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Perspective

Advanced atherosclerosis is associated with inflammation, vascular dysfunction and oxidative stress, but not hypertension

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ABSTRACT

Although hypertension may involve underlying inflammation, it is unknown whether advanced atherosclerosis – a chronic inflammatory condition – can by itself promote hypertension. We thus tested if advanced atherosclerosis in chronically hypercholesterolemic mice is associated with systemic and end-organ inflammation, vascular dysfunction and oxidative stress, and whether blood pressure is higher than in control mice. Male ApoE$^{-/-}$ and wild-type (C57Bl6J) mice were placed on a high fat or chow diet, respectively, from 5 to 61 weeks of age. Expression of several cytokines (including IL-6, TNF-α, IFN-γ and/or IL-1β) was elevated in plasma, brain, and aorta of ApoE$^{-/-}$ mice. Aortic superoxide production was ~3.5-fold greater, and endothelium-dependent relaxation was markedly reduced in aorta and mesenteric artery of ApoE$^{-/-}$ versus wild-type mice. There was no difference in blood pressure of aged ApoE$^{-/-}$ (104 ± 3 mmHg, n = 13) and wild-type mice (113 ± 1 mmHg, n = 18). To clarify any effects of aging alone, findings from 61 week-old wild-type mice were compared with those from young (8–12 weeks old) chow-fed wild-type mice. The data indicate that aging alone increased renal and aortic expression of numerous cytokines (including CCL2, CCL7 and IL-1β). Aging had no effect on blood pressure, systemic inflammation, oxidative stress or endothelial function. Despite systemic and end-organ inflammation, oxidative stress and endothelial dysfunction, advanced atherosclerosis does not necessarily result in elevated blood pressure.

1. Introduction

Hypertension is a complex condition with approximately 90% of cases classified as 'essential hypertension' in which the underlying cause is unknown [1]. While findings from human and animal studies have demonstrated an association between hypertension and inflammation [2], it remains controversial as to whether inflammation is a cause or an effect of hypertension. There is evidence to suggest that inflammation can precede hypertension. For example, the plasma level of the acute phase protein, C-reactive protein, which rises in response to inflammation [3], is elevated in pre-hypertensive patients [4], and is associated with a higher risk of developing hypertension [5].

Hypertension is also recognized as a major risk factor leading to atherosclerosis – in which conduit arteries develop thickened lesions that involve chronic inflammation throughout all stages of plaque development [6]. While there is much clinical evidence of an association between hypertension and atherosclerosis, a cause and effect relationship has not been definitively shown. Interestingly, while it is clear that stiffening of large conduit arteries: 1) contributes to elevated systolic blood pressure during hypertension [7]; and 2) is a strong predictor of future cardiovascular events in hypertensive patients [8]; the concept that vascular inflammation and stiffening in atherosclerosis may in itself lead to hypertension has received little consideration.

Abbreviations: ApoE$^{-/-}$, apolipoprotein E knock out; CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; CRP, C-reactive protein; DEA-NO, diethylamine NONOate; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; HDL, high-density lipoprotein; IFN, interferon; IL, interleukin; LDL, low-density lipoprotein; PEG-SOD, superoxide dismutase-polyethylene glycol; PdB, phorbol 12,13-dibutyrate; TNF, tumour necrosis factor.

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We have thus tested whether atherosclerosis may lead to hypertension by measuring arterial pressure over a period of 56 weeks of high-fat feeding in apolipoprotein E deficient ($ApoE^{-/-}$) mice. In addition, as oxidative stress and endothelial dysfunction occur in the early stages of atherogenesis [9] and are both associated with hypertension [10,11] we assessed whether vascular and systemic inflammation, oxidative stress and endothelial dysfunction are present during advanced atherosclerosis and whether their levels are correlated with arterial pressure.

2. Materials and methods

2.1. Model of atherosclerosis

The study was approved by the Monash University Animal Research Platform Animal Ethics Committee. Male wild-type C57Bl6J mice (WT, n = 37) and $ApoE^{-/-}$ mice (n = 28) were obtained from Monash Animal Research Platform. $ApoE^{-/-}$ mice were placed on a high fat diet for 56 weeks (22% fat, 0.15% cholesterol semi-pure rodent diet SF00-219, Specialty Feeds, Australia) from 5 weeks of age. Age-matched C57Bl6J mice on normal chow served as controls. Effects of aging were assessed by comparing young (8–12 weeks old; n = 10) and aged (15–16 months old; n = 27) WT mice. Mice were euthanized by overdose of inhaled isoflurane.

2.2. Blood pressure measurements

Systolic blood pressure was monitored in conscious mice via tail cuff plethysmography using the MC4000 Multichannel system (Hatteras Instruments). Blood pressure measurements commenced at age 19 weeks in WT and $ApoE^{-/-}$ mice (i.e. 14 weeks after starting the dietary regime), and were performed every 7 weeks according to the following procedure. On the week of measurement, mice were trained for 3 consecutive days for acclimatization to the procedure, and blood pressure was then recorded on the following 2 days. The average of these two measurements was then taken as the reading for that mouse. In separate cohorts of mice, systolic blood pressure was also measured in $ApoE^{-/-}$ mice after 54 weeks of high-fat feeding and in age-matched WT mice using radiotelemetry, for which mice were surgically implanted with a telemeter probe (Model TA11PA-C10, Data Sciences International, USA) as previously described [12], and allowed to recover for one week. Blood pressure was then recorded over a 2-week period.

2.3. Atherosclerotic lesions

The thoracic aorta was removed, cut open longitudinally and fixed in 60% isopropyl alcohol for 5 min, stained in oil red O (0.5% in 60% isopropyl alcohol) for 30 min, placed in 60% isopropyl alcohol for 5 min to remove excess stain, and then placed in PBS until *en face* images were photographed.

2.4. Plasma cholesterol and cytokines

At the time of euthanasia, whole blood was collected in a 1 ml syringe via the posterior vena cava, transferred to a heparinized tube, and centrifuged at 10,000 RPM for 10 min at 4 °C to obtain plasma. Total cholesterol, triglycerides, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol were measured by Gribbles Veterinary Pathology (Melbourne, Australia). Plasma cytokine (interferon (IFN)-γ, interleukin (IL)-12p70, IL-4, IL-5, IL-6, and tumour necrosis factor (TNF)-α) levels were measured using a ProcartaPlex™ Mouse Essential Th1/Th2 Cytokine Panel (eBioscience).

2.5. Inflammatory genes

Expression of pro- and anti-inflammatory genes in the brain, kidneys and aorta was determined using TaqMan® real-time PCR. Tissues were harvested and snap frozen in liquid nitrogen. RNA was extracted from brains and kidneys using RNeasy Mini kit (Qiagen), and from aortae using RNeasy Micro kit (Qiagen), and was quantified using a Nanodrop 1000D spectrophotometer (Thermo Scientific). Extracted RNA was then converted to 1st strand cDNA using a High Capacity cDNA RT Kit (Applied Biosystem). Commercially available primers (Applied Biosystems) were used to assess mRNA expression of inflammatory markers, and the house-keeping genes β-actin or Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), on a CFX96 Touch Real-Time PCR Detection machine (Bio-Rad). Differences in gene expression were analyzed using the comparative C_T method [13].

2.6. Endothelial function

Rings of abdominal aorta (2 mm in length) and second order mesenteric arteries (125–260 μm in diameter) were isolated. Aortic rings were mounted in a Mulvany-style small vessel wire myograph (Danish Myo Technology) containing Krebs-bicarbonate buffer (in mmol/L: D-glucose 11.1; $CaCl_2$ 2.5; NaCl 118; KCl 4.5; KH_2PO_4 1.03; $MgSO_4$ 0.45; $NaHCO_3$ 25) and bubbled with carbogen (95% O_2/5% CO_2), and after 20 min equilibration were stretched to a baseline tension of 5 mN and then maximally constricted using U46619 (U-max, 300 nmol/L). After washing 3 times and a further 20 min equilibration, aortic rings were then contracted submaximally (to ~50% of U-max) and concentration-relaxation responses to carbachol (1 nmol/L to 30 μmol/L) and then diethylamine NONOate (DEA-NO, 1 nmol/L to 30 μmol/L) were recorded in the same vessel with an ~30 min washout period between curves. Rings of mesenteric artery were mounted between two microcannulae at 60 mmHg in a pressure-myograph (Living Systems Instrumentation, Inc) and were superfused with carbogen-bubbled Krebs-bicarbonate solution (in mmol/L: D-glucose 11.1; $CaCl_2$ 2.5; NaCl 118; KCl 4.5; KH_2PO_4 1.03; $MgSO_4$ 0.45; $NaHCO_3$ 25) and bubbled with carbogen (95% O_2/5% CO_2) at 37 °C. After the arterial segment was submaximally constricted by U-46619 (to ~30% of baseline diameter), concentration-response curves to carbachol (1 nmol/L to 100 μmol/L) and then DEA-NO (1 nmol/L to 100 μmol/L) were constructed in the same vessel with an ~30 min washout period between curves.

2.7. Superoxide measurements

L-012-enhanced chemiluminescence was performed to measure superoxide levels in rings of freshly harvested thoracic aorta in the absence and presence of the NOX2 oxidase activator, phorbol 12,13-dibutyrate (PdB) (10 μM). In semi-darkness, aortic rings were placed in a white 96-well plate containing Krebs-HEPES buffer (in mM: D-glucose 11.1; Na-HEPES 20; $NaHCO_3$ 25; $CaCl_2$ 2.50; NaCl 99.01; KCl 4.69; KH_2PO_4 1.03; $MgSO_4$ 1.20; pH 7.4) and background photon counts measured using a Hidex Chameleon single photon counter (30 × 1 min cycles, 3 s per well). L-012 (100 μM) was then added to each well and measurements were made for another 30 cycles. PdB was then added to each well and measurements were made for another 30 cycles. The aortic rings were then transferred onto aluminium foil, where they were dried for 24 h at 37 °C and weighed. Superoxide production was expressed relative to the dry weight of tissue.