

## Effect of a Chronic Cholesterol-rich Diet on Vascular Structure and Oxidative Stress in LDLR<sup>-/-</sup> Mice

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### Key Words

LDLR<sup>-/-</sup> • Hypercholesterolemia • Oxidative Stress • Aorta

### Abstract

**Aims:** There is conflicting evidence regarding the relationship between hypercholesterolemia and oxidative stress in vessels. To test the potential relationship, a mouse model of hypercholesterolemia was used. **Methods:** Low density lipoprotein receptor-deficient (LDLR<sup>-/-</sup>) and control (C57Bl/6) mice were fed a normal or (1.25%) high-cholesterol (HC) diet for 8 weeks, and the incidence of this chronic diet was evaluated on the degree of vascular oxidative stress and vascular structure (collagen content and lipid infiltration expressed in arbitrary units: AU=%/mm<sup>2</sup>). **Results:** Animals treated with the HC diet presented an increase in lipid infiltration (0.35±0.13 vs. 1.7±0.18 control and 1.04±0.16 vs. 1.84±0.23 LDLR<sup>-/-</sup>, AU p<0.05) associated with higher collagen content (control: 2.13±0.40 vs. 3.46±0.36 and LDLR<sup>-/-</sup>: 2.37±0.36 vs. 3.79±0.60; AU p<0.05 red Sirius staining). Interestingly, ROS production in the aorta was only increased in the LDLR<sup>-/-</sup> +cholesterol group (0.17±0.04 and 0.16±0.05 in the control

groups, 0.14±0.02 vs. 0.34±0.06 in the LDLR<sup>-/-</sup> groups, p<0.05). C57Bl/6 and LDLR<sup>-/-</sup> mice presented altered vascular structure associated with the rich cholesterol diet, which was not necessarily associated with increased oxidative stress. **Conclusion:** These findings highlight the complex interrelation between oxidative stress and lipid metabolism in the circulatory tract.

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### Introduction

Impaired cholesterol metabolism is associated with a high risk of atherosclerosis and vascular events [1]. In many models, a high cholesterol diet has been shown to increase leukocyte adhesion, oxidative stress due to reduced oxidative defences, and impaired endothelium-dependent vasorelaxation, which could lead to vascular alterations [1-3]. Thus, a cholesterol-rich diet could induce modifications of the structural and oxidative status of blood vessels leading to atherosclerosis. In fact, the implication

of oxidative stress in atherogenesis is not clear [4-6], it seems that it plays an important role in the development of the pathology, but the relationship between oxidative stress and high circulating levels of LDL has never been tested.

The mouse is a very useful model in that reproduction and genetic manipulations are straightforward. The low density lipoprotein receptor-deficient mouse (LDLR<sup>-/-</sup>) is one of the most interesting models to study the impact of high circulating levels of cholesterol on metabolic and functional parameters of the organs [7]. These models have been developed to study atherosclerosis, and their physiological parameters are comparable to those found in familial hypercholesterolemia in humans. LDLR<sup>-/-</sup> mouse is prone to develop plaque and to present high levels of circulating cholesterol after a cholesterol-rich diet, but the relationship between plaque formation and oxidative stress has not yet been clearly established.

In the present study, we chose hypercholesterolemia as a pathological mouse model. LDLR<sup>-/-</sup> and C57Bl/6 mice were fed for 8 weeks with a high-cholesterol (HC: 1.25% or normal diet) to evaluate the impact of the cholesterol-rich diet on vascular oxidative stress and vascular structure.

## Materials and Methods

### *Animals*

The local ethics committee approved the experimental protocol and the investigators complied with authorization 6006 from the French government, which agrees with the Guide for the Care and use of Laboratory Animals published by US National Institutes for Health. Female LDLR<sup>-/-</sup> mice purchased from Jackson Institute and C57Bl/6 (4 months) mice (Wild-type), purchased from IFFA Credo (France), were fed for 8 weeks with either a high-cholesterol (HC, 1.25% cholesterol, 4% fat, 24 % protein, 5% cellulose and vitamin mix 1.2%) or normal diet (0.2 % cholesterol, 4% fat, 24 % protein, 5% cellulose and vitamin mix 1.2%). The mice were then anaesthetized with sodium thiopental (60 mg/kg) and heparin (500 UI/kg) was injected intraperitoneally. Blood samples were obtained to measure plasma levels of triglycerides and lipids. Levels of total and HDL plasma cholesterol were measured with a colorimetric device (Cobas Integra, Roche Diagnostics, Switzerland) and expressed in mM.

Once the mice were anesthetized, after sacrifice, the abdominal aorta was immediately placed in ice-cold Krebs solution at 4 °C (mM concentrations: NaCl 116, KCl 5.4, CaCl<sub>2</sub> 1.8, KH<sub>2</sub>PO<sub>4</sub> 0.6, MgSO<sub>4</sub> 0.8, NaHCO<sub>3</sub> 19, glucose 5.5). Sections from diaphragm to renal arteries were cut into 5 mm ring segments free of adventitia prior to OCT inclusion. For each analysis 10 to 15 slices (9 µm) of the same aortic portion were used.

### *Collagen content*

As described previously [8], collagen content was assessed on cryosections using two different methods. In the first method, all of the sections were stained with Masson trichrome, and the presence of collagen (green color) was scored at X 400 magnification (0, absence; 1, mild; 2, moderate; 3, marked). Three entire sectional areas were evaluated per artery, by two blinded investigators. For the first method, cryosections were fixed in acetone for 5 minutes then incubated with Mayer's Hemalun for 2 minutes and washed in water.

For the second method, cryosection were fixed in acetone for 5 minutes prior to a 5 minutes washing period in water. The slices were then incubated with sirius red (Polysciences) dissolved in 0.1% saturated picric acid for 30 minutes. After washing for 30 seconds, the sections were viewed under polarized light and captured with the same threshold for all specimens. Automatic computer-based analysis was performed with the same threshold for all specimens (Nikon Eclipse 600, Japan). The results are expressed as the percentage of collagen content per unit area: %/mm<sup>2</sup> corresponding to 1 arbitrary unit (AU).

### *Lipid infiltration*

Cryosections were stained with Oil Red O (Sigma, France). They were first incubated in 60% isopropanol (Prolabo, France) for 2 minutes and then in Oil Red O solution for 20 minutes and rinsed in water. The presence of intracellular lipid droplets was scored at X 400 magnification (yes / no score) corresponding to 1 arbitrary unit (AU). Three entire sectional areas were evaluated per artery, by two blinded investigators.

### *In situ detection of superoxide*

Dihydroethidium (DHE), an oxidative fluorescent probe, [9] purchased from invitrogen (France) was used to localize O<sup>•</sup>. Fresh-frozen slices were fixed for 10 min in acetone. The slices were incubated in a light-protected humidified chamber at room temperature with DHE (5 µM) for 5 minutes. To verify the specific detection of O<sub>2</sub><sup>•-</sup> by DHE, some slices were incubated with superoxide dismutase (300 IU/mL, Dako) before DHE incubation, and in this case no labeling was observed (data not shown). The slices were immediately analyzed with a computer-based digitizing image system (Microvision, Evry, France) using a fluorescent microscope (Eclipse 600, Nikon, Champigny-Sur-Marne, France) connected to a video camera (Tri CCD, Sony, Paris, France). Fluorescence was detected with 510-560 nm excitation and 590 nm emission filters. The nuclei were counted using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), which binds selectively to DNA. Automatic computer-based analysis was performed with the same threshold for all sections (X 500 magnification). Results are expressed as the percentage of fluorescence/nuclei.

### *Statistical analysis*

All data are expressed as means ± SEM; statistical analyses were performed with a nonparametric Mann-Whitney rank sum test (SigmaStat). Significance was established at a value of *P* < 0.05.

	C57Bl/6	C57Bl/6 HC	LDLR <sup>-/-</sup>	LDLR <sup>-/-</sup> HC
TGL (mM)	0.18±0.02 <sup>a</sup>	0.14±0.03 <sup>a</sup>	0.79±0.12 <sup>b</sup>	0.49±0.11 <sup>b</sup>
HDL (mM)	0.60±0.03 <sup>a</sup>	0.86±0.06 <sup>b</sup>	0.66±0.05 <sup>a</sup>	0.88±0.13 <sup>b</sup>
LDL (mM)	0.68±0.03 <sup>a</sup>	0.90±0.08 <sup>b</sup>	4.41±0.49 <sup>c</sup>	15.81±2.48 <sup>d</sup>
Chol (mM)	1.38±0.04 <sup>a</sup>	2.10±0.25 <sup>b</sup>	5.43±0.43 <sup>c</sup>	16.62±2.33 <sup>d</sup>

**Table 1.** Plasma lipids levels in the 4 groups after 8 weeks of a normal diet or a cholesterol-rich diet: (HC) (1.25%), values are Mean±SEM. Means with different letters (a, b, c, d) differ,  $p < 0.05$ .

## Results

### Physiological parameters

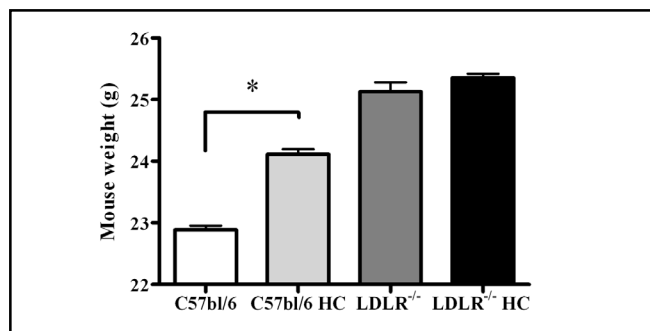
**Serum cholesterol levels.** As shown in Table 1 the HDL cholesterol levels in the C57Bl/6 and LDLR<sup>-/-</sup> groups were comparable. The cholesterol-rich diet in both groups led to a significant increase in these levels ( $p < 0.05$ ). The triglycerides (TGL) were higher in both the LDLR<sup>-/-</sup> and LDLR<sup>-/-</sup> HC groups compared with the C57Bl/6 ( $p < 0.05$ ). The C57Bl/6 group had the lowest cholesterol level (1.38±0.04 mM), but the 8-week cholesterol-rich diet led to an increase in cholesterol levels. In the LDLR<sup>-/-</sup> groups the cholesterol levels were even higher, and after 8 weeks of the cholesterol-rich diet it reached 16.62±2.33 mM in the LDLR<sup>-/-</sup> HC ( $p < 0.05$ ).

**Mouse weight.** As shown in Fig. 1, the cholesterol rich diet induced an increase in body weight in the C57Bl/6 groups (22.89±0.22 vs. 24.11±0.30 g, respectively,  $p < 0.05$ ), but did not affect the weight of the LDLR<sup>-/-</sup> mice (Fig. 1).

### Vessel characterization

**Collagen content.** The collagen content was evaluated on abdominal aorta cryosections using two methods: red Sirius (Fig. 2) and Masson's trichrome (Fig. 3) staining. Both methods demonstrated 1) at the beginning of the diet study, the two groups had similar vascular collagen contents, 2) a higher collagen content in both groups of HC aortas than in normal ones. For instance, in the C57Bl/6 groups 2.13±0.04 vs. 3.78±0.06 AU,  $p < 0.05$  for the red Sirius staining; and with the LDLR<sup>-/-</sup> 2.35±0.14 vs. 3.00±0.13 AU,  $p < 0.05$  with Masson's trichrome staining.

**Lipid infiltration.** Oil red O staining made it possible to score lipid infiltration into the media of the abdominal aorta (Fig. 4). Concerning the initial values C57Bl/6 showed the lowest level of lipid infiltration in comparison with the values obtained in LDLR<sup>-/-</sup> mice. The 8-week HC diet led to an increase in Oil Red O staining in this group. LDLR<sup>-/-</sup> aorta always presented a high level of



**Fig. 1.** Mouse weight after 8 weeks of normal diet or a cholesterol-rich diet HC (n = 12) for the C57Bl/6 □, C57Bl/6 HC □, LDLR<sup>-/-</sup> ■, and LDLR<sup>-/-</sup> HC ■. Values are Means±SEM. \*  $p < 0.05$ .

lipid infiltration, but this was far greater after an HC diet ( $p < 0.05$ ).

**Free radical production.** DHE reacts with the superoxide radical to form ethidium bromide, which intercalates with DNA to produce nuclear fluorescence. The aortas from group C57Bl/6, C57Bl/6 HC and LDLR<sup>-/-</sup> presented comparable DHE staining, while the aortas of LDLR<sup>-/-</sup> HC group presented a significantly higher production of O<sub>2</sub><sup>•-</sup> (Fig. 5,  $p < 0.05$ ).

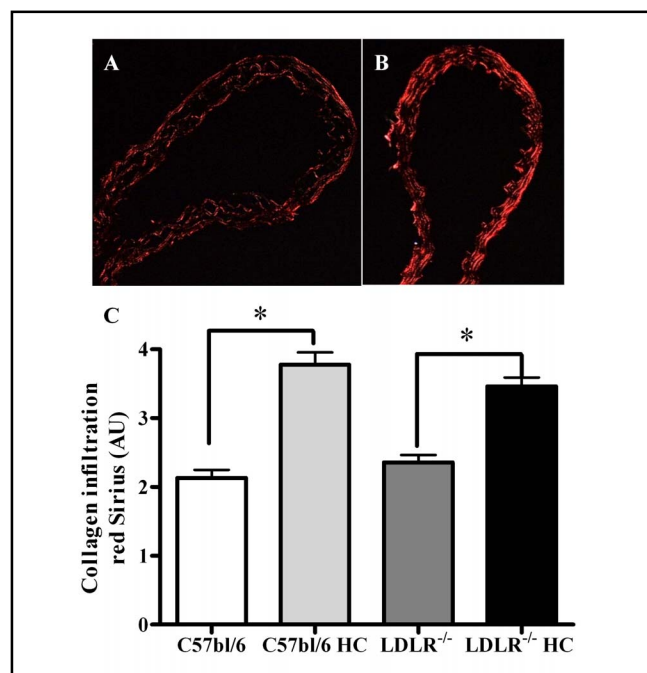
## Discussion

The low density lipoprotein receptor-deficient mouse (LDLR<sup>-/-</sup>) is a genetic model widely used to study obesity, lipid metabolism and atherosclerosis [1]. The LDLR<sup>-/-</sup> mouse develops hypercholesterolemia and arterial lesions when placed on a high-fat diet. In our study, the HC diet did not affect the weight of LDLR<sup>-/-</sup> mice but induced an increase in body weight in the C57Bl/6 group. Recently, Ngai et al. [10] reported that loss of LDLR decreased susceptibility to obesity. The role of LDLR in regulating weight and susceptibility to obesity appears not clear. In humans, LDLR polymorphisms have been associated with obesity [11]. Our results showed that the incidence of an HC diet on the weight was not the same in the

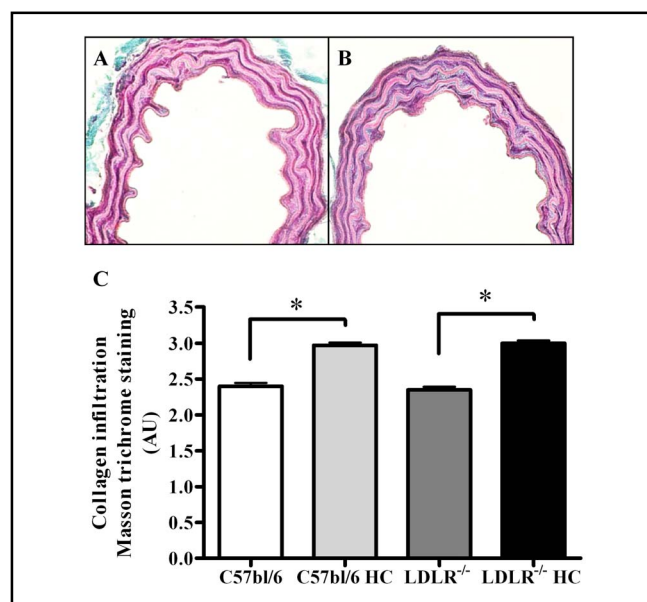
different strains; the rich cholesterol diet induced an increase in body weight in the C57Bl/6 mice, but did not affect the weight of the LDLR<sup>-/-</sup> mice. Yet the plasma cholesterol levels were increased by a cholesterol-rich diet (8 weeks), in the mice of both groups. This dissociation between the rise in concentrations of plasma cholesterol and the increase in mouse weight is of interest. Loss of the LDLR may indirectly affect the reduction of lipolysis in fat cells or the alteration of thermogenesis, which would also increase lipid storage in adipose tissue. Therefore, in these animals, the presence of lipid accumulation in adipose tissue suggests that the cellular processes involved in accumulation were saturated. Our results confirm that the alleles of the LDLR could be used to identify individuals prone to obesity [2, 12, 13].

An interesting point of this study is the impact of the mutation at the locus for the LDL receptor and of the HC diet on collagen and lipid infiltration into aortas. According to one hypothesis, the imbalanced metabolism induced by the mutation or the HC diet may lead to the uncontrolled accumulation of lipids that frequently result in arterial diseases [14]. In our study, the aortic collagen content and lipid infiltration were increased to a comparable extent by the HC in the C57Bl/6 and LDLR<sup>-/-</sup> groups, whereas circulating levels of cholesterol, LDL and TGL were much higher in the LDLR<sup>-/-</sup> group. These results are in accordance with the literature [12]. In fact, even though circulating levels of cholesterol rose to very high concentrations in the LDLR<sup>-/-</sup> HC groups, the alterations to the aorta were comparable in the C57Bl/6 and LDLR<sup>-/-</sup> HC groups. The LDLR<sup>-/-</sup> mouse was one of the earliest gene-targeted strains developed for the study of atherosclerosis and atherosclerotic plaque formation to be associated with a significant increase in collagen content [15]. Interestingly, it has been reported that a long-term cholesterol-rich diet leads to the activation of matrix metalloproteinase and the degradation of collagen content in the arterial wall [16]. In our study, an 8-week cholesterol-rich diet may have been too short to activate MMPs leading to collagen accumulation in the arterial wall. Previous studies have reported that a high-cholesterol (HC) diet for 8 weeks was associated with the initiation of enhanced leukocyte adhesion, and the magnitude of leukocyte-endothelial cell interactions increased with increased duration of high-cholesterol feeding. There is, however, a relationship between chronic inflammatory disease and the degree of vascular oxidative stress [17].

The oxidative fluorescent probe dihydroethidium (DHE) was used in our study to evaluate *in situ*



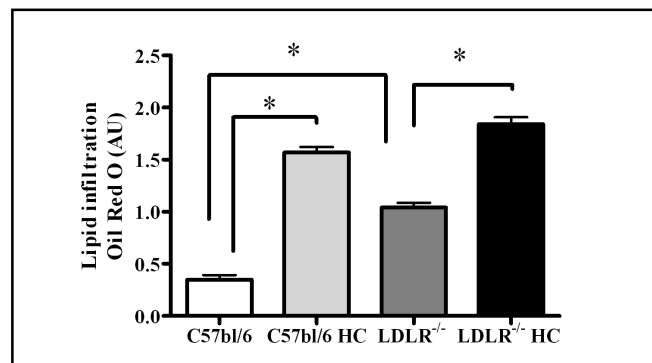
**Fig. 2.** Increased collagen content in aortas assessed with red Sirius staining after 8 weeks of a normal diet or a cholesterol-rich diet: HC (X 200 magnification, n = 6 to 8), photomicrographs are representative of experiments. A: C57Bl/6 normal diet B: LDLR<sup>-/-</sup> HC. C: Collagen content evaluated by red Sirius in C57Bl/6 □, C57Bl/6 HC ▤, LDLR<sup>-/-</sup> ▥, and LDLR<sup>-/-</sup> HC ■. Values are Means±SEM. \* p<0.05.



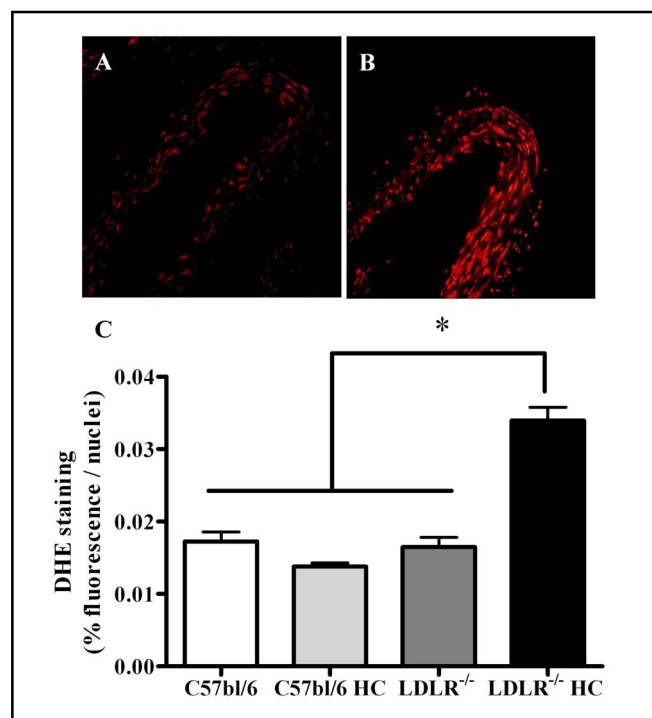
**Fig. 3.** Increased collagen content in aortas assessed (teal coloration in the media) with Masson trichrome staining after 8 weeks of a normal diet or a cholesterol-rich diet: HC (X200 magnification, n=6 to 8), photomicrographs are representative of experiments A: C57Bl/6 normal diet B: LDLR<sup>-/-</sup> HC. C: Collagen content evaluated by Masson trichrome staining in C57Bl/6 □, C57Bl/6 HC ▤, LDLR<sup>-/-</sup> ▥, and LDLR<sup>-/-</sup> HC ■. Values are Means±SEM. \* p<0.05.

superoxide anion production on histological sections [18-20]. In a preliminary study [21], we determined the specificity for superoxide by using Superoxide Dismutase (SOD, 300 IU/ml). DHE is a cell permeable dye that is oxidized by superoxide to ethidium bromide, which subsequently intercalates with DNA and is trapped within cell nuclei. In our study, morphological analyses revealed the intracellular formation of superoxide in vessel areas containing endothelial cells and medial smooth muscle cells. One important result of our work is that the aortas from C57Bl/6 and LDLR<sup>-/-</sup> presented comparable DHE staining. We documented a significant rise in oxidative vascular status only in the LDLR<sup>-/-</sup> HC group, while the cholesterol-rich diet did not modify DHE staining in C57Bl/6 mice. It seems that modifications in circulating levels of cholesterol are not linked to vascular oxidative stress, and that a major increase in cholesterol levels is needed to induce a significant increase in oxidative stress. Previous experimental investigations have been reported in which total tissue glutathione (GSH) was measured in the myocardium as an index of the oxidant status in LDLR<sup>-/-</sup> and wild-type mice 2 and 18 weeks after the initiation of a high-cholesterol diet. As in our results, the authors did not observe any changes in GSH levels in wild-type and LDLR<sup>-/-</sup> mice. The GSH levels were reduced after two weeks of a high-cholesterol diet and, in contrast, significantly increased after 12 weeks of this diet only in LDLR<sup>-/-</sup> but not in the wild-type mice. Because increased adiposity and elevated plasma lipoprotein levels is accompanied by systemic increases in inflammation and oxidative stress, some studies fed antioxidants such as vitamin E to LDLR<sup>-/-</sup> animals to determine the impact of such a diet on vascular parameters. Hasty et al. [22] reported that 8-iso-prostaglandin F2alpha (a reliable marker of overall oxidative stress in urine samples) was not modified in LDLR<sup>-/-</sup> with vitamin E supplementation.

Our results using the LDLR<sup>-/-</sup> model did not address the causal or temporal relationship between oxidative stress, lipid accumulation and disease progression. In our study, it appears that HC diet led to a significant increase in LDL levels in C57Bl/6 and LDLR<sup>-/-</sup> groups but this increase was associated with oxidative stress only in LDLR<sup>-/-</sup> HC. Thus, an important increase in cholesterol levels was needed to induce a significant rise of oxidative stress. According to recent studies exploring the mechanisms underlying atherosclerosis, one can suggest an important role of the chemotactic activity and recruitment of macrophages. Qiao et al. [23] reported that oxidative stress provides an additional mechanism for the enhanced chemotactic activity of macrophages in



**Fig. 4.** Increased lipid infiltration in aortas assessed with Oil-red-O staining after 8 weeks of a normal diet or a cholesterol-rich diet: HC (n = 6 to 8), C57Bl/6 □, C57Bl/6 HC □, LDLR<sup>-/-</sup> ■, and LDLR<sup>-/-</sup> HC ■. Values are Means±SEM. \* p<0.05.



**Fig. 5.** ROS generation within the aortas from control and after 8 weeks of a normal diet or a cholesterol-rich diet: HC (n=6 to 8) aortas determined with dihydroethidium (DHE) fluorescence (X 200 magnification). Photomicrographs are representative of experiments A: C57Bl/6 normal diet B: LDLR<sup>-/-</sup> HC. C: Superoxide anion production in C57Bl/6 □, C57Bl/6 HC □, LDLR<sup>-/-</sup> ■, and LDLR<sup>-/-</sup> HC ■. Values are Means±SEM. \* p<0.05.

metabolically-stressed mice. But on the other hand, inflammation and monocytes dysfunction induced by metabolic diseases [24] must be taken into consideration in the dissociation observed in our study between plasma cholesterol levels and the extent of lipid deposition in the vessels. Taken together, it appears that development of atherosclerosis is multifactorial, and the expression of

immunoregulatory molecules by vascular wall components is thought to contribute to the ongoing atherosclerotic process. Epigenetic mechanisms may also be involved in the regulation of the pathogenesis [25].

In conclusion, C57Bl/6 and LDLR<sup>-/-</sup> mice presented altered vascular structure associated with the cholesterol-rich diet. These alterations were not necessarily associated with increased oxidative stress. These findings highlight the complexity of the relationship between oxidative stress

and lipid metabolism in the circulatory tract.

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