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Critical Limb Ischaemia Exacerbates Mitochondrial Dysfunction in ApoE−/− Mice Compared with ApoE+/+ Mice, but N-acetyl Cysteine still Confers Protection

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WHAT THIS PAPER ADDS

The study shows that critical limb ischaemia (CLI) exacerbates mitochondrial dysfunction and that antioxidant therapies such as N-acetyl cysteine (NAC) still confer protection, even if comorbidities are known to reduce the protective effects of many drugs. Clinical studies using antioxidant strategies have been mostly disappointing and antioxidant therapies are still far from being integrated into treatment algorithms for peripheral arterial disease, but these results suggest that oxidative stress needs to be considered as a target for protective strategies in the setting of CLI.

Objectives: The current study was performed in order to determine the influence of hypercholesterolaemia on critical limb ischaemia (CLI) and whether targeting oxidative stress by antioxidant therapies such as N-acetyl cysteine (NAC), considered to be a direct scavenger of reactive oxygen species, could confer muscle protection.

Methods: Apolipoprotein E (ApoE)−/− mice ($n = 9$, 29 weeks old) and their genetic controls ApoE+/+ mice ($n = 9$, 29 weeks old) were submitted to sequential right femoral and iliac ligations; the left limb served as control. ApoE+/+ mice were divided into two groups: Group 1 ($n = 4$) and Group 2 ($n = 5$); as well as ApoE−/− mice: Group 3 ($n = 3$), and Group 4 ($n = 6$). NAC treatment was administered to Groups 2 and 4 in drinking water. Mice were sacrificed on Day 40 and gastrocnemius muscles were harvested to study mitochondrial respiration by oxygraphy, calcium retention capacity by spectrofluorometry, and production of reactive oxygen species by electron paramagnetic resonance.

Results: CLI associated with ApoE deficiency resulted in more severe mitochondrial dysfunction: maximum oxidative capacity and calcium retention capacity were decreased (−42.9% vs. −25.1%, $p = .010$; and −73.1% vs. −40.3%, $p = .003$ respectively) and production of reactive oxygen species was enhanced (+63.6% vs. +41.4%, $p = .03$) in ApoE−/− mice compared with ApoE+/+ mice respectively. Antioxidant treatment restored oxidative capacity, calcium retention capacity and decreased production of reactive oxygen species in both mice strands.

Conclusions: In this small murine study, hypercholesterolaemia exacerbated mitochondrial dysfunction, as clinically expected; but antioxidant therapy appeared protective, which is counter to clinical experience. Further work is clearly needed.

Keywords: Ischaemia, Atherosclerosis, Mitochondria, Oxidative stress, Muscle, Peripheral arterial disease
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INTRODUCTION

Critical limb ischaemia (CLI) is associated with poor outcomes in terms of survival or limb salvage.[1] In order to better understand CLI pathophysiology, a murine model, stable over time, was developed based on sequential arterial ligations.[2,3] This murine model, which mimics

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human pathology well, allowed assessment of the key pathophysiological mechanisms of CLI, such as mitochondrial dysfunction and oxidative stress.[2,4] Indeed, suboptimal energy production from defective mitochondria in addition to reduced oxygen supply enhanced the production of reactive oxygen species (ROS).[3] Increased ROS production and an impaired mitochondrial antioxidant defence system led to high oxidative stress.[3]

The understanding of CLI pathophysiology allowed the development of alternative therapeutic approaches, especially for the "no option" patients, since up to a third of CLI patients are not amenable to conventional interventions and will ultimately require major amputation.[1] Indeed, increased ROS production occurs before mitochondrial dysfunction, suggesting that strategies aiming to reduce ROS production might be successful.[2,3] Such antioxidant strategies have thus been considered as promising therapeutic targets, but clinical results have been mostly disappointing and antioxidant therapies are still far from being integrated into treatment algorithms for peripheral arterial disease, despite promising preclinical efficacy.[1,5,6] This discrepancy has brought into question the relevance of the preclinical hind limb ischaemia models that were used for efficacy testing.[7]

Thus, although this CLI model can be considered to be relevant to CLI, it does not entirely reproduce the complex human condition and there are important limitations that should be taken into account.[7] In particular, the surgical protocol was performed on healthy young wild type mice, constituting a main limitation. Indeed, CLI generally occurs in old patients presenting with cardiovascular risk factors leading to the build up of atherosclerotic plaque.

Targeting oxidative stress through N-acetyl cysteine (NAC) administration has been shown to confer muscle protection in the setting of CLI.[8,9] The antioxidant effect of NAC is due to its ability to act as a reduced glutathione precursor, which is a well known direct antioxidant and a substrate of several antioxidant enzymes. Moreover, NAC can act as a direct antioxidant for some oxidant species such as nitrogen dioxide. The antioxidant activity of NAC is also due to the breaking of thiolated proteins, thus releasing free thiols and reduced proteins, which have important antioxidant activity.[10] However, comorbidities, particularly hypercholesterolaemia, have been shown to reduce cardioprotection related to antioxidants during cardiac ischaemia reperfusion.[11] Consequently, the efficacy of antioxidant therapy deserved to be questioned in the setting of CLI, owing to associated comorbidities.

The aim of the present study was therefore to investigate first whether CLI would lead to a more severe mitochondrial dysfunction ApoE knockout (ApoE−/−) mice, commonly used to simulate the effects of atherosclerosis and dyslipidaemia,[12,13] and second whether targeting oxidative stress through NAC administration in such mice would still confer muscle protection as previously observed in healthy mice.[8,9]

MATERIALS AND METHODS

Animals

The protocol was approved by the Ethics Committee for Animal Research of the university (reference number AL/70/77/02/13). ApoE−/− mice ($n = 9$, 29 weeks old) and their genetic controls ApoE+/+ mice ($n = 9$, 29 weeks old) were fed a high fat diet and handled while observing the French criteria for the care and use of laboratory animals in research.

CLI model

Surgery was performed in all animals under general anaesthesia, according to an established model.[2] In rodents, ligation of the femoral artery just distal to the origin of the profunda femoris is most commonly used, but this method leaves most of the collateral circulation to the lower limb intact and blood flow to the lower limb is restored within 7 days. The model is thereby based on a two step procedure performed under a microscope. The first step consisted of ligation of the right femoral artery, performed midway between the superficial epigastric artery and bifurcation of the popliteal and saphenous arteries, and of three collateral vessels. The second step consisted of ligation of the right common iliac artery 0.5 cm distal to its origin, since collateral vessels in the mouse arise mostly from the internal iliac artery. By performing sequential ligations, a sustainable ischaemia was thereby obtained. The left limb was considered as control[2,14] (Fig. S1).

Subcutaneous buprenorphine (1.5 mg/kg) was administered daily to all animals until Day 7. During follow up once CLI was established, clinical evaluation of all animals was conducted twice a day, to ensure their well being and health status. Indicators of pain such as no grooming, vocalisations, isolation of an animal from the rest of the group, changes in posture were searched for, according to the French criteria for the care and use of laboratory animals in research. Buprenorphine was administered accordingly.

Antioxidant treatment

NAC was administered by dissolution in drinking water for four weeks, starting on Day 7.[15,16] A dose of 1.5 g/kg/day was chosen since it had been previously shown that this dose reduced oxidative stress and triggered mitochondrial biogenesis.[8]

Mitochondrial respiratory chain complex activities

The study of mitochondrial complex activity was measured by oxygraphy through oxygen consumption in skinned fibres, assessing the functional oxidative capacity of the skeletal muscle in its cellular environment. Substrates were added in order to activate or inhibit the different complexes of the respiratory chain. Adenosine diphosphate (2 mM) was added in order to study complex I, III, and IV activity. Succinate (25 mM) was then added to study complex I, II, III, and IV activity, which determined the maximum oxidative

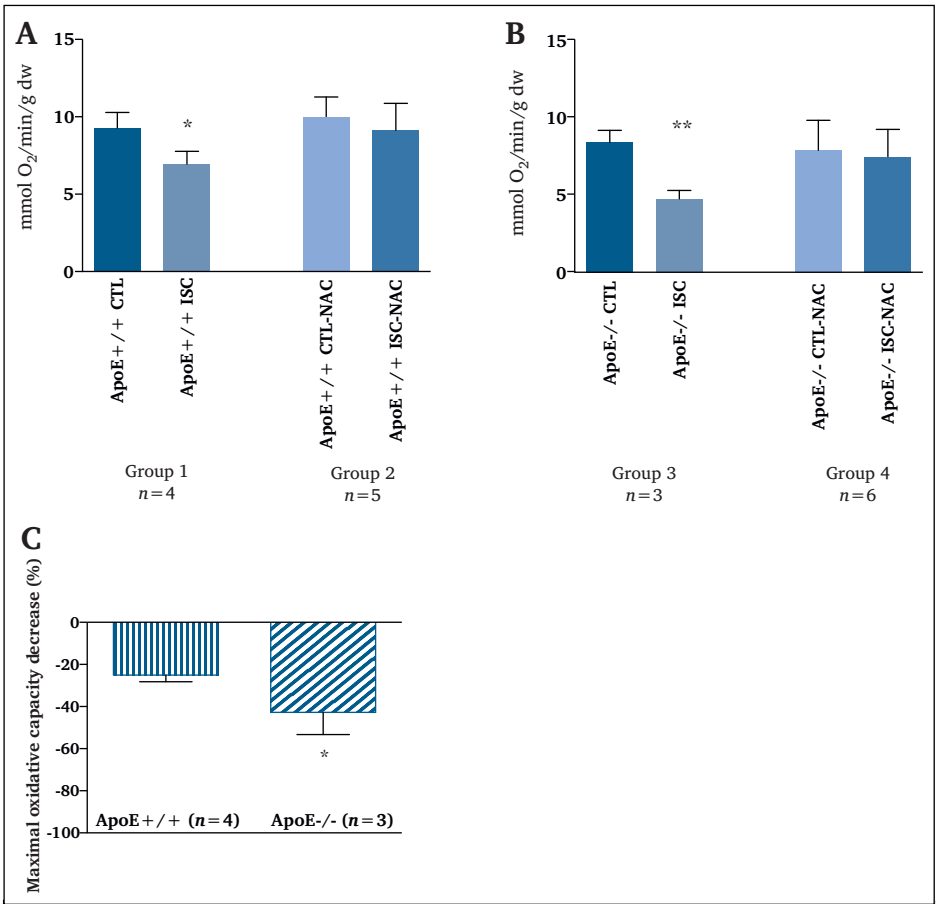

Figure 1. Maximum oxidative capacity was impaired in ischaemic muscles compared with control muscles in ApoE+/+ mice (group 1, $n = 4$) and in ApoE−/− mice (group 3, $n = 3$), but was restored in ischaemic muscles compared with control muscles in ApoE+/+ mice (group 2, $n = 5$) and in ApoE−/− mice (group 4, $n = 6$) after NAC treatment (blue columns, A and B). Maximum oxidative capacity decrease was greater in ApoE−/− mice than in ApoE+/+ mice (C). Results are expressed as mean ± standard deviation. CTL = control limb; ISC = ischaemic limb; NAC = N-acetyl cysteine; ApoE = apolipoprotein E. *$p < .05$; **$p < .01$.

capacity (Vmax). The addition of amytal (0.02 mM) subsequently inhibited complex I; and the addition of N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (0.5 mM) and ascorbate (0.5 mM) specifically activated complex IV.

Calcium retention capacity

Calcium retention capacity was measured by spectrofluorometry. It represents the high cut off amount of calcium that determines the opening of the mitochondrial transition pore, which allows the release of calcium that will in turn lead to apoptosis. Calcium pulses (20 µM) were applied to skinned fibres of gastrocnemius muscle. Thereafter the mitochondrial calcium uptake was assessed after the addition of a single calcium pulse (20 µM), by measuring the decrease of the extramitochondrial calcium concentration. The measure was monitored using a fluorescence probe (Calcium Green-5N, Invitrogen, Carlsbad, CA, USA). When mitochondrial calcium release started, when the calcium uptake showed an inflection point, it was considered that the mitochondrial transition pore opening was observed. Mitochondrial calcium retention capacity, which is a reliable index of mitochondrial transition pore sensitivity, was calculated as the total amount of calcium taken up by mitochondria before calcium release.

Production of ROS

Production of ROS was assessed by electron paramagnetic resonance. Muscles (1 mm^3 fragments) were incubated with a 200 µM molecular probe (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine HCl), which was oxidised in the presence of unpaired electrons of ROS. The amount of oxidised probe was measured through the intensity of the resonance signal.

Study design and group assignment

Eighteen 29 week old mice were allocated to the experiment: ApoE−/− ($n = 9$) and their genetic control ApoE+/+ ($n = 9$). The model showed a good reproducibility over time and an important aim was to determine the potential protective effects of NAC; it was thereby decided in agreement with the statistician to assign more mice to the NAC groups.

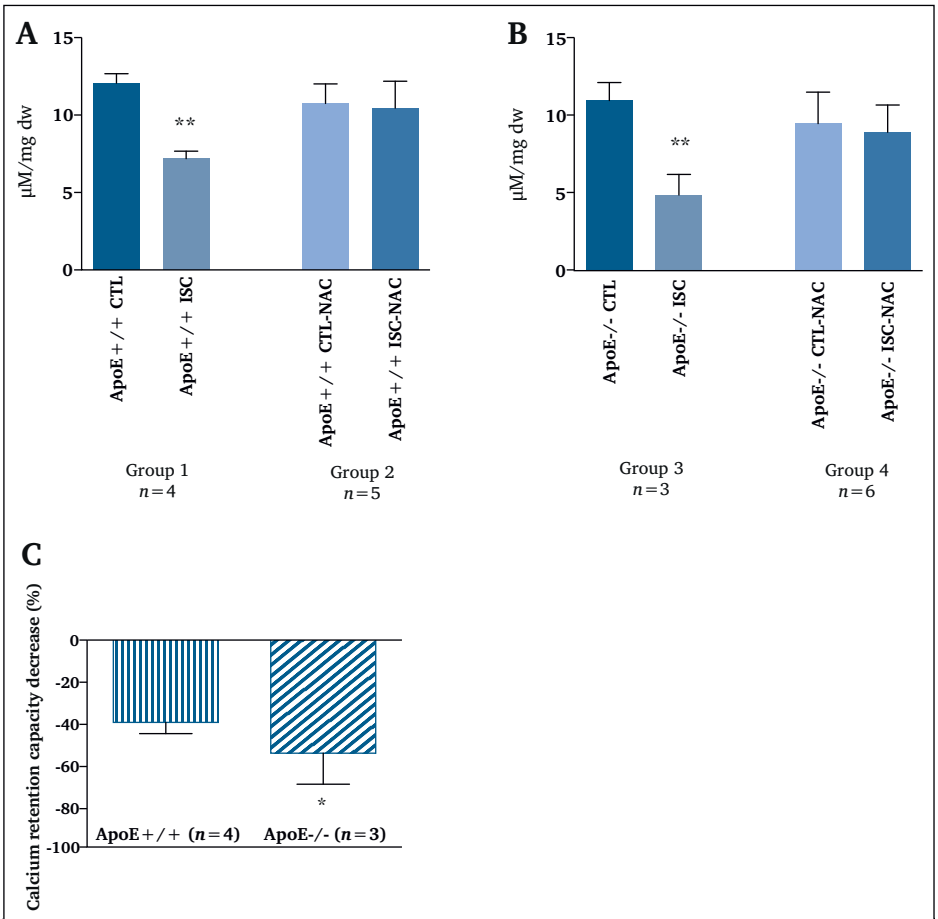

Figure 2. Calcium retention capacity was impaired in ischaemic muscles compared with control muscles in ApoE+/+ mice (group 1, $n = 4$) and in ApoE−/− mice (group 3, $n = 3$), but restored in ischaemic muscles compared with control muscles in ApoE+/+ mice (group 2, $n = 5$) and in ApoE−/− mice (group 4, $n = 6$) after NAC treatment (blue columns, A and B). Calcium retention capacity decrease was higher in ApoE−/− mice than in ApoE+/+ mice (C). Results are expressed as mean ± standard deviation. CTL = control limb; ISC = ischaemic limb; NAC = N-acetyl cysteine; ApoE = apolipoprotein E. *$p < .05$; **$p < .01$.

Surgery was performed in all animals. ApoE+/+ mice were then divided into two groups: Group 1 ($n = 4$) and Group 2 ($n = 5$); ApoE−/− mice were also divided into two groups: Group 3 ($n = 3$) and Group 4 ($n = 6$). NAC treatment was administered to Groups 2 and 4. All animals were sacrificed on Day 40. Gastrocnemius muscles were collected in order to investigate mitochondrial respiratory chain complex activities, calcium retention capacity and ROS production.

Statistical analysis

The number of animals was approved by the Ethics Committee for Animal Research of the university. Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Normal distribution of the variables was confirmed by the Shapiro–Wilk test. Inter-individual comparison was assessed using the paired *t* test. Inter-individual comparison was assessed with a one way ANOVA test. Results are expressed as means and standard deviation. A *p* value $< .05$ was considered to be statistically significant.

RESULTS

Critical limb ischaemia in ApoE−/− mice

Maximum oxidative capacity was impaired in ischaemic muscles compared with control muscles in ApoE+/+ mice: 6.95 ± 0.81 vs. 9.27 ± 1.01 mmol O_2/min/g dw ($p = .001$) as well as in ApoE−/− mice: 4.76 ± 0.55 vs. 8.43 ± 0.77 mmol O_2/min/g dry weight (dw) ($p = .011$) (Fig. 1A,B). Such decrease was higher in ApoE−/− mice than in ApoE+/+ mice (−42.9% vs. −25.1%, $p = .021$) (Fig. 1C).

Calcium retention capacity was impaired in ischaemic muscles compared with control muscles in ApoE+/+ mice: 7.28 ± 0.56 vs. 12.23 ± 0.70 µM/mg dw ($p = .001$) and 4.86 ± 1.46 vs. 11.07 ± 1.33 µM/mg dw in ApoE−/− mice ($p = .001$) (Fig. 2A and B). The decrease was also higher in ApoE−/− mice than in ApoE+/+ mice (−73.1% vs. −56.7%, $p = .03$) (Fig. 2C).

Production of ROS was increased in ischaemic muscles compared with control muscles in ApoE+/+ mice: 0.105 ± 0.019 vs. 0.074 ± 0.009 mmol/min/mg dw

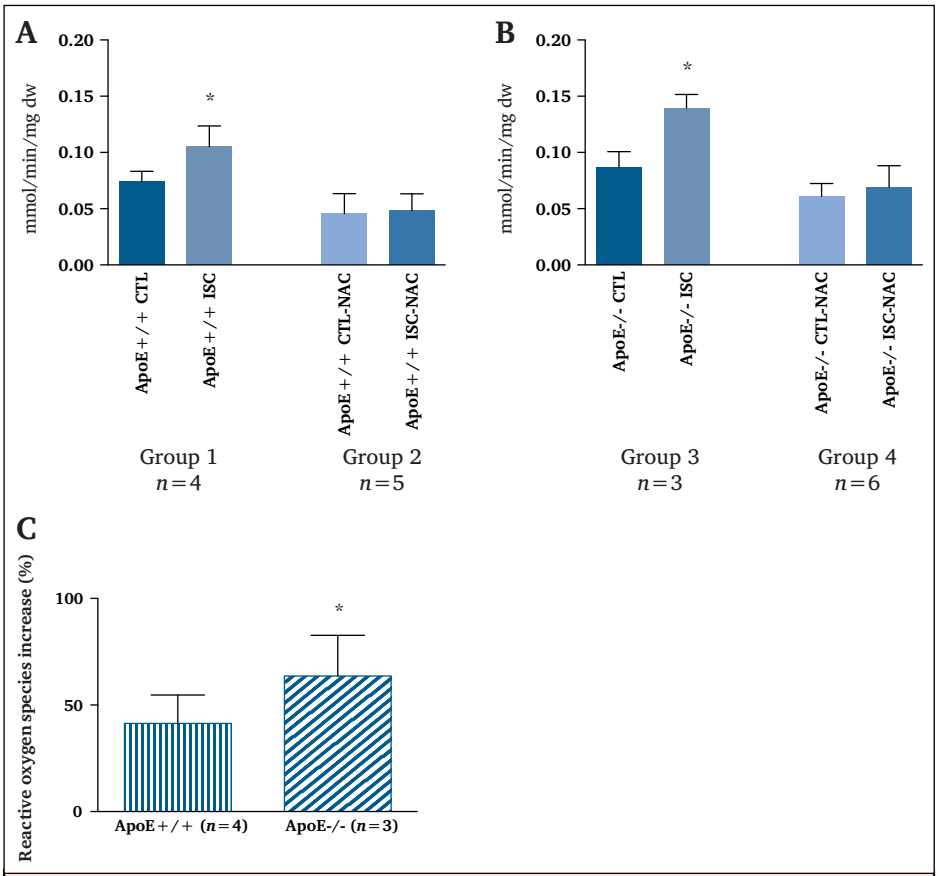

Figure 3. Production of ROS was increased in ischaemic muscles compared with control muscles in ApoE+/+ mice and in ApoE−/− mice, but comparable in ischaemic muscles and in control muscles in ApoE+/+ mice (group 1, $n = 4$) and in ApoE−/− mice (group 3, $n = 3$) after NAC treatment (blue columns, A and B). Reactive oxygen species was higher in ApoE−/− mice than in ApoE+/+ mice (C). Results are expressed as mean ± standard deviation. CTL = control limb; ISC = ischaemic limb; NAC = N-acetyl cysteine; ApoE = apolipoprotein E; ROS = reactive oxygen species. *$p < .05$.

($p = .011$), and 0.139 ± 0.011 vs. 0.087 ± 0.013 mmol/min/mg dw ($p = .039$) in ApoE−/− mice (Fig. 3A,B). ROS increase was greater in ApoE−/− mice than in ApoE+/+ mice (63.6% vs. 41.4%, $p = .031$) (Fig. 3C).

NAC treatment in ApoE−/− mice

Maximum oxidative capacity was restored in ischaemic muscles compared with control muscles in ApoE+/+ mice: 9.13 ± 1.75 vs. 10.02 ± 1.26 mmol O_2/min/g dw (p = NS) and 7.51 ± 1.77 vs. 7.91 ± 1.94 mmol O_2/min/g dw (p = NS) in ApoE−/− mice (Fig. 1A and B).

Calcium retention capacity was restored in ischaemic muscles compared with control muscles in ApoE+/+ mice: 10.56 ± 1.89 vs. 10.90 ± 1.39 µM/mg dw (p = NS) and 9.07 ± 1.79 vs. 9.58 ± 2.16 µM/mg dw (p = NS) in ApoE−/− mice (Fig. 2A and B).

Production of ROS was similar in ischaemic muscles and control muscles in ApoE+/+ mice: 0.048 ± 0.015 vs. 0.046 ± 0.017 mmol/min/mg dw (p = NS) and 0.069 ± 0.018 vs. 0.061 ± 0.011 mmol/min/mg dw (p = NS) in ApoE−/− mice (Fig. 3A and B).

DISCUSSION

The main finding of this study is that CLI leads to more severe mitochondrial dysfunction under hypercholesterolaemic conditions in ApoE−/− mice, since maximum oxidative and calcium retention capacities decreases and production of ROS increases were enhanced compared with ApoE+/+ mice. Further, targeting oxidative stress through antioxidant therapy such as NAC in such mice still conferred muscle protection, not only in ApoE+/+ but also in ApoE−/− mice.

Intensive research was performed to understand the pathophysiology of ischaemia/reperfusion, but surprisingly little effort has been made to uncover the cellular mechanisms by which major cardiovascular risk factors may interfere with ischaemia/reperfusion. Moreover, many studies investigated the myocardium and little is known concerning skeletal muscle in the setting of CLI. In epidemiological studies, there is a well-recognised relationship between serum total cholesterol concentrations and morbidity and mortality due to myocardial infarction.[11] Previously, this was attributed solely to the development

of coronary atherosclerosis as a result of hypercholesterolaemia. However, the majority of preclinical studies, together with some small scale clinical studies, have shown that hypercholesterolaemia *per se*, and not atherosclerosis, leads to a significant aggravation of myocardial ischaemia/reperfusion injury.[11] The mechanisms by which hypercholesterolaemia may influence the severity of ischaemia/reperfusion injury is however not fully understood, but increased oxidative stress, deactivation of protein kinase G, and enhanced apoptotic cell death have been demonstrated as consequences of hypercholesterolaemia in the myocardium.[11] This study confirms that hypercholesterolaemia further potentiates the deleterious effects of CLI on skeletal muscle. The mechanisms involved combine enhanced mitochondrial dysfunction with reduced oxidative capacity and premature opening of the mitochondrial permeability pore, thereby favouring apoptosis, together with increased oxidative stress.

Whether NAC administration might lose its protective effects in CLI, as previously observed in cardiac ischaemia reperfusion, deserved to be investigated. Indeed, hypercholesterolaemia attenuates the protective effect of pharmacological conditioning via changes in protective signalling pathways.[11] Again, studies mainly investigated the myocardium. It has thus been demonstrated that pharmacological activation of the NO—cGMP—PKG pathway significantly reduced infarct size in normal rat hearts, but was ineffective in hearts of rats fed a cholesterol enriched diet.[17,18] The loss of cardioprotection was possibly due to inactivation of PKG by oxidative dimerisation of the kinase under dyslipidaemic conditions. These results indicated that drug targets upstream of PKG might not provide cardioprotection under hypercholesterolaemic conditions.[19] Similarly concerning ATP sensitive potassium (K_{ATP}) channel activation, it was shown that the infarct size limiting effect of either the non-selective K_{ATP} activator cromakalim or the selective mitochondrial K_{ATP} activator diazoxide was abrogated in dyslipidaemic cholesterol fed rats.[20] Moreover, in dyslipidaemic Zucker obese rats, the mPTP inhibitor cyclosporine A could not exert cardioprotection.[21] These results indicated that either activation of the NO—cGMP—PKG pathway or K_{ATP} channels, or the inhibition of the mPTP may not be ideal cardioprotective drug targets, because they were ineffective in the presence of dyslipidaemia. Consequently, knowing that the beneficial effects of NAC observed in both acute lower limb ischaemia reperfusion and CLI were obtained in healthy animals, such results could not preclude efficacy in the case of hypercholesterolaemia.[5,8,9,11]

Importantly, NAC still conferred muscle protection in hypercholesterolaemic mice. Indeed, in the present study, targeting oxidative stress by antioxidant administration restored mitochondrial function, in both ApoE+/+ and ApoE—/— mice, supporting the hypothesis that antioxidant interventions would be beneficial in terms of vascular disease prevention.[5] However, the results of clinical studies regarding potential effects of antioxidant administration are disappointing, questioning the relevance of the preclinical hind limb ischaemia models in other situations.[5,6] Conversely, recent studies revealed that some pharmacological targets remain effective even in the presence of hypercholesterolaemia.[19] Thus, pharmacological inhibition of GSK3β allowed muscle protection in both normal and hypercholesterolaemic rats.[22] Consequently, even if the majority of clinical studies show that antioxidant strategies are ineffective, it seems mandatory to clearly define drug targets. By identifying the ideal drug target, the increased susceptibility of skeletal muscle to CLI could be reversed, even in hypercholesterolaemic situations.

This study suffers from several limitations. The role of hypercholesterolaemia on seven month old ApoE—/— mice was investigated, commonly used to simulate the effects of atherosclerosis and dyslipidaemia, but other risk factors and comorbidities, including hypertension, diabetes, and ageing should also be investigated. Another limitation is the small sample size. However, normally distributed continuous data results with small variance between animals in the same groups coupled with valid within animal data pairing statistics provided very high probabilities of rejecting the null hypothesis. Consequently, significant conclusions may be drawn from the data despite small sample size. Another important point is that fast glycolytic muscles were specifically investigated. This choice was based on the fact that muscle susceptibility to ischaemia/reperfusion injury depends on fibre type specific antioxidant level, explaining the greater injuries observed in glycolytic than oxidative muscles.[23] Muscle impairment had to be observed in order to determine eventual protection by NAC. Thus, the data apply only to glycolytic muscles, but this is pertinent since they are key targets to protect. Finally, further study will be useful to determine the molecular mechanisms and the signalling pathways involved in both the hypercholesterolaemia related exacerbation of CLI mitochondrial dysfunction and in muscle protection afforded by NAC.

In conclusion, the current study highlights that hypercholesterolaemia exacerbates CLI mitochondrial dysfunction. Interestingly, NAC kept its protective properties despite hypercholesterolaemia being known to reduce the efficacy of protective pathways. Complementary studies will be useful to further identify the underlying mechanisms at a molecular level allowing precise determination of the "ideal" antioxidant target.

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CONFLICTS OF INTEREST

None.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejvs.2019.03.028.

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