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Short report

Spontaneous experimental atherosclerosis in hypercholesterolemic mice advances with ageing and correlates with mitochondrial reactive oxygen species

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ABSTRACT

Ageing and atherosclerosis are associated with oxidative stress. Mitochondrial redox function declines with ageing. Here we tested whether ageing LDL receptor knockout mice ($LDLr^{-/-}$) develop spontaneous atherosclerosis and whether mitochondrial reactive oxygen species (mtROS) correlate with atherosclerosis. Compared with young mice, aged $LDLr^{-/-}$ mice exhibited 20-fold larger aortic lesion size, although the plasma cholesterol levels did not vary between age groups. The lesion sizes increased exponentially from 3 to 24 months of age ($r = 0.92$, $p = 0.0001$) and were correlated with mtROS across the age range ($r = 0.81$, $p = 0.0001$). Thus, $LDLr^{-/-}$ mice develop spontaneous diet-independent atherosclerosis, that advances exponentially with ageing. We propose that age related increases in mtROS contribute to accelerate atherosclerosis development in hypercholesterolemic mice.

1. Introduction

Atherosclerosis constitutes the pathogenic process of ischemic heart disease and of stroke that are known as the major causes of mortality around the world (Mozaffarian et al., 2015). The strongest unchangeable independent risk factor for the development of atherosclerosis is ageing (Ferrari et al., 2003). Cellular oxidative stress seems to be a common denominator in many age-related diseases, including atherosclerosis. The current view on atherogenesis proposes that the initiation steps are triggered by a local vascular oxidative stress that involves LDL oxidation and subsequent foam cell formation. However, the mechanisms that drive in vivo oxidative stress are still largely unclear (Steinberg, 2009; Yurdagul et al., 2016). Mitochondrial respiration is one of the major sources of cellular reactive oxygen species (ROS) (Boveris and Chance, 1973). These reactive species are important signaling molecules for several cell processes, including differentiation, adaptation, and senescence (Hamanaka and Chandel, 2010). The levels of ROS are controlled through efficient mitochondrial and cell antioxidant systems. High ROS generation rates or failure of antioxidant defenses induce cellular oxidative stress observed in many degenerative and age-related diseases (Figueira et al., 2013; Hamanaka and Chandel, 2010). Mitochondrial function declines with ageing. This may be due to accumulation of oxidative damage observed in different model systems and organisms (Liu et al., 2002; Bratic and Larsson, 2013). Thus, mitochondria play an important role in age-related diseases, however, whether mitochondrial deterioration is a cause or a consequence of the ageing process remains elusive (Sanz, 2016). Mitochondrially derived ROS seems to play a relevant role in the context of atherosclerosis, since they are involved in endothelial dysfunction, infiltration and activation of inflammatory cells and apoptosis of endothelial and vascular smooth muscle cells (Hulsmans et al., 2012). Our group has previously shown that mitochondria from various tissues of hypercholesterolemic atherosclerosis-prone LDL receptor knockout mice ($LDLr^{-/-}$) release more ROS than wild type derived mitochondria (Oliveira et al., 2005). Furthermore, we recently reported that mitochondrial reactive oxygen species (mtROS) is a novel independent risk factor for the development of spontaneous atherosclerosis in this familial hypercholesterolemia mouse model (Dorighello et al., 2016). Thus, the objectives of the present study were: 1 - to evaluate whether there is spontaneous (not diet induced) atherosclerosis development in aged $LDLr^{-/-}$ mice, and 2 - whether mtROS levels were associated with atherosclerosis severity in the ageing context.

2. Material and methods

2.1. Animals

Male LDL receptor–knockout mice founders were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the

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University animal facility (CEMIB/Unicamp). The animal protocols were approved by the University's Committee for Ethics in Animal Experimentation (CEUA/UNICAMP, protocol #1101-1). Mice had free access to standard laboratory rodent chow diet (Nuvital CR1, Colombo, Paraná, Brazil) and were housed at 22 ± 1 °C on a 12 h light/dark cycle. At the age range of 2 to 24 months, 1 to 5 mice were anesthetized with ketamine/xylazine (100 and 10 mg/kg body weight, respectively) for heart perfusion followed by heart and liver excision.

2.2. Plasma cholesterol analysis

Blood samples were drawn from the retro-orbital plexus of anesthetized and overnight fasted mice. Total cholesterol was measured in fresh plasma using a standard commercial kit (Roche-Hitachi®, Germany and Wako®, Germany).

2.3. Mouse liver mitochondria preparation and reactive oxygen species release (ROS)

Liver mitochondria were isolated by conventional differential centrifugation at 4 °C. The experiments were done in a standard medium containing: 125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, and 10 mM Hepes buffer, pH 7.2, as previously described (Oliveira et al., 2005). Isolated mitochondria were kept on ice and used within 90 min from preparation. ROS levels derived from mitochondria were monitored using the membrane-permeable fluorescent dye 2′,7′ dichloro-dihydro-fluorescein diacetate (H_2DCF-DA) as previously described (Oliveira et al., 2005). A calibration curve was obtained with known concentrations of dichlorofluorescein (DCF) (Sigma-Aldrich, Inc., St Louis, MO, catalog # D6665).

2.4. Histological analysis of atherosclerosis lesions

In situ perfused hearts were excised and embedded in Tissue-Tek® OCT compound (Sakura, USA), frozen at −80 °C, cut in 10 μm-sections along 480 μm aorta length from the aortic valve leaflets and stained with Oil red O as previously described (Dorighello et al., 2016). The lipid-stained lesions were quantified using the *Image J* (1.45 h) software.

2.5. Statistical data analyses

The results are presented as the means ± SEM. The comparisons between the groups were analyzed by unpaired Student's *t*-test and the correlation analyses by Spearman's correlation test. The level of significance was set at $P < 0.05$.

3. Results and discussion

LDL receptor knockout (LDLr$^{-/-}$) mice, a model of human familial hypercholesterolemia, are widely used to study diet-induced atherosclerosis. It is generally accepted that LDLr$^{-/-}$ mice do not develop atherosclerosis, unless high fat or cholesterol containing diets are employed (Jawień et al., 2004). However, a few previous studies including ours (Dorighello et al., 2016; Mortensen et al., 2002) have reported the presence of small and moderate spontaneous atherosclerotic lesions in young adult (4–7 months of age) LDLr$^{-/-}$ mice fed with standard (low fat) diets. High fat and high cholesterol diets induce atherosclerosis very fast and potently in this model. However, these unbalanced diets also induce a range of secondary factors such as inflammation, insulin resistance and obesity, which interact synergistically to increase atherosclerosis. Thus, in this study we aimed at investigating whether spontaneous atherosclerosis lesions would increase along with ageing in

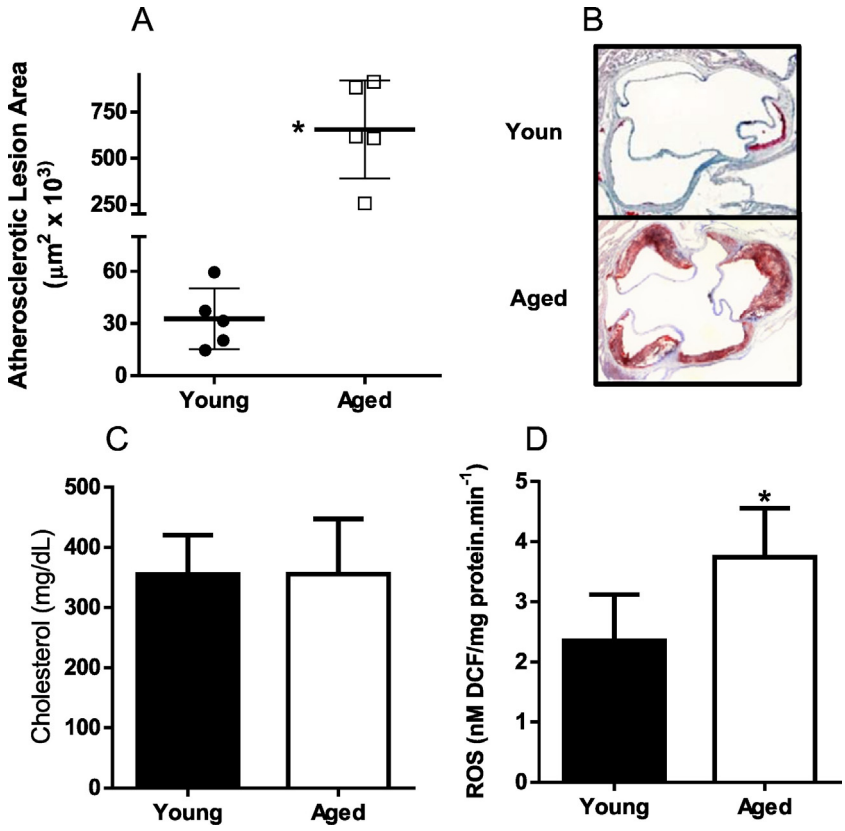

Fig. 1. Atherosclerosis, plasma cholesterol levels and mitochondrially derived reactive oxygen species (mtROS) in standard chow diet fed young (4–5 month-old) and aged (16–18 month-old) LDLr$^{-/-}$ mice. (A) Lipid stained areas of atherosclerotic lesions in the aorta root ($n = 5$, $^*P < 0.001$). (B) Representative images of aorta root from young and aged mice. (C) Fasting plasma cholesterol levels ($n = 12$–13). (D) Liver mitochondrial ROS (mtROS) levels as measured by DCF oxidation ($n = 4$, $^*P < 0.05$).

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LDLr$^{-/-}$ mice fed with a standard chow diet (4 g% fat). Initially, we compared the aorta root (atherosclerosis predilection site) of 16–18 month-old with 4–5 month-old LDLr$^{-/-}$ mice. The aged mice presented a 20-fold increase in the size of the fat stained lesions compared with lesions of young mice (Fig. 1A and B). The plasma cholesterol levels were similarly high in both young and aged LDLr$^{-/-}$ mice (Fig. 1C), and liver mitochondrial reactive oxygen species levels (mtROS) were increased 40% in aged LDLr$^{-/-}$ mice compared with levels of young mice (Fig. 1D).

In healthy humans and some wild type rodent models, plasma cholesterol levels increase with ageing (Celermajer et al., 1994; Fujihara et al., 1992). However, most mouse strains have no or very mild increases of plasma cholesterol with ageing (Rivnay et al., 1979; Weibust, 1973, Nakashima et al., 1994). Here, the standard chow diet fed LDLr$^{-/-}$ mice exhibited stably high levels of plasma cholesterol in early and advanced ages. The high levels of plasma cholesterol since birth certainly contribute to the development of spontaneous lesions in this model. Elevated LDL-cholesterol levels constitute a bulk of oxidisable substrate, without which ROS induced lesions would develop very slowly.

Primary culture of aorta smooth muscle cells derived from aged wild type C57BL6 mice (16 vs 4 month-old) have increased global ROS levels that were associated with decreased endogenous antioxidant activity, increased lipid peroxidation, and mitochondrial DNA damage (Moon et al., 2011). By using specific mitochondrial probes or isolated mitochondria, more recent studies have shown that mtROS are associated with ageing (Martínez-Cisuelo et al., 2016; Vendrov et al., 2015) and with atherosclerosis (Vendrov et al., 2015; Dorighello et al., 2016). Hyperlipidemic aged apo E knockout mice (16 month-old) present increased atherosclerosis lesion area, higher cellular and mitochondrial superoxide radical levels and oxidatively damaged nuclear DNA in their aortic wall compared with 4 month-old mice (Vendrov et al., 2015). The authors also showed that cellular and mtROS were increased in the aortic vascular smooth muscle cells of aged wild type and NOX1/2 knockout mice, indicating that this feature is related with the ageing process rather than with the hyperlipidemic context (Vendrov et al., 2015). Compared with young wild type mice (4 month-old CB6F1 hybrid), aged mice (16 month-old) presented higher ROS levels from isolated liver mitochondria (120%), and other signs of oxidative damage such as mitochondrial protein and lipid oxidation (Martínez-Cisuelo et al., 2016).

To confirm that augmented atherosclerosis and mtROS in LDLr$^{-/-}$ mice would be a function of ageing, we analyzed a detailed age range from 2 to 24 month-old. Small atherosclerotic lesions appeared as early as 3 months of age and advanced exponentially up to the age of 24 months (Fig. 2A). The Spearman correlation test showed a significant direct relationship between atherosclerosis lesion area and ageing ($r = 0.92$, $P < 0.0001$). Accordingly, epidemiological studies have clearly shown that the atherosclerotic process in humans increases exponentially with ageing (Simons, 1989).

The free radical theory of ageing, proposed >60 years ago (Harman, 1956), postulates that ageing reflects an accumulation of oxidative damage in macromolecules and cell structures. The relevance of the mitochondria as a source of free radical production was later suggested by Harman (1972). This “mitochondrial theory of ageing” has been reinforced by several subsequent studies (Barja, 2013). Therefore, we hypothesized that in LDLr$^{-/-}$ mice the levels of mitochondrially derived ROS would be a major mechanism linked to the progression of the disease. Fig. 2B shows that mtROS levels indeed correlate with ageing (Spearman correlation $r = 0.88$, $P < 0.0001$). Furthermore, the extent

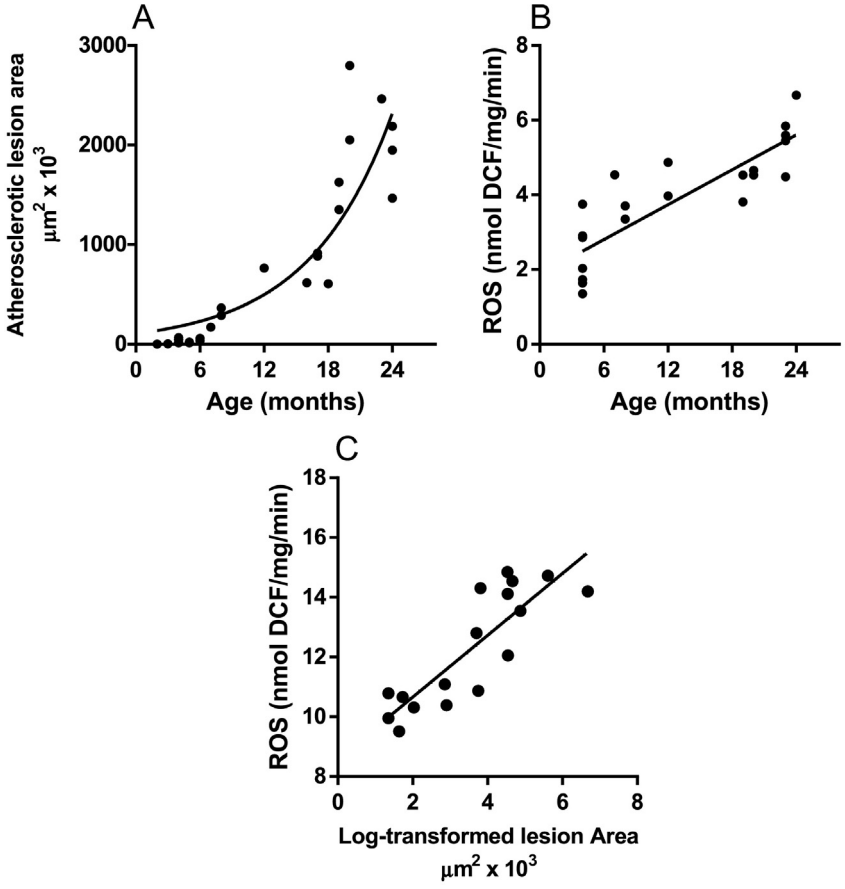

Fig. 2. Progress of atherosclerosis and mitochondrially derived reactive oxygen species (mtROS) along with ageing in LDLr$^{-/-}$ mice fed with standard chow diet. (A) Atherosclerotic lesion areas (Spearman correlation of log-transformed areas and age: $r = 0.92$, $P < 0.0001$, $n = 34$). (B) Liver mitochondrial ROS (mtROS) levels as measured by DCF oxidation ($r = 0.88$, $P < 0.0001$, $n = 22$). (C) Spearman correlation between mtROS levels and atherosclerosis (log-transformed lesion areas): $r = 0.81$, $P = 0.0001$, $n = 17$.

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of atherosclerosis (log transformed lesion area) correlates with mtROS levels ($r = 0.81$, $P = 0.0001$, Fig. 2C) in this context of ageing. Although this correlation does not imply in causation, it evidences a degree of dependency between atherosclerosis development and increases in mitochondria derived ROS during ageing. Unfortunately, studies with antioxidant interventions to reduce atherosclerosis have shown conflicting results and disappointing outcomes. While some studies in humans and animals models reported reduced atherosclerosis, others have shown that antioxidant treatments actually increased atherosclerosis (Lonn et al., 2012). This critical question is complicated because of the involvement of ROS in normal signaling pathways and because of spatial compartmentalization of cellular oxidative signaling and/or damage (Lonn et al., 2012). A good approach to confirm a causal relationship between mtROS and atherosclerosis in the context of hypercholesterolemia would be to treat $LDLr^{-/-}$ mice with effective mitochondrial targeted antioxidants to reduce mtROS and consequently, the severity of atherosclerosis (Victor et al., 2009).

In conclusion, these data show that $LDLr^{-/-}$ mice develop spontaneous atherosclerosis that worsens exponentially along with ageing and correlates with age related increases in mitochondrial ROS levels.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Authors' contribution

The authors' contributions are as follows: GGD: conducted the research, analyzed the data, wrote the paper and bears primary responsibility for its final content; BAP and ACRL: conducted the research and analyzed the data; HCFO and AEV were co-responsible for obtaining the grants, project conception and research design, data analyses, and paper writing. All authors read and approved the final manuscript.

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