ***$p < 0.001$ compared to their corresponding matched control curves (two-way ANOVA; Newman-Keuls *post hoc* test; n = 5 rats/group). **(E)** Endogenous levels of H_2O_2 in aortas from *lean* and *fa/fa* animals expressed as nmol/total protein amount in mg. **$p < 0.01$ *vs lean* AL, #$p < 0.05$ *vs fa/fa* AL (one-way ANOVA, Newman-Keuls *post hoc* test; n = 5 rats/group). All data are means ± S.E.M.

CR = 92.28 ± 3.92%). As shown in a previous study of our group, relaxation to ACh was significantly lower in *fa/fa* AL animals compared to the *lean* AL group ($EMax_{fa/faAL}$ = 82.2 ± 2.6% *vs.* $EMax_{leanAL}$ = 92.1 ± 1%; two-way ANOVA, $p < 0.001$). CR enhanced this relaxation in *fa/fa* aortic rings to values similar to rings from *lean* animals AL ($EMax_{fa/faCR}$ = 93.3 ± 0.6% *vs.* $EMax_{leanAL}$ = 92.1 ± 1%) because of an increased NO availability [10]. No effect of CR was observed in the *lean* group. To test the involvement of H_2O_2 on the restoration of endothelial function after CR, cumulative concentration-response curves to ACh were performed in presence of catalase (n = 5; 1000 U/mL), which induces H_2O_2 degradation. Catalase significantly reduced relaxations to ACh in *lean* AL (Fig. 1A; two-way ANOVA, $p < 0.001$), but not in *fa/fa* AL rings (Fig. 1B). CR restored the inhibitory effect of catalase in *fa/fa* rings (Fig. 1C; two-way ANOVA, $p < 0.001$), whereas no effect was observed in the *lean* group (Fig. 1D). To confirm the increase in H_2O_2 in aorta in response to CR, direct measurements of H_2O_2 tissue levels were performed. Levels of H_2O_2 were lower in *fa/fa* AL compared to *lean* AL rings, but were significantly increased by CR up to *lean* AL values (Fig. 1E; one-way ANOVA, $p < 0.05$). No effect of CR was observed on H_2O_2 levels of *lean* rats.

3.2. CR increases SOD but not NADPH oxidase (NOX) activity in aorta from fa/fa rats

The mechanism leading to vascular H_2O_2 increase by CR was further analyzed. Aortic protein levels of $p22^{phox}$ and $p47^{phox}$ were not modified by CR (Fig. 2A and B). In addition, no difference between groups was found neither in NOX activity (Fig. 2C) nor in expression levels of xanthine oxidase (Fig. 2D). Mn-SOD expression, the mitochondrial isoform of SOD and main $O_2^{\cdot-}$ scavengers within the cell, was increased by CR although the effect was lower in the *fa/fa* group (Fig. 3A). Total aortic SOD activity (Fig. 3B) was markedly enhanced by CR in both *lean* and *fa/fa* animals (one-way ANOVA, $p < 0.01$ and $p < 0.05$, respectively). However, both catalase expression (Fig. 3C) and activity (Fig. 3D) were similar in both AL groups, and were not modified by CR.

3.3. Serum deprivation or 2-deoxy-D-glucose induce H_2O_2 and $O_2^{\cdot-}$ production in endothelial cells

Since CR increases H_2O_2 in yeast cultures [34], we used a cell culture approach to further corroborate the causal implication of CR on H_2O_2 increase, as well as the signaling mechanism. We used serum deprivation (-FBS, 16–20 h) or 2-DG (10^{-3} M, 10 min) to mimic CR in BAECs. 2-DG is an energy restriction mimetic leading to CR phenotypes in cells and rodents [45,46]. It is a glucose analog that cannot undergo further glycolysis competitively inhibiting the production of glucose-6-phosphate and reducing the amount of available energy [47]. On the other hand, serum restriction induces similar proteomic changes to 2-DG in cancer cells [48].

As shown in Fig. 4, both -FBS and 2-DG markedly increased DCF fluorescence. This increase was abolished in presence of catalase (1000 U/mL) demonstrating that both approaches increase H_2O_2 production in BAECs. To further analyze if the source of H_2O_2 increase was an enhancement in $O_2^{\cdot-}$ production, cells were incubated with DHE. As observed in Fig. 5, -FBS or 2-DG markedly increased DHE fluorescence in BAECs.

3.4. Serum deprivation or 2-deoxy-D-glucose induce NO production in endothelial cells

To confirm the stimulatory effect of CR on NO availability, the action of serum deprivation (-FBS) and 2-DG addition on DAF fluorescence was determined. Both approaches markedly increased DAF fluorescence, although the effect of 2-DG was more intense than -FBS (Fig. 6). As expected, DAF fluorescence was substantially reduced by incubation with catalase (1000 U/mL).

3.5. CR induces AMPK and NOS activation in endothelial cells

We have previously demonstrated that 20% CR for 2 weeks improves aortic relaxation through endothelial AMPK activation in this obesity model [10]. Our next step was, therefore, to analyze the possible link between H_2O_2 and AMPK on CR-induced NO increase.

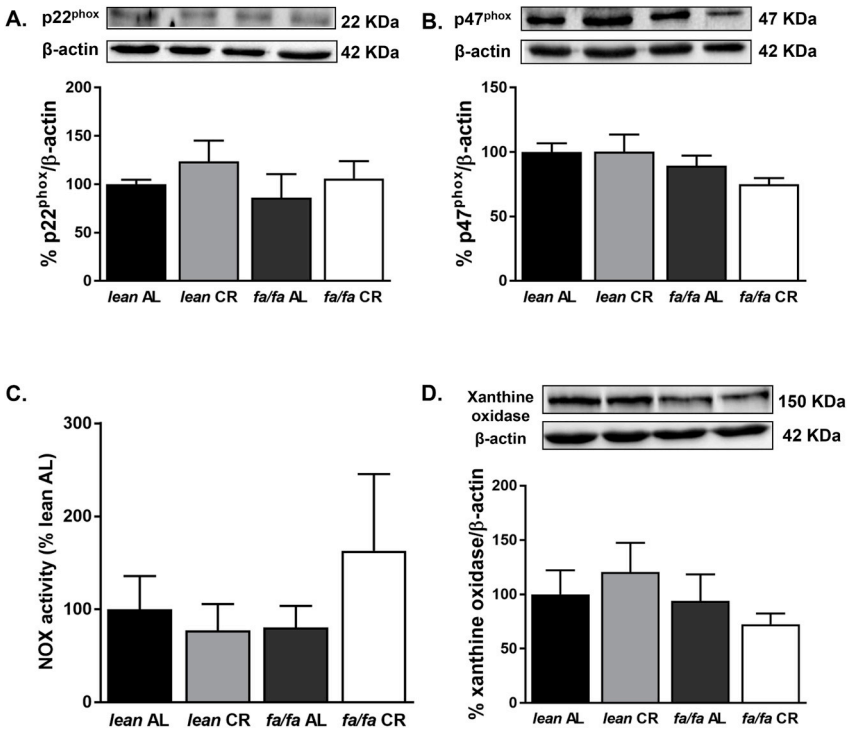


Fig. 2. CR does not change NADPH oxidase activity in aorta from *fa/fa* rats. **(A, B)** Representative immunoblots of NOX subunits $p22^{phox}$ **(A)** and $p47^{phox}$ **(B)** in aorta (upper panel) and densitometric analysis (lower panel) expressed as percentage of $p22^{phox}$/β-actin and $p47^{phox}$/β-actin, respectively, in *lean* AL group (n = 5). **(C)** NOX activity in aorta. Results are expressed as percentage compared to *lean* AL group (n = 5). **(D)** Representative immunoblots of xanthine oxidase in aorta (upper panel) and densitometric analysis (lower panel) expressed as percentage of xanthine oxidase/β-actin in the *lean* AL group. No statistical differences were detected (one-way ANOVA, Newman-Keuls *post hoc* test; n = 6 rats/group). All data are means ± S.E.M.

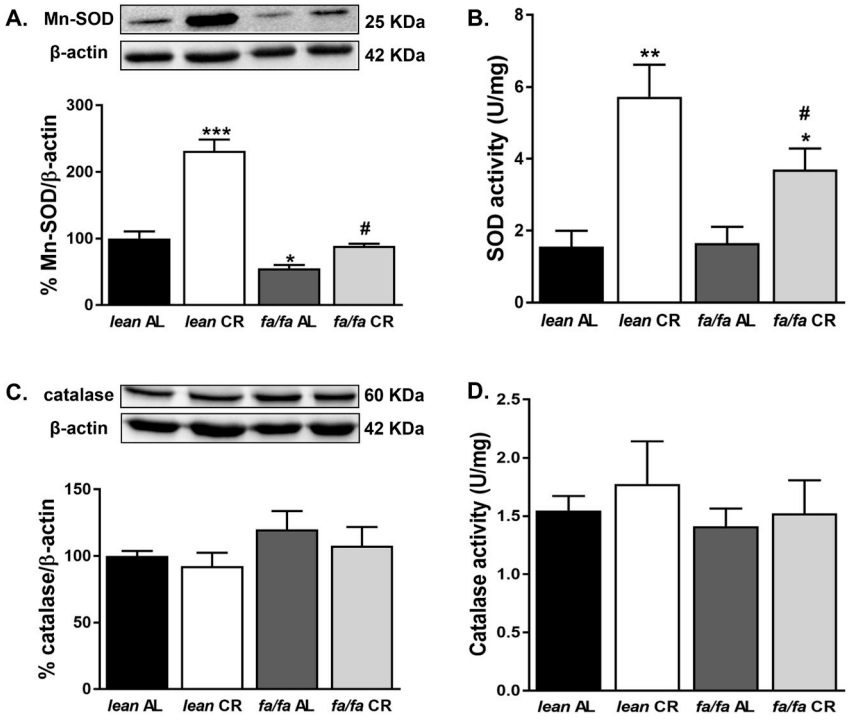


Fig. 3. CR increases superoxide dismutase activity in aorta from *fa/fa* rats. **(A)** Representative immunoblots of Mn superoxide dismutase (Mn-SOD) in aorta (upper panel) and densitometric analysis (lower panel) expressed as percentage of Mn-SOD/β-actin in *lean* AL group. **(B)** SOD activity in aorta. *p < 0.05, *p < 0,01 and ***p < 0,001 *vs lean* AL; #p < 0.05 *vs fa/fa* AL; (one-way ANOVA, Newman-Keuls *post hoc* test; n = 5). **(C)** Representative immunoblots of catalase in aorta (upper panel) and densitometric analysis (lower panel) expressed as percentage of catalase/β-actin in *lean* AL group (n = 6). **(D)** Catalase activity in aorta (n = 5). All data are means ± S.E.M.

Preincubation of BAECs with the AMPK inhibitor, compound C (comp C) completely abolished the increase of DAF fluorescence after 2-DG treatment (Fig. 7A). p-AMPK/AMPK expression (Fig. 7B) was significantly higher in BAECs after 2-DG treatment and increased by AMTZ, a catalase inhibitor that prevents H_2O_2 degradation. Similarly, incubation with exogenous H_2O_2 (10^{-4} M) significantly increased p-AMPK/AMPK levels. Pretreatment with catalase, to increase H_2O_2 degradation, reversed p-AMPK/AMPK levels to control values.

Similar results were observed on p-eNOS/eNOS levels (Fig. 7C), which were significantly higher after 2-DG, AMTZ or H_2O_2 stimulation, and reversed to control values with catalase. All these data suggest that mimicking CR stimulate $O_2^{\cdot-}$ and H_2O_2 formation leading to an increase in p-AMPK and p-eNOS, thus stimulating NO release.

3.6. CAMKII silencing strongly inhibits NO production in endothelial cells

To elucidate the upstream role of CAMKII in mediating the effects of H_2O_2, *CamkII* was silenced in BAECs by 65% (Supplemental Fig. 1). NO production was determined with DAF-2 DA in both control and *CamkII* silent cells. As shown in Fig. 8, *CamkII* silencing strongly inhibited NO production induced by 2-DG, serum deprivation (-FBS) and H_2O_2, thus demonstrating that CR-induced NO release in endothelial cells is modulated, at least in part by CAMKII.

4. Discussion

The novel finding of this study is that a mild short-term CR induces an increase of H_2O_2 levels in aortas from young Zucker obese *fa/fa* rats leading to an improvement of endothelial function through the activation of CAMKII-AMPK-eNOS-NO pathway. Since CR mediates life span extension in yeast and invertebrate models of aging by a moderate increase in ROS production, including H_2O_2 [20,21,34,49], we suggest that this mechanism may represent an evolutionary conserved process to trigger signaling pathways in which H_2O_2 functions as a protective second messenger [21,25].

To our knowledge, this is the first demonstration for a CR-induced H_2O_2 production in blood vessels. We confirmed increased H_2O_2 production by its direct measurement in aorta, as well as by indirect evidence based on functional experiments with and without catalase. Zucker *fa/fa* rats exhibit significantly lower levels of H_2O_2 in aorta, which are increased by CR to Zucker *lean* levels, thus enhancing ACh-evoked relaxation. A high-fat/low-carbohydrate diet similar to the Atkins diet improves microvessel endothelial function through the increase of a hyperpolarizing factor, which might be H_2O_2 [23,50]. In the same line, a recent study aimed to determine whether the impairment of ACh-evoked relaxation in aortas from $LDLr^{-/-}$ is prevented by aerobic exercise training. This report showed an increase in H_2O_2 contribution together with an enhancement in the inhibitory effect of catalase after the exercise [33]. In our study, the H_2O_2 contribution to endothelial-dependent relaxation was lost in *fa/fa* obese rats and restored after the CR. However, in the study of Guizoni et al. [33], H_2O_2 still plays an important role in ACh-induced relaxation in sedentary $LDLr^{-/-}$ mice, pointing out the difference between animal models used. The fact that reduced NO bioavailability is reversed after both CR [10] and exercise training [33] suggests a partly common mechanism for both non-pharmacological strategies.

Some studies have shown that CR decreases or does not modify H_2O_2 formation in a number of tissues from rodents [51,52]. These discrepancies might be due to differences in the severity and duration of the CR protocol, which in these studies reduces the daily intake by 40% for several months [51,52]. There is no agreement in the literature on CR protocol features (i.e. severity, duration, age of initiation or composition of the diet formulation) to exert its maximal benefits [53]. However, we believe that a mild 20% calorie reduction better resembles dietary restrictions in overweight/obese subjects without limiting its practicability in patients [54]. To note, here we already observe the benefits of CR after two weeks.

Mesquita et al. [34] mimic CR in yeast cultures by reduction of glucose levels from 2 to 0.5 and 0.05% in the culture medium. Here, CR was mimicked by both 2-DG treatment, a widely used CR mimetic,

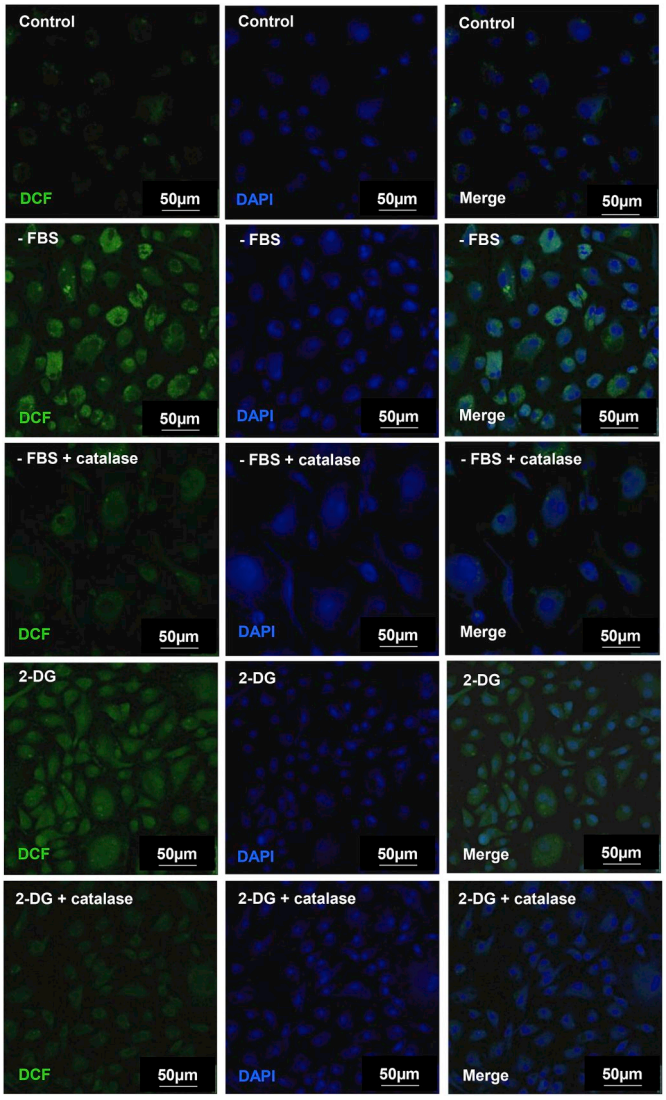


Fig. 4. Mimicking CR *in vitro* induces H_2O_2 production in endothelial cells. Representative confocal images of H_2O_2 fluorescent indicator diacetate 2′,7′-dichlorofluorescein (DCF, first column, green) and DAPI (second column, blue) in cultured endothelial cells in basal conditions (control), after fetal bovine serum depletion (-FBS) in absence or presence of catalase (1000U/ml) and after 2-deoxy-D-glucose (2-DG, 10^{-3} M) addition in absence or presence of catalase (1000 U/ml) (n = 3).

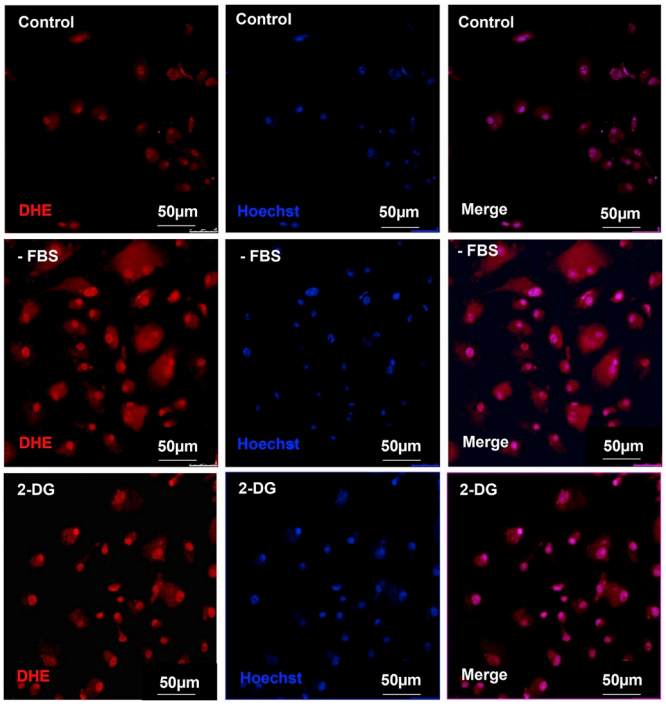


Fig. 5. Mimicking CR *in vitro* induces O2- production in endothelial cells. Representative confocal images of $O_2^{\cdot-}$ fluorescent indicator dihydroethidium (DHE, first column, red) and Hoechst (second column, blue) in cultured endothelial cells in basal conditions (control), after fetal bovine serum depletion (-FBS) and after 2-deoxy-D-glucose (2-DG, 10^{-3} M) addition (n = 3).

which decreases glycolysis [46,47] or by serum depletion, which deprives the cells from several growth factors and proteins mimicking 2-DG proteomic changes[49]. We show that both approaches are beneficial in terms of endothelial cell protection by increasing both H_2O_2 and NO levels in BAECs. Early studies have shown that a reduction in protein content leads to an increased life span in rodents [49,55,56], although little is known about the underlying mechanisms. Our results suggest that both H_2O_2 and NO might be upregulated in both caloric and protein restriction strategies, emphasizing the need of more studies to further investigate this fact.

The enhancement of H_2O_2 levels in aortic rings from *fa/fa* animals by CR might be related to an increase in (a) SOD activity and/or (b) $O_2^{\cdot-}$ production, and/or to (c) a decrease in catalase activity. Although catalase activity is upregulated by CR in adipose tissue from diet-induced obese mice [57] and aorta from aged rats [58], the latter possibility was excluded in our study since aortic catalase expression and activity were similar between groups. An upregulation of aortic Mn-SOD is involved in Sirt1-mediated effects of CR in mice subjected to a long-term (26 months) 40% caloric intake reduction [8] and in the reduction of age-associated oxidative stress in old mice (6 weeks under food intake restriction up to 3.2 g, defined as mild and short) [59]. Whereas some studies find no changes in total SOD activity after CR (40% for either 18 or 90 weeks [58]), this is increased in yeast [34] and in aortas from OLETF rats after severe (70%) and long (13 weeks) CR [18]. In accordance, we show that the *fa/fa* AL strain exhibited reduced Mn-SOD levels which were upregulated by CR together with an increase in total SOD activity in both lean and *fa/fa* animals. The increase in total SOD activity even in *lean* animals, where there is no vascular impairment, suggests that weight loss, white adipose tissue reduction or lower triglyceride levels, as observed in both groups after CR [10], might be involved in SOD regulation [57].

Effects of CR on vascular $O_2^{\cdot-}$ formation in the literature are contradictory. Moderate CR for 8 weeks markedly attenuates vascular $O_2^{\cdot-}$ overproduction in diet-induced obese mice [5]. However, vascular sources of $O_2^{\cdot-}$ (NOX and xanthine oxidase) were not significantly upregulated in the aorta of 12-week-old Zucker *fa/fa* animals, since increased $O_2^{\cdot-}$ levels in the vasculature of this strain are first observed at the age of 17 and 28 weeks [60–63]. At 12-weeks of age, we can conclude that CR increases $O_2^{\cdot-}$ dismutation to H_2O_2 through an upregulation of SOD without changes in $O_2^{\cdot-}$ formation. On the other hand, serum deprivation or 2-DG increased DHE staining, indicating an increased $O_2^{\cdot-}$ formation in BAECs which will be accordingly dismutated

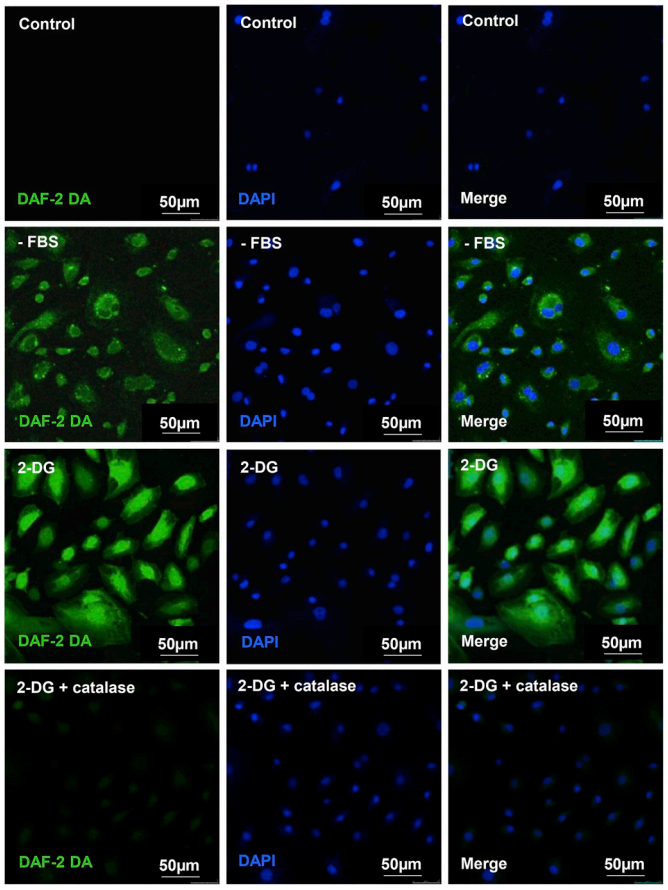


Fig. 6. Mimicking CR *in vitro* induces NO production in endothelial cells. Representative confocal images of NO fluorescent indicator 4,5-diaminofluorescein diacetate (DAF-2 DA, first column, green) and DAPI (second column, blue) in cultured endothelial cells in basal conditions (control), after fetal bovine serum depletion (-FBS) and after 2-deoxy-D-glucose (2-DG, 10^{-3} M) addition in absence or presence of catalase (1000 U/ml) (n = 3).

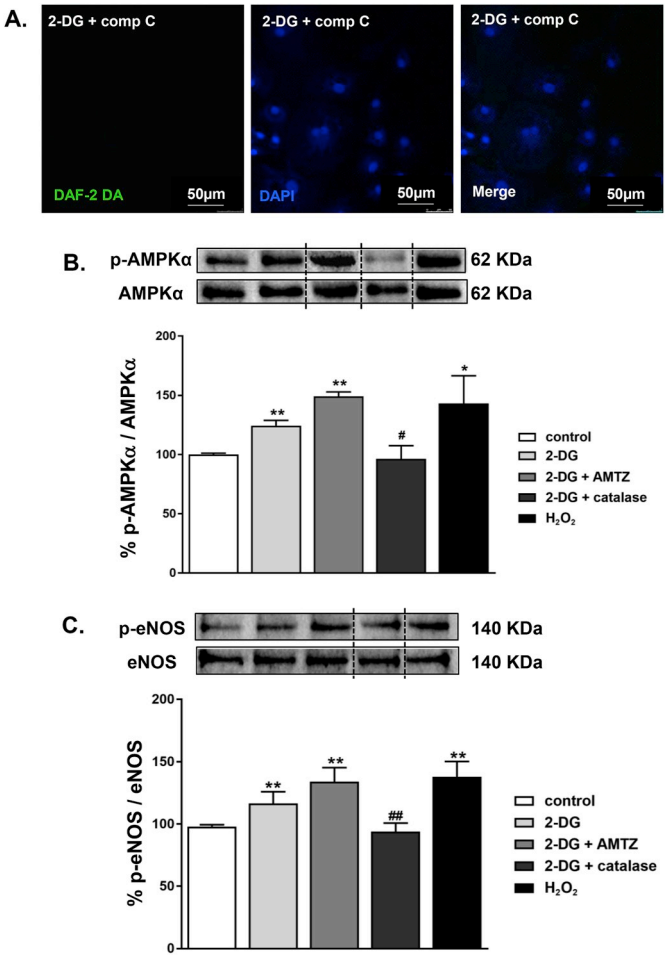


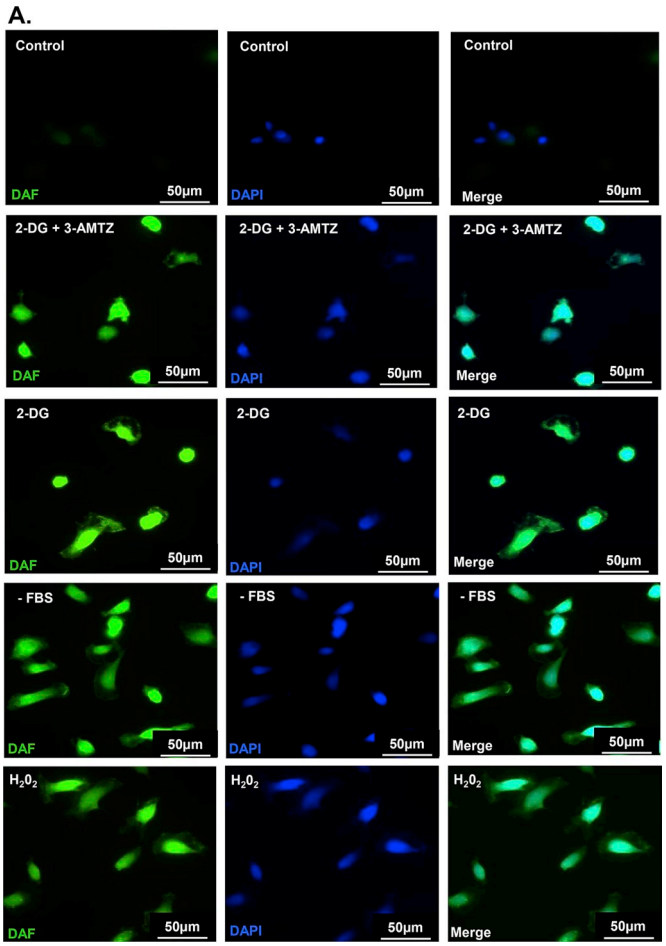
Fig. 7. Mimicking CR *in vitro* induces AMPK and NOS activation in endothelial cells. (A) Representative confocal images of NO fluorescent indicator 4,5-diaminofluorescein diacetate (DAF-2 DA, first column, green) and DAPI (second column, blue) in cultured endothelial cells after 2-DG (10^{-3} M) and AMPK inhibitor (compound C, 10^{-5} M, 10 min) addition (n = 3). **(B, C)** Representative immunoblots of p-AMPKα (Tyr^{172}) **(B)** and p-eNOS (Ser^{1177}) **(C)** in BAEC. Diagram bars show the result of densitometric analysis expressed as percentage of p-AMPKα/AMPKα and p-eNOS/eNOS, respectively, in the control group. *p < 0.05 and **p < 0,01 *vs* control; #p < 0.05 and ##p < 0,01 *vs*. 2-DG + AMTZ group (Student *t*-test; n = 5). All data are means ± S.E.M.

to H_2O_2 by SOD. This opens the question, whether upregulation of $O_2^{\cdot-}$ formation is an acute effect shortly observed after CR and lost at longer periods.

We previously demonstrated that CR restores impaired endothelial function and normalizes systolic blood pressure in Zucker *fa/fa* rats through the activation of endothelial AMPK and a subsequent activation of the PI3K-Akt-eNOS pathway [10]. The significant increase in DAF-2 DA fluorescence induced by serum deprivation and especially by 2-DG observed in the present study suggests that H_2O_2 activates the AMPK-NO pathway. This is confirmed by the increase in pAMPK/AMPK levels induced by the increase of H_2O_2 levels in BAECs (i.e. 2-DG plus AMTZ or exogenous addition of H_2O_2) or by the reduction in pAMPK/AMPK levels elicited by catalase. Similar results are observed for peNOS/eNOS, further substantiating a critical role of AMPK-eNOS-NO pathway activated by CR. Both glucose deprivation and 2-DG have been shown to activate PI3K-Akt due to the increase in H_2O_2 production by NOX4 in HepG2 cells [64]. This mechanism of cellular adaptive responses to changes in metabolic stress might be an upstream cascade for the observed AMPK activation. Furthermore, the Mn-SOD overexpression after CR in our model might point to an increase in the intracellular steady-state production of H_2O_2, which might be blocking the activation of cellular apoptotic processes [65] activating pro-survival signaling pathways mediated by p53, NF-κappaB, and activator protein AP-1 [19].

AMPK cascades are very sensitive to oxidative stress. In this direction, a study performed in NIH-3T3 cells have demonstrated that H_2O_2 induces the activation of AMPK though its phosphorylation at Thr^{172} [66]. Interestingly, CAMKII has been shown to be involved in AMPK activation [37] and to play a role in eNOS activation [67]. In addition, CAMKII plays also a critical upstream role in regulating H_2O_2-mediated effects on ERK1/2, PKB, and IGF-1R phosphorylation in vascular smooth muscle cells [68]. Consistent with these studies and since *CamkII* silencing significantly blunts H_2O_2-mediated NO production, we propose herein a role for CAMKII in modulating, at least in part, CR-induced effects of H_2O_2 on AMPK activation and consequent NO production and beneficial vascular effects.

In conclusion, we propose that mild short-term CR might be a trigger of mechanisms aimed at protecting the vascular wall. CR acutely



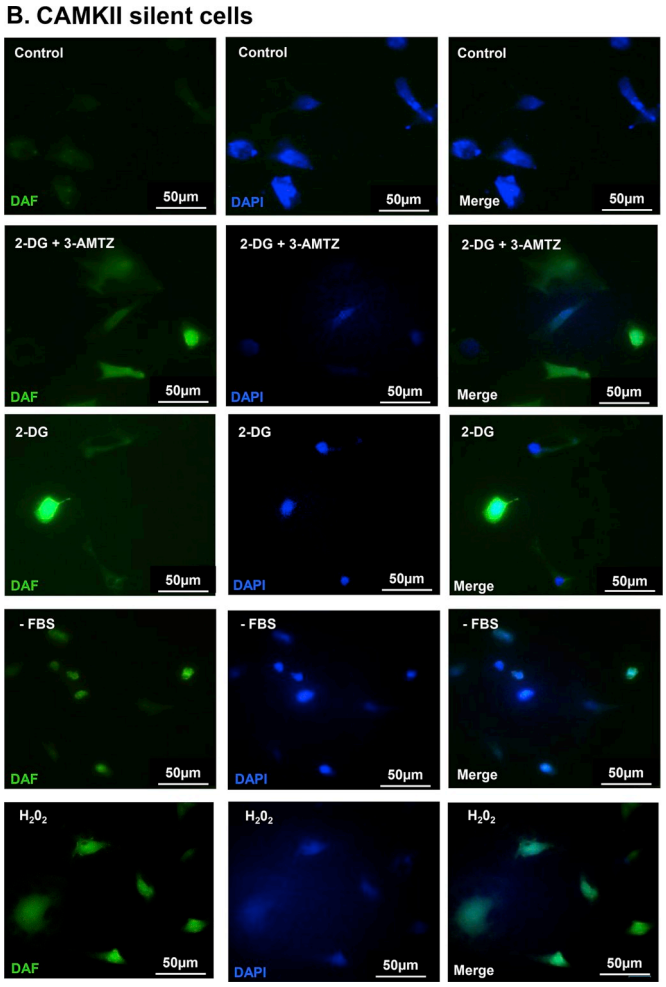


Fig. 8. CAMKII silencing strongly inhibits NO production in endothelial cells. Representative confocal images of NO fluorescent indicator (DAF-2 DA, first column, green) and DAPI (second column, blue) in cultured control **(A)** or CAMKII silent **(B)** endothelial cells in basal conditions (control), after 2-deoxy-D-glucose(2-DG, 10^{-3} M) in presence or absence of 3-AMTZ (10^{-2} M), after fetal bovine serum depletion (-FBS) and after H_2O_2 (10^{-4} M) addition (n = 3).

Fig. 8. (*continued*)

increases $O_2^{\cdot-}$ levels and SOD activity leading to an enhancement of H_2O_2, which then activates AMPK and NO release through CAMKII, thus improving endothelium-dependent relaxation. Although it is well established that excessive $O_2^{\cdot-}$ production from mitochondria is the driving, initial cellular response underlying metabolic complications [69], recent evidence shows that they are produced in a regulated manner and are essential signaling messengers in many cellular processes, i.e. immune response, cell proliferation and aging [20,69], exerting also protective effects at vascular level [70]. Moreover, results of antioxidant-based trials have been largely negative, supporting hormesis theories related to an adequate ROS balance and highlighting the dark-side of blunting ROS production. Efforts should be then focused on slightly reshape their production rather than their total elimination, and moderate CR seems to be a good candidate to achieve that aim.

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Disclosures

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2019.05.016.

References

[1] C.F. Garcia-Prieto, M.S. Fernandez-Alfonso, Caloric restriction as a strategy to improve vascular dysfunction in metabolic disorders, Nutrients 8 (6) (2016).
[2] Z.E. Gillespie, J. Pickering, C.H. Eskiw, Better living through chemistry: caloric restriction (CR) and CR mimetics alter genome function to promote increased health and lifespan, Front. Genet. 7 (2016) 142.
[3] G.S. Kopeina, V.V. Senichkin, B. Zhivotovsky, Caloric restriction - a promising anticancer approach: from molecular mechanisms to clinical trials, Biochim. Biophys. Acta 1867 (1) (2017) 29–41.
[4] K. Shinmura, K. Tamaki, K. Saito, Y. Nakano, T. Tobe, R. Bolli, Cardioprotective effects of short-term caloric restriction are mediated by adiponectin via activation of AMP-activated protein kinase, Circulation 116 (24) (2007) 2809–2817.
[5] J. Ketonen, T. Pilvi, E. Mervaala, Caloric restriction reverses high-fat diet-induced endothelial dysfunction and vascular superoxide production in C57Bl/6 mice, Heart Vessel. 25 (3) (2010) 254–262.
[6] S.T. Baker, G. Jerums, L.A. Prendergast, S. Panagiotopoulos, B.J. Strauss, J. Proietto, Less fat reduction per unit weight loss in type 2 diabetic compared with nondiabetic obese individuals completing a very-low-calorie diet program, Metabolism 61 (6) (2012) 873–882.
[7] J.N. Cooper, M.L. Columbus, K.J. Shields, J. Asubonteng, M.L. Meyer, K. Sutton-Tyrrell, B.H. Goodpaster, J.P. DeLany, J.M. Jakicic, E. Barinas-Mitchell, Effects of an intensive behavioral weight loss intervention consisting of caloric restriction with or without physical activity on common carotid artery remodeling in severely obese adults, Metabolism 61 (11) (2012) 1589–1597.
[8] A.J. Donato, A.E. Walker, K.A. Magerko, R.C. Bramwell, A.D. Black, G.D. Henson, B.R. Lawson, L.A. Lesniewski, D.R. Seals, Life-long caloric restriction reduces oxidative stress and preserves nitric oxide bioavailability and function in arteries of old mice, Aging Cell 12 (5) (2013) 772–783.
[9] N. Nordstrand, E. Gjevestad, J.K. Hertel, L.K. Johnson, E. Saltvedt, J. Roislien, J. Hjelmesaeth, Arterial stiffness, lifestyle intervention and a low-calorie diet in morbidly obese patients-a nonrandomized clinical trial, Obesity 21 (4) (2013) 690–697.
[10] C.F. Garcia-Prieto, H. Pulido-Olmo, G. Ruiz-Hurtado, M. Gil-Ortega, I. Aranguez, M.A. Rubio, M. Ruiz-Gayo, B. Somoza, M.S. Fernandez-Alfonso, Mild caloric restriction reduces blood pressure and activates endothelial AMPK-PI3K-Akt-eNOS pathway in obese Zucker rats, Vasc. Pharmacol. 65–66 (2015) 3–12.
[11] T. Yamamoto, K. Tamaki, K. Shirakawa, K. Ito, X. Yan, Y. Katsumata, A. Anzai, T. Matsuhashi, J. Endo, T. Inaba, K. Tsubota, M. Sano, K. Fukuda, K. Shinmura, Cardiac Sirt1 mediates the cardioprotective effect of caloric restriction by suppressing local complement system activation after ischemia-reperfusion, Am. J. Physiol. Heart Circ. Physiol. 310 (8) (2016) H1003–H1014.
[12] Z. Guo, F. Mitchell-Raymundo, H. Yang, Y. Ikeno, J. Nelson, V. Diaz, A. Richardson, R. Reddick, Dietary restriction reduces atherosclerosis and oxidative stress in the aorta of apolipoprotein E-deficient mice, Mech. Ageing Dev. 123 (8) (2002) 1121–1131.
[13] M. Takatsu, C. Nakashima, K. Takahashi, T. Murase, T. Hattori, H. Ito, T. Murohara, K. Nagata, Calorie restriction attenuates cardiac remodeling and diastolic dysfunction in a rat model of metabolic syndrome, Hypertension 62 (5) (2013) 957–965.
[14] G. Ruiz-Hurtado, C.F. Garcia-Prieto, H. Pulido-Olmo, J.P. Velasco-Martin, P. Villa-Valverde, M.E. Fernandez-Valle, L. Bosca, M. Fernandez-Velasco, J. Regadera, B. Somoza, M.S. Fernandez-Alfonso, Mild and short-term caloric restriction prevents obesity-induced cardiomyopathy in young zucker rats without changing in metabolites and fatty acids cardiac profile, Front. Physiol. 8 (2017) 42.
[15] M. Zanetti, R. Barazzoni, M. Vadori, M. Stebel, G. Biolo, G. Guarnieri, Lack of direct effect of moderate hyperleptinemia to improve endothelial function in lean rat aorta: role of calorie restriction, Atherosclerosis 175 (2) (2004) 253–259.
[16] E. Ozbek, A. Simsek, M. Ozbek, A. Somay, Caloric restriction increases internal iliac artery and penil nitric oxide synthase expression in rat: comparison of aged and adult rats, Arch. Ital. Urol. Androl. 85 (3) (2013) 113–117.
[17] V.W. Dolinsky, J.S. Morton, T. Oka, I. Robillard-Frayne, M. Bagdan, G.D. Lopaschuk, C. Des Rosiers, K. Walsh, S.T. Davidge, J.R. Dyck, Calorie restriction prevents hypertension and cardiac hypertrophy in the spontaneously hypertensive rat, Hypertension 56 (3) (2010) 412–421.
[18] Y. Minamiyama, Y. Bito, S. Takemura, Y. Takahashi, S. Kodai, S. Mizuguchi, Y. Nishikawa, S. Suehiro, S. Okada, Calorie restriction improves cardiovascular risk factors via reduction of mitochondrial reactive oxygen species in type II diabetic rats, J. Pharmacol. Exp. Ther. 320 (2) (2007) 535–543.
[19] G. Groeger, C. Quiney, T.G. Cotter, Hydrogen peroxide as a cell-survival signaling molecule, Antioxidants Redox Signal. 11 (11) (2009) 2655–2671.
[20] V.M. Labunskyy, V.N. Gladyshev, Role of reactive oxygen species-mediated signaling in aging, Antioxidants Redox Signal. 19 (12) (2013) 1362–1372.
[21] M. Molin, A.B. Demir, Linking peroxiredoxin and vacuolar-atpase functions in calorie restriction-mediated life span extension, Int J Cell Biol 2014 (2014) 913071.
[22] T. Matoba, H. Shimokawa, M. Nakashima, Y. Hirakawa, Y. Mukai, K. Hirano, H. Kanaide, A. Takeshita, Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice, J. Clin. Investig. 106 (12) (2000) 1521–1530.
[23] H.C. Hercule, W.H. Schunck, V. Gross, J. Seringer, F.P. Leung, S.M. Weldon, A. da Costa Goncalves, Y. Huang, F.C. Luft, M. Gollasch, Interaction between P450 eicosanoids and nitric oxide in the control of arterial tone in mice, Arterioscler. Thromb. Vasc. Biol. 29 (1) (2009) 54–60.
[24] F.M. Faraci, S.P. Didion, Vascular protection: superoxide dismutase isoforms in the vessel wall, Arterioscler. Thromb. Vasc. Biol. 24 (8) (2004) 1367–1373.
[25] A.W. Linnane, M. Kios, L. Vitetta, The essential requirement for superoxide radical and nitric oxide formation for normal physiological function and healthy aging, Mitochondrion 7 (1–2) (2007) 1–5.
[26] W. Droge, Free radicals in the physiological control of cell function, Physiol. Rev. 82 (1) (2002) 47–95.
[27] T.M. Paravicini, C.G. Sobey, Cerebral vascular effects of reactive oxygen species: recent evidence for a role of NADPH-oxidase, Clin. Exp. Pharmacol. Physiol. 30 (11) (2003) 855–859.
[28] J. Gil-Longo, C. González-Vázquez, Characterization of four different effects elicited by H2O2 in rat aorta, Vasc. Pharmacol. 43 (2) (2005) 128–138.
[29] G.R. Drummond, H. Cai, M.E. Davis, S. Ramasamy, D.G. Harrison, Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide, Circ. Res. 86 (3) (2000) 347–354.
[30] S. Thomas, S. Kotamraju, J. Zielonka, D.R. Harder, B. Kalyanaraman, Hydrogen peroxide induces nitric oxide and proteosome activity in endothelial cells: a bell-shaped signaling response, Free Radic. Biol. Med. 42 (7) (2007) 1049–1061.
[31] A. Zembowicz, R.J. Hatchett, A.M. Jakubowski, R.J. Gryglewski, Involvement of nitric oxide in the endothelium-dependent relaxation induced by hydrogen peroxide in the rabbit aorta, Br. J. Pharmacol. 110 (1) (1993) 151–158.
[32] V.A. Barbosa, T.F. Luciano, S.O. Marques, M.F. Vitto, D.R. Souza, L.A. Silva, J.P. Santos, J.C. Moreira, F. Dal-Pizzol, F.S. Lira, R.A. Pinho, C.T. De Souza, Acute exercise induce endothelial nitric oxide synthase phosphorylation via Akt and AMP-activated protein kinase in aorta of rats: role of reactive oxygen species, Int. J. Cardiol. 167 (6) (2013) 2983–2988.
[33] D.M. Guizoni, G.G. Dorighello, H.C. Oliveira, M.A. Delbin, M.H. Krieger, A.P. Davel, Aerobic exercise training protects against endothelial dysfunction by increasing nitric oxide and hydrogen peroxide production in LDL receptor-deficient mice, J. Transl. Med. 14 (1) (2016) 213.
[34] A. Mesquita, M. Weinberger, A. Silva, B. Sampaio-Marques, B. Almeida, C. Leão, V. Costa, F. Rodrigues, W.C. Burhans, P. Ludovico, Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H2O2 and superoxide dismutase activity, Proc. Natl. Acad. Sci. U. S. A. 107 (34) (2010) 15123–15128.
[35] Z. Ungvari, C. Parrado-Fernandez, A. Csiszar, R. de Cabo, Mechanisms underlying caloric restriction and lifespan regulation: implications for vascular aging, Circ. Res. 102 (5) (2008) 519–528.
[36] M. Barton, O. Baretella, M.R. Meyer, Obesity and risk of vascular disease: importance of endothelium-dependent vasoconstriction, Br. J. Pharmacol. 165 (3) (2012) 591–602.
[37] X. Zhu, K. Wang, F. Zhou, L. Zhu, Paeoniflorin attenuates atRAL-induced oxidative stress, mitochondrial dysfunction and endoplasmic reticulum stress in retinal pigment epithelial cells via triggering Ca(2+)/CaMKII-dependent activation of AMPK, Arch Pharm. Res. (Seoul) 41 (10) (2018) 1009–1018.
[38] J. Palomeque, O.V. Rueda, L. Sapia, C.A. Valverde, M. Salas, M.V. Petroff, A. Mattiazzi, Angiotensin II-induced oxidative stress resets the Ca2+ dependence of Ca2+-calmodulin protein kinase II and promotes a death pathway conserved across different species, Circ. Res. 105 (12) (2009) 1204–1212.
[39] C. Steireif, C.F. Garcia-Prieto, G. Ruiz-Hurtado, H. Pulido-Olmo, I. Aranguez, M. Gil-Ortega, B. Somoza, G. Schonfelder, A. Schulz, M.S. Fernandez-Alfonso, R. Kreutz, Dissecting the genetic predisposition to albuminuria and endothelial dysfunction in a genetic rat model, J. Hypertens. 31 (11) (2013) 2203–2212 discussion 2212.
[40] K.C. Nielsen, C. Owman, Contractile response and amine receptor mechanisms in isolated middle cerebral artery of the cat, Brain Res. 27 (1) (1971) 33–42.
[41] B. Somoza, R. Guzman, V. Cano, B. Merino, P. Ramos, C. Diez-Fernandez, M.S. Fernandez-Alfonso, M. Ruiz-Gayo, Induction of cardiac uncoupling protein-2 expression and adenosine 5'-monophosphate-activated protein kinase phosphorylation during early states of diet-induced obesity in mice, Endocrinology 148 (3) (2007) 924–931.
[42] H. Pulido-Olmo, C.F. Garcia-Prieto, G. Alvarez-Llamas, M.G. Barderas, F. Vivanco, I. Aranguez, B. Somoza, J. Segura, R. Kreutz, M.S. Fernandez-Alfonso, L.M. Ruilope, G. Ruiz-Hurtado, Role of matrix metalloproteinase-9 in chronic kidney disease: a new biomarker of resistant albuminuria, Clin. Sci. 130 (7) (2016) 525–538.
[43] H. Kuthan, H.J. Haussmann, J. Werringloer, A spectrophotometric assay for superoxide dismutase activities in crude tissue fractions, Biochem. J. 237 (1) (1986) 175–180.
[44] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (6836) (2001) 494.
[45] K. Hu, Y. Yang, L. Lin, Q. Ai, J. Dai, K. Fan, P. Ge, R. Jiang, J. Wan, L. Zhang, Caloric restriction mimetic 2-deoxyglucose alleviated inflammatory lung injury via suppressing nuclear pyruvate kinase M2-signal transducer and activator of transcription 3 pathway, Front. Immunol. 9 (2018) 426.
[46] D.K. Ingram, M. Zhu, J. Mamczarz, S. Zou, M.A. Lane, G.S. Roth, R. deCabo, Calorie restriction mimetics: an emerging research field, Aging Cell 5 (2) (2006) 97–108.
[47] A.N. Wick, D.R. Drury, H.I. Nakada, J.B. Wolfe, Localization of the primary metabolic block produced by 2-deoxyglucose, J. Biol. Chem. 224 (2) (1957) 963–969.
[48] M.M. Schroll, X. Liu, S.K. Herzog, S.B. Skube, A.B. Hummon, Nutrient restriction of glucose or serum results in similar proteomic expression changes in 3D colon cancer cell cultures, Nutr. Res. 36 (10) (2016) 1068–1080.
[49] M. Ristow, S. Schmeisser, Extending life span by increasing oxidative stress, Free Radic. Biol. Med. 51 (2) (2011) 327–336.
[50] M. Focardi, A. Picchi, S. Donnini, M. Cameli, M. Ziche, M. Marzilli, S. Mondillo,

Hydrogen peroxide mediates endothelium-dependent dilation of coronary arterioles in obese rats on a low-carbohydrate diet, Microcirculation 20 (7) (2013) 599–608.
[51] J.J. Ramsey, K. Hagopian, T.M. Kenny, E.K. Koomson, L. Bevilacqua, R. Weindruch, M.E. Harper, Proton leak and hydrogen peroxide production in liver mitochondria from energy-restricted rats, Am. J. Physiol. Endocrinol. Metab. 286 (1) (2004) E31–E40.
[52] B. Colom, J. Oliver, P. Roca, F.J. Garcia-Palmer, Caloric restriction and gender modulate cardiac muscle mitochondrial H2O2 production and oxidative damage, Cardiovasc. Res. 74 (3) (2007) 456–465.
[53] L.T. Robertson, J.R. Mitchell, Benefits of short-term dietary restriction in mammals, Exp. Gerontol. 48 (10) (2013) 1043–1048.
[54] J.R. Speakman, S.E. Mitchell, Caloric restriction, Mol. Asp. Med. 32 (3) (2011) 159–221.
[55] G. Fernandes, E.J. Yunis, R.A. Good, Influence of diet on survival of mice, Proc. Natl. Acad. Sci. U. S. A. 73 (4) (1976) 1279–1283.
[56] G. Stoltzner, Effects of life-long dietary protein restriction on mortality, growth, organ weights, blood counts, liver aldolase and kidney catalase in Balb/C mice, Growth 41 (4) (1977) 337–348.
[57] H.R. Lijnen, M. Van Hul, B. Hemmeryckx, Caloric restriction improves coagulation and inflammation profile in obese mice, Thromb. Res. 129 (1) (2012) 74–79.
[58] J.W. Kim, Y. Zou, S. Yoon, J.H. Lee, Y.K. Kim, B.P. Yu, H.Y. Chung, Vascular aging: molecular modulation of the prostanoid cascade by calorie restriction, J Gerontol A Biol Sci Med Sci 59 (9) (2004) B876–B885.
[59] C. Rippe, L. Lesniewski, M. Connell, T. LaRocca, A. Donato, D. Seals, Short-term calorie restriction reverses vascular endothelial dysfunction in old mice by increasing nitric oxide and reducing oxidative stress, Aging Cell 9 (3) (2010) 304–312.
[60] J.K. Folkmann, L.K. Vesterdal, M. Sheykhzade, S. Loft, P. Moller, Endothelial dysfunction in normal and prediabetic rats with metabolic syndrome exposed by oral gavage to carbon black nanoparticles, Toxicol. Sci. 129 (1) (2012) 98–107.
[61] M.L. Justo, C. Claro, E. Vila, M.D. Herrera, R. Rodriguez-Rodriguez, Microvascular disorders in obese Zucker rats are restored by a rice bran diet, Nutr. Metabol. Cardiovasc. Dis. 24 (5) (2014) 524–531.
[62] S.A. Phillips, F.A. Sylvester, J.C. Frisbee, Oxidant stress and constrictor reactivity impair cerebral artery dilation in obese Zucker rats, Am. J. Physiol. Regul. Integr. Comp. Physiol. 288 (2) (2005) R522–R530.
[63] N. Tajbakhsh, E.M. Sokoya, Sirtuin 1 is upregulated in young obese Zucker rat cerebral arteries, Eur. J. Pharmacol. 721 (1–3) (2013) 43–48.
[64] S. Owada, Y. Shimoda, K. Tsuchihara, H. Esumi, Critical role of H2O2 generated by NOX4 during cellular response under glucose deprivation, PLoS One 8 (3) (2013) e56628.
[65] J. Dasgupta, S. Subbaram, K.M. Connor, A.M. Rodriguez, O. Tirosh, J.S. Beckman, D. Jourd'Heuil, J.A. Melendez, Manganese superoxide dismutase protects from TNF-alpha-induced apoptosis by increasing the steady-state production of H2O2, Antioxidants Redox Signal. 8 (7–8) (2006) 1295–1305.
[66] S.L. Choi, S.J. Kim, K.T. Lee, J. Kim, J. Mu, M.J. Birnbaum, S. Soo Kim, J. Ha, The regulation of AMP-activated protein kinase by H(2)O(2), Biochem. Biophys. Res. Commun. 287 (1) (2001) 92–97.
[67] S. Murthy, O.M. Koval, J.M. Ramiro Diaz, S. Kumar, D. Nuno, J.A. Scott, C. Allamargot, L.J. Zhu, K. Broadhurst, V. Santhana, W.J. Kutschke, K. Irani, K.G. Lamping, I.M. Grumbach, Endothelial CaMKII as a regulator of eNOS activity and NO-mediated vasoreactivity, PLoS One 12 (10) (2017) e0186311.
[68] A. Bouallegue, N.R. Pandey, A.K. Srivastava, CaMKII knockdown attenuates H2O2-induced phosphorylation of ERK1/2, PKB/Akt, and IGF-1R in vascular smooth muscle cells, Free Radical Biol. Med. 47 (6) (2009) 858–866.
[69] K. Sharma, Mitochondrial hormesis and diabetic complications, Diabetes 64 (3) (2015) 663–672.
[70] S.P. Gray, E. Di Marco, K. Kennedy, P. Chew, J. Okabe, A. El-Osta, A.C. Calkin, E.A. Biessen, R.M. Touyz, M.E. Cooper, H.H. Schmidt, K.A. Jandeleit-Dahm, Reactive oxygen species can provide atheroprotection via NOX4-dependent inhibition of inflammation and vascular remodeling, Arterioscler. Thromb. Vasc. Biol. 36 (2) (2016) 295–307.