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High intrinsic aerobic capacity and pomegranate juice are protective against macrophage atherogenecity: studies in high- vs. low-capacity runner (HCR vs. LCR) rats

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Abstract

We studied the rat model system of high- vs. low-capacity runner (HCR vs. LCR) rats to question the atherogenic properties (oxidative stress, triglycerides and cholesterol metabolism) in the rat macrophages, serum, liver and heart. Half of the LCR or HCR rats consumed pomegranate juice (PJ; 15 μmol of gallic acid equivalents/rat/day) for 3 weeks and were compared to placebo-treated rats. At the end of the study blood samples, peritoneal macrophages (RPM), livers, and hearts were harvested from the rats. RPM harvested from HCR vs. LCR demonstrated reduced cellular oxidation (21%), increased paraoxonase 2 activity (28%) and decreased triglycerides mass (44%). Macrophage uptake rates of fluorescein–isothiocyanate-labeled low-density lipoprotein (LDL) or oxidized LDL were significantly lower, by 37% or by 18%, respectively, in HCR vs. LCR RPM. PJ consumption significantly decreased all the above atherogenic parameters with more substantial beneficial effects observed in the LCR vs. the HCR rats (~80% vs. ~40% improvement, respectively). Similar hypo-triglyceridemic pattern was noted in serum from HCR vs. LCR. In contrast to the above results, liver oxidation and triglycerides mass were both minimally increased in HCR vs. LCR rats by 31% and 28%, respectively. In the heart, lipid content was very low, and interestingly, an absence of any significant oxidative stress, along with modest triglyceride accumulation, was observed.

We conclude that HCR vs. LCR rats demonstrate reduced atherogenicity, mostly in their macrophages. PJ exerts a further improvement, mostly in macrophages from LCR rats.

Keywords: HCR rats; LCR rats; Rat peritoneal macrophages; Oxidative stress; Liver; Heart; Pomegranate juice

1. Introduction

Cardiovascular patients are characterized by high levels of lipids (cholesterol, triglycerides) and increased oxidative stress in their blood and tissues [1–4]. Lifestyle, including exercise, is an important feature for cardiovascular protection, and according to the *aerobic hypothesis*, variations in capacity for oxygen metabolism are an important determinant of heart and blood vessels health and for attenuation of atherosclerosis development [5]. Macrophages play a major role in the pathogenesis of atherosclerosis as they can take up oxidized low-density lipoprotein (Ox-LDL) at enhanced rate, leading to cholesterol and oxidized lipids accumulation and foam cell formation, the hallmark of early atherogenesis [6,7]. Arterial macrophage foam cells are characterized by increased cellular levels of oxidative stress, triglycerides and cholesterol [8]. A similar pattern is shared by blood monocytes, the precursors of macrophages. In contrast to arterial macrophages, the liver was shown to accumulate lipids and oxidized lipids by their removal from the circulation [9], which decreases their atherogenicity in the blood. As for the heart, it is known to accumulate only small amount of lipids, mainly for the purpose of energy metabolism.

Protection from oxidative stress may be achieved by endogenous antioxidants, including the paraoxonase (PON) enzymes [10] and exogenous antioxidants, mainly from dietary origin. The most nutritionally potent antioxidant and antiatherosclerotic agent is the pomegranate juice (PJ), which is rich in ellagitannin compounds, such as punicalagin [11]. Previous studies have demonstrated that PJ consumption by healthy volunteers significantly decreased their LDL, high-density lipoprotein (HDL), macrophages and atherosclerotic lesion oxidative status [12]. Moreover, consumption of PJ by patients with carotid artery stenosis significantly increased serum PON1 activity; decreased serum oxidative stress, lesion oxidative stress and lesion cholesterol content; and even attenuated atherosclerotic plaque development in the carotid arteries [13]. Similarly, daily consumption

Abbreviations: HCR, high-capacity runner; LCR, low-capacity runner; RPM, rat peritoneal macrophages; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Ox-LDL, oxidized LDL; PON1, paraoxonase 1; PON2, paraoxonase 2; PJ, pomegranate juice; GAE, gallic acid equivalents; DCFH-2′,7′, dichlorofluorescin diacetate; DHC, dihydrocoumarin; AAPH, 2.2′-azobis, 2-amidinopropane hydrochloride; TBARS, thiobarbituric acid reactive substances; IP, intraperitoneal; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; MFI, mean fluorescence intensity.

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of PJ improved stress-induced myocardial ischemia in patients with coronary artery disease [14].

The strong clinical association between low aerobic capacity and all-cause morbidity and mortality [15] led us to formulate the idea that variation in capacity for aerobic metabolism is a central mechanistic determinant of the divide between complex diseases and health, which we termed the *aerobic hypothesis* [16]. By the early 1990s, we proposed that the aerobic hypothesis could be tested by applying two-way artificial selection to develop rat lines of low-capacity runner (LCR) and high-capacity runner (HCR) rats [17]. That is, if the aerobic hypothesis is true, we expected that susceptibility to disease would segregate with LCR rats, and resistance to disease would segregate in HCR rats and simultaneously provide uniquely contrasting models for study. Consistent with the hypothesis, the LCR harbor and the HCR are relatively free from numerous disease risks including metabolic syndrome, hepatic steatosis, body weight gain on a high fat diet, intracerebral hemorrhage, inducible cancer, unsuccessful aging and diminished longevity [18]. These contrasting rat lines are currently at 35 generations of selection and differ by over 10-fold for treadmill running capacity [19]. Conceptually, exercise capacity can be considered to derive from two parts: (1) an intrinsic component that operates in the sedentary (nontrained state) and (2) an extrinsic component that follows as an adaptive response that accrues from all activities above the sedentary state [20]. The LCR/HCR model system mostly, but not purely, represents intrinsic capacity.

Here we used this rat model system to evaluate the possible role of intrinsic aerobic running capacity in atherogenesis, in the absence or presence of the antioxidant agent — PJ. Our results clearly demonstrate reduced atherogenicity in HCR vs. LCR rats, mostly in their macrophages. In addition, PJ retrieves atherogenesis in both groups, but more strongly in LCR compared to HCR rats.

2. Experimental procedures

2.1. Materials

Fluorescein–isothiocyanate (FITC), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), dihydrocoumarin (DHC), phenyl acetate and thiobarbituric acid were all purchased from Sigma-Aldrich (St. Louis, MO, USA). BBL thioglycolate medium brewer modified was purchased from Becton, Dickinson and Company (Sparks, MD, USA). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, nystatin, L-glutamine and sodium pyruvate were all purchased from Biological Industries (Beth Haemek, Israel). PJ was obtained from PomWonderful, Los Angeles, CA, USA. PJ concentrate was diluted 5× with double-distilled water to get the single-strength PJ.

2.2. The LCR/HCR rat model system

The development of LCR and HCR rats has been described previously [17]. Briefly, starting in 1996, two-way artificial selective breeding was used to create LCR and HCR strains that were divergent for treadmill running capacity. The selection criterion was the distance run to exhaustion on a graded treadmill test performed on Monday, Wednesday and Friday at 11 weeks of age. The best run of the three trials was considered the trial most closely associated with the heritable component of endurance running capacity. The founder population was the genetically heterogeneous N: The National Institutes of Health out crossed stock of rats that we obtained from Hansen and Spuhler [21]. The 13 lowest and 13 highest running capacity rats of each sex were selected from the founder population and randomly paired for mating. At each subsequent generation, within-family selection from 13 mating pairs was practiced for each line: this number of families maintains a relatively low coefficient or inbreeding (<0.01/generation) and maximizes the retention of genetic variation. Run-tested rats from generation 34 were shipped via overnight flight from the University of Michigan to Technion–Israel Institute of Technology for further study.

The study was conducted on male LCR (weight 405±30 g) and HCR (weight 332±25 g) rats. When tested at 11 weeks of age, the LCR ran to exhaustion at 157±28 m and the HCR exhausted at 2103±235 m. The rats were 5 months old at the time of study and were on a chow diet. Ten LCR rats and nine HCR rats were included in the study. Four LCR rats and four HCR rats consumed for 3 weeks, in their drinking water, 15 μmol of gallic acid equivalents (GAE)/rat/day of PJ and compared to placebo-treated rats. At the end of the study, the rats were anesthetized with isoflurane, injected intraperitoneally (IP) with thioglycolate to induce inflammatory reaction and the differentiation of blood monocytes into macrophages in the peritoneum. Four days post-injection blood samples, peritoneal macrophages, livers and hearts were harvested from the rats. This protocol was approved by the Committee for the Supervision of Animal Experiments and complied with the Guidance for Care and Use of Laboratory Animals at the Technion–Israel Institute of Technology, Haifa, Israel.

2.3. Lipoprotein isolation

LDL and HDL were isolated from fresh plasma derived from healthy normolipidemic volunteers (Rambam Hospital Helsinki Committee number 30572-10-RBM), by discontinuous density gradient ultracentrifugation [22]. The LDL was separated at $d=1.063$ g/ml, and the HDL at $d=1.210$ g/ml. Both lipoproteins were dialyzed against 150 mmol/l NaCl, 1 mmol/l Na_2EDTA (pH 7.4) at 4°C, and then sterilized by filtration (0.45 μM), kept under nitrogen in the dark at 4°C, and used within 2 weeks. The LDL and HDL protein concentration was determined by the Lowry method [23].

2.3.1. Copper ion-induced LDL oxidation

Prior to oxidation, the LDL was dialyzed against EDTA-free, PBS solution, pH 7.4, at 4°C. LDL (1 mg of protein/ml) was incubated with 5 μmol/l of $CuSO_4$ for 20 h at 37°C. At the end of the incubation, the extent of LDL oxidation was determined by measuring the generated amount of thiobarbituric acid reactive substances (TBARS) [24] and the amount of lipid peroxides [25].

2.4. Serum analyzes

2.4.1. Serum lipids and glucose concentrations

Serum cholesterol, triglyceride and glucose concentrations were determined using commercially available diagnostic kits.

2.4.2. Serum PON1 arylesterase activity

Arylesterase activity was measured using phenyl acetate as the substrate [26]. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 5 μl serum (diluted 1:10), 1.0 mmol/l phenyl acetate and 1 mmol/l $CaCl_2$ in 50 mmol/l Tris–HCl, pH 8.0. The E_{270} for the reaction is 1310 M^{-1} cm^{-1}. One unit of arylesterase activity is equal to 1 μmol of phenyl acetate hydrolyzed/min/ml.

2.4.3. Serum lipid peroxidation

Serum samples were diluted 4× in PBS and were incubated with or without 100 mmol/l of 2.2′-azobis, 2-amidinopropane hydrochloride (AAPH; Wako, Japan) for 2 h at 37°C [27]. The extent of lipid peroxidation was measured by the TBARS assay [24].