Gender-specific effects of caloric restriction on the balance of vascular nitric oxide and superoxide radical

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Aims
Caloric restriction (CR) and female gender attenuate oxidative damage and improve vascular endothelium-dependent relaxation (EDR). Multiple mechanisms that ameliorate vascular O2²⁻ could enhance the NO*/O2²⁻ balance and thus improve EDR. The aim of this study is to compare the effects of short-term (2 weeks) CR and gender on molecular mechanisms involved in NO*/O2²⁻ balance and EDR.

Methods and results
Wistar rats (8 weeks old) of both genders were fed ad libitum (control) or were subjected to CR (60% of food intake of controls) for 2 weeks. Plasma levels of NO*, insulin, and ghrelin, EDR, vascular NO* and O2²⁻ production, as well as endothelial NO* synthase (eNOS) and NADPH oxidase (Nox) expression were examined and analysed. CR improved EDR and vascular NO* levels and ameliorated NADPH-sensitive O2²⁻ production in male rats more than in females. Both CR and female gender reduced mRNA expression of Nox1 and Nox p22phox only in males. Protein expression studies showed that CR enhanced eNOS and reduced Nox4 only in males.

Conclusion
Short-term CR improved the NO*/O2²⁻ balance by lowering vascular O2²⁻ production through decreased expression of Nox in males, thus enhancing bioactive NO* levels and EDR. In this regard, CR shifted the state of vascular NO*/O2²⁻ balance in males to a state similar to that in females.

Keywords
Caloric restriction · Gender · Endothelial function · Nitric oxide · NADPH oxidase

1. Introduction
Caloric restriction (CR) can prevent or delay ageing-associated diseases and attenuate oxidative damage in various tissues and organs.¹⁻³ In the vasculature, a major effect of oxidative stress is the reaction between nitric oxide (NO*) and excessive superoxide (O2²⁻), which results in a reduction in the bioavailability of NO*.⁴ In addition, short-term (1–2 weeks) CR has been shown to improve endothelium-dependent relaxation (EDR) in healthy rats² and obese patients,⁵ suggesting that CR increases bioactive NO* within the vasculature. Recent findings suggest that NADPH oxidases (Nox) are important source of O2²⁻ production, which reduces NO* levels in the vasculature including rat aortas.⁶ These enzymes are membrane bound and generate O2²⁻ by transferring electrons from NADPH to molecular oxygen through a flavin-containing ‘Nox’ cata-lytic subunit.⁸ Although it has been suggested that CR reduces O2²⁻ production in endothelial cells from aortic tissues of aged rats,⁹ short-term (2 weeks) CR also decreases muscle mitochondrial H2O2 production in young rats.¹⁰ However, it is not known whether short-term CR augments vascular bioactive NO* by reducing O2²⁻ production via Nox.

In addition, CR exhibits greater effects on physical growth¹¹ and lifespan¹² in males than in females. On the other hand, there is a greater incidence of cardiovascular disease in men and post-menopausal women when compared with premenopausal women suggesting that oestrogen may provide vascular protective

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Ovariectomy (OVX), OVX + transition to the 40% CR. They were then maintained at a daily 40% of food on the first day and 20% on the second day for a smooth measurement every 2 days and then averaged to determine daily food restriction from the food intake level of the control animals until the end of the 2-week experiment, all groups of animals were fasted for 8 h and then sacrificed using sodium pentobarbital (50 mg/kg intraperitoneal). Before thoracic aortas were removed, blood samples were collected aorta (Ach; 30 μM) for functional assessment of endothelial integrity. EDR was evaluated with cumulative concentrations of Ach (10⁻⁹–3 × 10⁻⁵ mol/L) in aortic rings pre-contracted with PE (1 μmol/L). Endothelium-independent relaxation was determined by analysing the relaxant effects of sodium nitroprusside (SNP, 10⁻⁹–10⁻⁶ mol/L) in pre-contracted aortic rings. The pre-contracted tension elicited by PE was statistically identical among experimental groups (data not shown).

**2.4 Measurement of Nox1 concentration**

We measured NO⁺ content in plasma and in Ach-stimulated aortas using a chemiluminescence-based assay with an NO analyser (NOA280i, Sievers Instruments, USA) as previously described (see Supplementary material online).

**2.5 Measurement of NADPH-sensitive superoxide production**

Isolated aortas were cut into rings (2 mm) and immediately placed in Krebs–Hepes solution (see Supplementary material online) for 20 min. Superoxide production was measured, using lucigenin-enhanced chemiluminescence, and validated as described previously (see Supplementary material online).

**2.6 Quantitative reverse transcriptase–polymerase chain reaction**

Total RNA from aortas were extracted using TRIzol reagent (Invitrogen, USA). Total RNA (1 μg) was reverse-transcribed using iScript cDNA synthesis kits (Bio-Rad, USA) in a total volume of 20 μL. cDNA encoding eNOS, Nox1, Nox2, Nox4, gp91phox, and p47phox were amplified using real-time polymerase chain reaction (PCR). Gene-specific primers were designed using Beacon Designer 7.5 software (Premier Biosoft International, USA) (see Supplementary material online, Table S1). Real-time PCR was performed twice using the intercalating dye SYBR Green. Each sample (25 μL) contained 12.5 μL of iQ SYBR Green SuperMix (Bio-Rad), 0.4 μM of each primer, and 1 of 50 of the RT product. PCR cycles were programmed and recorded using the iQ5 Real-Time PCR detection system (Bio-Rad). The cycle number (CT) was compared with that of β-actin, referred to as ΔCT. The relative gene expression level was expressed as 2^[−(ΔΔCT)]$, where ΔΔCT = ΔCT of female minus the ΔCT of the male rats or ΔCT of CR rats minus the ΔCT of the control rats.

**2.7 Protein expression of eNOS and Nox4**

Protein expression of eNOS and Nox4 in aortic tissues was detected using western blotting. In brief, aortas were homogenized in ice-cold lysis buffer (see Supplementary material online) with protease inhibitors and centrifuged (25,000 g for 30 min) at 4 °C. Equal amounts of protein underwent polyacrylamide SDS–PAGE (7.5%) and electrophoretically transferred to a nitrocellulose membrane. These membranes were blocked in milk (5%), incubated overnight (4°C) with anti-eNOS (Santa Cruz, USA), anti-Nox4 (Abcam, USA), and anti-GAPDH (Santa Cruz, USA) primary antibodies, and then incubated with horseradish peroxidase-conjugated immunoglobulin for 1 h at room temperature. After the final wash, immunoreactive bands were detected using enhanced chemiluminescence (Millipore, USA) and exposed to X-ray films. Protein expression levels were quantified using ImageJ densitometry software (National Institutes of Health, USA).

**2.8 Statistical analysis**

All results are expressed as mean ± SEM values. Differences between genders or between control and CR groups were analysed by Student’s t-test. Interactions between gender and CR were analysed by two-way ANOVA. Simple linear regression was used to determine the correlation.
Table 1 Physiological and biochemical parameters of all animal groups after 2 weeks of treatment with and without caloric restriction

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Male + CR</th>
<th>Female</th>
<th>Female + CR</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>351.1 ± 3.7G</td>
<td>342.9 ± 2.8G</td>
<td>215.0 ± 4.4</td>
<td>210.9 ± 1.4</td>
<td>G×R</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>373.0 ± 8.3G</td>
<td>350.4 ± 2.5G</td>
<td>221.5 ± 3.9R</td>
<td>197.1 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Plasma insulin (µg/L)</td>
<td>2.52 ± 0.55G</td>
<td>0.98 ± 0.14</td>
<td>0.66 ± 0.07</td>
<td>0.73 ± 0.14</td>
<td>G×R</td>
</tr>
<tr>
<td>Plasma ghrelin (pg/mL)</td>
<td>172.47 ± 20.56G</td>
<td>235.62 ± 14.78</td>
<td>309.93 ± 31.20</td>
<td>363.35 ± 53.55</td>
<td>G×R</td>
</tr>
<tr>
<td>Plasma NO (µmol/L)</td>
<td>15.21 ± 0.83G</td>
<td>18.92 ± 0.63</td>
<td>20.11 ± 0.32</td>
<td>19.46 ± 0.85</td>
<td>G×R</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM values (n = 10–12 animals per group). Differences between both genders (G) or between control and caloric restriction (CR; R) groups were analysed by Student’s t-test. Superscript G indicates P < 0.05 compared with respective female group; superscript R indicates P < 0.05 compared with respective CR group. Interactions between gender and CR (G × R, if present, indicates P < 0.05) were analysed by two-way ANOVA.

3. Results

3.1 Physiological and biochemical profiles of animals

Body weights of females were significantly lower than those of males (P < 0.05, Table 1). In both sexes, 2-week CR resulted in a significantly lower gain in body weights compared with control rats (P < 0.05, Table 1). Plasma insulin, acetylated ghrelin, and NO* concentrations were determined at the end of the experiments. Insulin was significantly lower, whereas acetylated ghrelin and NO* levels were significantly higher in control females than in males (P < 0.05, Table 1). CR lowered the plasma insulin, but increased both ghrelin and NO* significantly in male rats only (P < 0.05, Table 1).

To explore the role of oestrogen further, we injected oestrogen (10 µg/kg body weight) daily to male rats with or without CR for 2 weeks. In various groups, serum oestrogen level was significantly higher in female than in male and OVX groups (see Supplementary material online, Figure S2A). Oestrogen injection in male and OVX groups significantly raised serum oestrogen concentration compared with male and OVX groups (see Supplementary material online, Figure S2A). Serum testosterone level was higher in male groups compared with female groups (see Supplementary material online, Figure S2B). Oestrogen treatment drastically decreased serum testosterone in male rats compared with control male and female groups (see Supplementary material online, Figure S2B). OVX rats exhibited lower plasma NO* concentration compared with sham-operated rats (P < 0.05, see Supplementary material online, Figure S3A). Both CR and oestrogen supplementation significantly elevated the plasma NO* level in OVX rats (P < 0.05, see Supplementary material online, Figure S3A).

3.2 Assessment of vascular reactivity

In PE-induced pre-contracted rings, ACh elicited a dose-dependent relaxation (EDR). Maximal EDR was higher in control females than in males (P < 0.05, Figure 1A), whereas CR significantly enhanced maximal EDR in males but not in females (P < 0.05, Figure 1A). OVX significantly reduced maximal EDR compared with sham-operated rats (P < 0.05, see Supplementary material online, Figure S3B). Both CR and E2 supplementation significantly improved maximal EDR in OVX rats (P < 0.05, see Supplementary material online, Figure S3B). To evaluate endothelium-independent relaxation, pre-contracted aortic rings were treated with SNP and the maximal response was identical in all groups (Figure 1B and see Supplementary material online, Figure S3C).

3.3 Aortic NO* and NADPH-sensitive O2− production

ACh-stimulated NO* production in aortic tissue was higher in control females than in male rats (P < 0.05, Figure 1C) and CR caused a significant increase in ACh-stimulated NO* production in males, but not in females (P < 0.05, Figure 1C). Because aortic O2− production reduces bioavailable NO*, we next determined NADPH-sensitive O2− production and found that control male aortic rings exhibited significantly greater O2− levels than those of females (P < 0.01, Figure 1D). Further, CR significantly reduced NADPH-sensitive O2− production to a greater extent in males than in females (P < 0.01, Figure 1D), indicating that bioactive levels of NO* produced by aortas were greater in females than in males. We found that oestrogen treatment did not significantly alter NO* output, vascular O2− production in males with or without CR (see Supplementary material online, Figure S1A and B). O2− production was higher in OVX rats (see Supplementary material online, Figure S3D) and both CR and E2 supplementation significantly reduced aortic NADPH-sensitive O2− production in OVX rats (P < 0.05, see Supplementary material online, Figure S3D). To clarify the enzymatic source of aortic O2− production, several pharmacological inhibitors were used. NADPH-sensitive O2− production was suppressed by diphenyleneiodonium (10 µmol/L) and superoxide dismutase (SOD; 300 U/mL); however, it was not significantly altered by oxypurinol (100 µmol/L), indomethacin (10 µmol/L), Nω-nitro-l-arginine (100 µM), or rotenone (10 µmol/L) (see Supplementary material online, Figure S2).

3.4 Expression of Nox and eNOS

Nox mRNA levels were examined. Aortic Nox1, Nox2, Nox4, p22phox, and p47phox mRNA expression were detected by quantitative reverse transcriptase–PCR after normalizing to the β-actin mRNA expression level. The mRNA expression of aortic Nox1, Nox2, Nox4, p22phox, and p47phox was significantly higher in control males than in females (P < 0.05, Figure 2A–E). In male rats, CR significantly decreased the mRNA expression of all Nox subunits, except that of Nox2 (Figure 2B). While in females, CR only significantly diminished aortic Nox1 and p22phox mRNA expression.
The gender difference in CR response was particularly pronounced in Nox4 (Figure 2C) and p47phox (Figure 2E). Considering that Nox4 is a major component in vascular tissues of Nox,24,25 we determined the protein expression of Nox4 and eNOS. Control females exhibited higher aortic eNOS expression than males (P < 0.05, Figure 3A), whereas Nox4 protein expression was significantly higher in males (P < 0.05, Figure 3B). CR significantly increased aortic eNOS and decreased Nox4 expression in males only (Figure 3A and B). We further found that oestrogen treatment did not significantly alter eNOS and Nox4 expression in males with or without CR (see Supplementary material online, Figure S1C and D).

3.5 Correlational study of NO\textsuperscript{•}/O2\textsuperscript{•−}

We have determined vascular ACh-induced EDR (Figure 1A), NO\textsuperscript{•} production (Figure 1C), eNOS expression (Figure 3A), vascular O2\textsuperscript{•−} production (Figure 1D), and Nox4 (Figure 3B) from the same animal. Variation in both NO\textsuperscript{•} and O2\textsuperscript{•−} levels thus provided an opportunity to investigate the biochemical and functional correlations characterizing the state of NO\textsuperscript{•}/O2\textsuperscript{•−} balance of that animal. Linear regression analysis was used to examine the relation between eNOS and Nox4 expression in all animals. In male rats, the relationship (R\textsuperscript{2} = 0.52, P < 0.05) showed a significant negative correlation (Figure 4A), but the correlation was not significant in females (Figure 4B). Moreover, CR caused a shift from a low NO\textsuperscript{•}/O2\textsuperscript{•−} status (open circle) due to high O2\textsuperscript{•−} production and low maximal ACh-induced EDR (bioactive NO\textsuperscript{•}) in control males to a higher NO\textsuperscript{•}/O2\textsuperscript{•−} status (solid circle), closer to that in control females (triangles) (Figure 4C).

4. Discussion

This study investigates the gender-specific impact of short-term CR on the balance of vascular NO\textsuperscript{•} and O2\textsuperscript{•−} production in young rats. To our knowledge, this work provides the first evidence that CR results in a reduction of vascular O2\textsuperscript{•−} production through decreased
expression of Nox to a greater extent in male rats than in females. This reduction causes CR-treated rats to maintain more vascular bioactive NO \(^\dagger\) and exhibit greater EDR.

4.1 Gender differences in NO \(^\dagger\)/O₂ \(^\dagger\) balance in control rats

Oestrogen is known to produce gender dimorphism in cardioprotective actions by enhancing eNOS activity and NO \(^\dagger\) production in the vascular wall, resulting in enhanced EDR.\(^{26-28}\) This difference may also be due to less vascular production of O₂ \(^\dagger\) in females\(^{29}\) with lower NADPH-sensitive O₂ \(^\dagger\) production of various arteries in an oestrogen-dependent manner,\(^{30}\) and is consistent with the observation that more NADPH-sensitive O₂ \(^\dagger\) is produced in ovariectomized animals.\(^{31}\) Here, the aortas of control female rats exhibited greater EDR (Figure 1A and B), higher bioactive NO \(^\dagger\) in aortic tissues and plasma (Table 1 and Figure 1C), and less NADPH-sensitive O₂ \(^\dagger\) production than males (Figure 1D). In an exploration of the role of oestrogen in NO\(^\dagger\)/O₂ \(^\dagger\) balance, we employed oestrogen treatment to male rats for 2 weeks and found that oestrogen treatment did not significantly alter vascular NO \(^\dagger\) output, O₂ \(^\dagger\) production, eNOS, and Nox4 expression (see Supplementary material online, Figure S1A–D). However, oestrogen greatly decreased serum testosterone in male rats with or without CR compared with control (see Supplementary material online, Figure S2B). Testosterone deficiency in males is associated with elevated levels of total cholesterol, low-density lipoprotein, enhanced production of proinflammatory cytokines, increased thickness of the arterial wall, and endothelial dysfunction.\(^{32}\) The observed drop of serum testosterone level may thus offset the beneficial effects of oestrogen in our model. Nevertheless, in an oestrogen deficiency model, OVX caused an increase in vascular O₂ \(^\dagger\) production and impaired EDR (see Supplementary material online, Figure S3A–D).
Oestrogen supplementation improved the OVX-induced alterations (see Supplementary material online, Figure S3B–D). Furthermore, the nitrite level in plasma has been suggested to reflect eNOS activity in mammals including rats. Our data showed that plasma and acetylcholine-stimulated aortic NO\(^{\dagger}\) levels, as well as eNOS expression, were higher in control female than in male rats (Table 1). In addition, the E2 supplement elevated plasma NO\(^{\dagger}\) concentration in OVX rats (see Supplementary material online, Figure S3A). Taken together, these results suggest that oestrogen is at least partially responsible for gender dimorphism in the NO\(^{\dagger}\)/O\(^{2-}\) balance.

In vasculature, Nox generates O\(^{2-}\) by transferring an electron from NADPH to molecular oxygen via the Nox catalytic subunit, and Nox1, Nox2, and Nox4-containing isoforms are important for O\(^{2-}\) generation. Female cerebrovascular Nox activity has exhibited...
lower Nox1 and Nox4 expression than in males. In support of that observation, our data showed that N ox1, Nox2, Nox4, p22phox, and p47phox in aortic tissue were expressed at lower levels in control females than in males (Figure 2A–E). Also, a negative correlation exists between eNOS and Nox4 protein expression in males (Figure 4A), suggesting that Nox4 may be partly responsible for gender dimorphism in NO\(^*\)/O\(_2^\cdot\) balance.

The level of O\(_2^\cdot\) within vasculature is not regulated by Nox alone, and anti-oxidant enzymes, such as SOD, are also involved. In porcine femoral arteries, the expression of CuZnSOD and MnSOD is lower in males than in females; however, the levels of all SOD isoforms in the basilar arteries did not differ between genders. Thus, the role of SOD in gender-specific O\(_2^\cdot\) production is not clear. Other factors are also contributors to this gender difference. Here, we found that plasma acylated ghrelin content in females was higher than in males, consistent with previous investigations in humans. In addition, ghrelin can increase NO\(^*\) production in vascular tissue; thus, it may affect the NO\(^*\)/O\(_2^\cdot\) balance in a gender-specific manner.

4.2 Gender-specific effects of NO\(^*\)/O\(_2^\cdot\) balance in CR rats

Several studies in male rats have demonstrated that ageing impairs vascular NO\(^*\) production and lifelong CR prevents age-induced endothelial dysfunction. CR causes significant improvement of NO-mediated dilation of resistance arteries from the skeletal muscle of aged rats. Short-term (1 week) CR also increases EDR, NO\(^*\) production, and eNOS expression in aortic tissue of healthy male Fischer 344 rats. In support, we found that CR for 2 weeks enhanced EDR, plasma and Ach-stimulated NO\(^*\) production (Figure 1A and Table 1) and eNOS expression (Figure 3A) in aortas of male rats but not in females; given that control females already exhibited >90% of maximal relaxation (Figure 1A).

Bioavailability of NO\(^*\) can be enhanced by diminishing O\(_2^\cdot\) production in vascular tissues, and CR can reduce reactive oxygen species in a wide range of species and tissues. Here, CR significantly reduced aortic NADPH-sensitive O\(_2^\cdot\) production in young rats in a gender-dependent manner (by 47% in males, 21% in females) (Figure 1D). Furthermore, CR decreased aortic mRNA expression of Nox1, Nox4, p22phox, and p47phox in males, but only Nox1 and p22phox mRNA expression in females (Figure 2), suggesting that Nox4 and p47phox could be the molecular entities responsible for the observed gender difference. Several lines of evidence support this notion. First, females and CR rats both have exhibited lower oxidative damage in heart tissue compared with respective males and control rats. Secondly, Nox4 is considered to be the main source of O\(_2^\cdot\) in endothelial cells and vascular smooth muscle cells, suggesting that Nox4-derived O\(_2^\cdot\) could effectively antagonize the effect of NO\(^*\). Further, the expression of Nox4 is significantly higher than Nox1 in aortic tissue. Thirdly, although both p22phox and p47phox are essential for the Nox activity in vascular tissues, Nox1, Nox2, and Nox4 are all needed to form a functional complex; moreover, depletion of p47phox has caused enhanced endothelium derived O\(_2^\cdot\) production. Our findings that CR affected neither Nox4 nor p47phox expression in females suggest that they could be the major targets of CR in males.

CR also alters the expression and activities of anti-oxidant enzymes. Long-term CR has increased glutathione peroxidase and catalase expression but did not alter SOD activities in aged aortic tissue; it has prevented age-dependent decline in glutathione synthesis in aortic tissue. We have employed relatively short-term (6 weeks) CR on young rats and did not observe significant effects on anti-oxidant enzymes (unpublished results). Instead, the suppression of Nox by CR and the differential expression of Nox subunits, at least in part, explains our finding that CR reduced O\(_2^\cdot\) production more in males than in females. Interestingly, resveratrol, a CR mimetic, binds to oestrogen receptors in vitro and attenuates hepatic injury after trauma-haemorrhage via oestrogen receptor signalling. Furthermore, resveratrol not only mimics the transcriptional profile of CR mice in various organs, but also enhances vascular bioavailable NO\(^*\) levels by decreasing O\(_2^\cdot\) production. Our correlation results showed that CR appears to shift the state of the vascular balance of NO\(^*\)/O\(_2^\cdot\) in male rats to the state in female rats (Figure 4C).

A simple mechanism to shift NO\(^*\)/O\(_2^\cdot\) balance from male to female status would be to alter the levels of sex steroids. However, CR did not change the serum levels of either oestrogen or testosterone (see Supplementary material online, Figure S2A). Moreover, the mRNA expression of oestrogen receptors in both genders is not altered by CR (data not shown). Thus, oestrogen and/or oestrogen receptors were not the major factors for the observed beneficial effects of CR in males. Although the underlying mechanism for the effects of CR on males remains unclear, the gender-specific alterations of ghrelin and insulin (Table 1) suggest a interesting lead, because previous study has indicated that 1-week CR improves EDR in males and is attributable to an increased plasma ghrelin level, decreased insulin concentration, and unchanged glucose concentration. Unaltered glucose level in CR has also been documented in human studies. Here, CR only elicited enhanced EDR and ghrelin with reduced insulin in young male rats (Figure 5).

In summary, greater EDR in female than male rats was associated with both increased NO\(^*\) production via eNOS and lower vascular \(\text{O}_2^\cdot\) production. However, it is not clear how CR affects the balance between NO\(^*\) and \(\text{O}_2^\cdot\) in males.
O$_2^-$ production via Nox. Moreover, male rats subjected to short-term CR exhibited a favourable NO$^*$/O$_2^-$ balance by lowering vascular O$_2^-$ production through a decreased expression of Nox, thus enhancing bioactive NO$^*$. The CR-induced attenuations of both arms of the vascular NO$^*$/O$_2^-$ balance resulted in more pronounced benefits in male than female rats.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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58. Caloric restriction on vascular NO•/O2•− balance