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Original article

Antioxidant effect of sildenafil: Potential hepatoprotection *via* differential expression of mitochondrial proteins in apolipoprotein E knockout mice

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ABSTRACT

Background: High plasma cholesterol levels are able to trigger several pathophysiological events, including inflammation, cell damage and especially oxidative stress. Previously, studies have shown that sildenafil exhibited antioxidant effects in several experimental models. Here we evaluate the role of sildenafil in liver redox equilibrium of apolipoprotein E knockout (apoE-KO) mice.

Methods: ApoE-KO mice were divided in two groups: one group received the PDE5 inhibitor sildenafil (40 mg/kg/day) for 3 weeks (apoE-KO + Sil) and was compared to a second group of apoE-KO mice, which received only the vehicle (water) for 3 weeks (apoE-KO). Control group (C57 mice) received only a standard chow diet. At the age of 18 weeks, mice livers were collected for the measurement of intracellular ROS levels and apoptotic cells by flow cytometry analysis, and mitochondria isolation for proteomic analysis.

Results: Compared to the control group, liver cells from apoE-KO presented some typical redox imbalance features: higher levels of intracellular ROS (global oxidative stress ↑60%, superoxide anion ↑82%, and peroxynitrite/hydroxyl radical ↑53%), higher amounts of apoptotic cells (up to ↑19%) and higher mitochondrial intensity of catalase (+339%) and transferrin spots (+914%). After treatment with sildenafil, apoE-KO presented ROS levels and the number of apoptotic cells similar to those observed in C57. In addition, when compared to apoE-KO, apoE-KO + Sil showed lower spots volumes of catalase (-23%) and transferrin (-71%) and up-regulation of urate oxidase (+94%).

Conclusion: The treatment with sildenafil is able to induce beneficial changes in liver mitochondrial protein dynamics, which restores the redox homeostasis contributing to a potential hepatoprotection.

Introduction

Hypercholesterolemia is one of the most important risk factors for cardiovascular morbidity and/or mortality [1–4], being aggravated by nonalcoholic fatty liver diseases [5]. Both hypercholesterolemia and hepatic steatosis are strongly associated with insulin resistance, visceral obesity and high blood pressure, which are considered hallmarks of metabolic syndrome [6,7].

The most widely used murine model for the study of hypercholesterolemia is the apolipoprotein E knockout mouse (apoE-KO), which spontaneously displays accumulation of

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cholesterol ester-enriched particles in the blood. It has been shown that the progression of the hyperlipidemic process in apoE-KO can be accelerated and aggravated under a Western-type diet ([1] for review).

It is well known that high plasma cholesterol levels are able to trigger several pathophysiological events, including inflammation [8], cell damage [9,10] and increase of oxidative stress [11], the latter being the result of imbalance between the production of oxidant species (mainly reactive oxygen species - ROS) and antioxidant defenses [12]. High levels of ROS can lead to damage in cell components [13], with the mitochondria – as the main source of cellular ROS production [14] – playing a pivotal role in this process.

In previous studies using apoE-KO mice we have shown that hypercholesterolemia increases oxidative stress in different cell types [8–10]. Also, while investigating antioxidant alternative strategies to restore redox homeostasis in this mouse model, we were able to show that sildenafil – a phosphodiesterase 5 (PDE5) inhibitor widely associated with the treatment of erectile dysfunction and pulmonary hypertension – restores endothelial function [15] and prevents Cox-1/TXA2 pathway-mediated vascular hypercontractility [15]. We have also observed that this drug ameliorates biomarkers of genotoxicity in the liver [9], in a renovascular hypertension model [16] and in bone marrow cells [17]. Additionally, sildenafil prevented renal dysfunction in a Wistar rat model of nephropathy induced by contrast media [18].

The effects of sildenafil on the NO-cGMP pathway have been directly implicated in some of the beneficial outcomes aforementioned [21]. However, considering the complexity of mitochondrial oxidative stress processes associated with these disturbances, it stands to reason that a complete mechanism of action for this drug could involve additional pathways. Thus, the understanding of mitochondrial protein dynamics is a crucial step towards the unveiling of the events involved not only in the anti-oxidant effects of sildenafil but also in the overall redox homeostasis of this organelle. Mitochondrial proteomics rises as a powerful tool in the study of metabolic diseases, since it can cover mitochondrial proteins from several metabolic pathways, as the citric acid cycle, fatty acid oxidation and respiratory chain, as well as protein networks involved in stress responses [19].

Therefore, in this study we applied global proteomic profiling to identify changes in the liver mitochondrial proteomes of apoE-KO mice upon treatment with sildenafil. We have also attempted to further explore the effects of sildenafil on liver cells exposed to a non-physiological serum lipid profile in this mouse model. We believe our results could lead to a better understanding of the mechanism of action of sildenafil where redox homeostasis is concerned, potentially contributing also to the search for novel therapeutic strategies for pathophysiological processes elicited by hyperlipidemia.

Material and methods

Animals

Experiments were performed in male wild type (C57Bl/6) and apolipoprotein E knockout (apoE-KO) mice. Animals were bred and kept at automatic controlled temperature (22 °C), humidity (60%) and exposed to a 12/12 h light-dark cycle. All experimental procedures are in accordance with the National Institute of Health guidelines and approved by the Ethics Committee for Animal Research of the university (#07/2010).

At the age of 8 weeks, weighing on average 23 g, the mice were randomly selected as previously described [9,11,15,20]. ApoE-KO mice were fed a Western-type diet (Rhoster, Brazil) from the 8th to the 18th week to accelerate and aggravate the spontaneous hyperlipidemia. On the 16th week apoE-KO mice were divided into two groups: apoE-KO + Sil, in which the PDE5 inhibitor sildenafil (Viagra®, 40 mg/kg/day, by oral gavage) was administered for 3 weeks, and apoE-KO, which received only the vehicle (water) for 3 weeks. C57 mice (control group) received only standard chow diet. At the age of 18 weeks, samples (blood and liver) were collected from all animals for specific analysis.

Measurement of plasma lipids

Blood samples (N = 6) were obtained by puncturing the left ventricle of the anesthetized animals (thiopental sodium, 50 mg/kg). Plasma was separated immediately (800*g* for 10 min) and kept at −20 °C until analysis. Total plasma cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL) and triglycerides levels were determined using commercial colorimetric assay kits (Bioclin, Brazil). Very low-density lipoproteins (VLDL) were estimated by subtracting HDL and LDL from total serum cholesterol.

Determination of intracellular ROS, cell viability, apoptosis and necrosis from liver cells

Liver cells samples were prepared according to [9] and stored at −80 °C until flow cytometry analysis. Although the liver cell pellets were not further purified to remove macrophages and T cells, the Forward Scatter (FSC) *vs*. Side Scatter (SSC) analysis indicated a pattern of size and internal complexity of the cells. A predetermined gate (considering a specific FSC *vs*. SSC for hepatocytes) was applied in the liver cell analysis to reduce the possibility of contamination.

Global oxidative stress and superoxide anion were measured using 2′,7′-dichlorofluorescein diacetate (DCF, 20 mM) and dihydroethidium (DHE, 160 μM) respectively, according to [10]. The measurement of nitric oxide was performed according to [16], using the NO-sensitive fluorescent probe 4,5-diaminofluorescein-2/diacetate (DAF, 2 μM). Highly reactive oxygen species (hROS), as hydroxyl radical and peroxynitrite, were selectively detected by 2-[6-(4′-hydroxy) phenoxy-3Hxanthen-3-on-9-yl] benzoic acid (HPF) according to [21]. Detailed protocols are in supplementary data.

Apoptotic liver cells were quantified by annexin V-FITC and propidium iodide (PI) double staining using the annexin V-FITC apoptosis detection kit (BD Biosciences). Briefly, mice liver cells were washed twice with PBS and the volume adjusted to 500 μL of binding buffer (5×10^5 cells). Annexin V–FITC and PI were added to the cell suspension, which was gently vortexed and incubated for 15 min at room temperature (25 °C) in the dark. Necrotic cells were defined as annexin V−/PI+, late apoptotic or secondary apoptotic as annexin V+/PI+, and annexin V+/PI − cells were recognized as early or primary apoptotic.

In both cases, liver cells were analyzed by flow cytometry using a FACSCanto II (BD Biosciences).

Subcellular fractionation and validation by immunoblotting

Subcellular fractionation was performed to yield isolated mitochondria according to [22], resulting in a "mitochondrial fraction" and a "cytosolic fraction". Both samples were analyzed to evaluate their purity. For more details, see supplementary data.

Two-dimensional electrophoresis (2-DE), gel image analysis and protein spots identification by mass spectrometry

Samples of mitochondrial fraction were used for proteomic approach. The main features of 2-DE and gel image analysis, as well

as spots identification by mass spectrometry, were performed according to [23]. Details of these protocols can be found in the supplementary data.

Statistical analysis

Data are expressed as mean ± SEM. Gaussian distribution of the variables was analyzed using the D'Agostino-Pearson omnibus normality test. Statistical comparisons between the different groups were performed by one-way analysis of variance (ANOVA), followed by Tukey *post hoc* test. Differences between means with a value of $p < 0.05$ were considered statistically significant. Statistical analysis was performed with Prism 7.0, GraphPad Software Inc.

Results

Sildenafil does not influence serum lipid profile of apoE-KO mice

Table 1 presents the plasma lipid profile of the experimental groups. As expected, apoE-KO mice exhibited higher levels of triglycerides (3-fold), total plasma cholesterol (14-fold), LDL (5-fold), VLDL (35-fold) and lower levels of HDL (2-fold) when compared to C57 mice. These results validate the hyperlipidemic animal model used in this work. In accordance with our previous studies [11,15,20] there were no significant alterations on these parameters upon treatment with sildenafil.

Sildenafil reduces intracellular ROS levels and apoptotic cells in the liver of apoE-KO mice

Intracellular ROS levels and apoptotic liver cells were measured by flow cytometry. We observed higher levels of ROS production in apoE-KO mice. Global oxidative stress (Fig. 1A), superoxide anion (Fig. 1B) and hROS (peroxynitrite/hydroxyl, Fig. 1D) in hyperlipidemic animals (1.6, 1.8 and 1.5-fold, respectively) when compared to healthy mice. The number of apoptotic cells (annexin V+/PI- cells) of apoE-KO mice was higher than those observed in C57 group (18.71 ± 1.23% and 2.02 ± 0.19%, respectively) (Fig. 2).

Treatment with sildenafil brought about a clear protective effect, for ROS levels and the number of apoptotic cells were lower than those detected in the apoE-KO group, being similar to the patterns found in the control group.

Mitochondrial proteomics: robust alterations in oxidative stress-related proteins

Before proceeding with the analysis of the mitochondrial proteomes we ensured the accuracy of the enrichment protocol and purity of the mitochondrial fractions. For this, samples were examined by western blotting using antibodies against specific house keeper protein markers, revealing a single band at approximately 17 kDa for Cox-IV in the mitochondrial fraction and also a single band representing GAPDH at approximately 37 kDa in the cytosolic sample (Fig. 3A).

Table 1
Serum lipid profiles of C57, apoE-KO and apoE-KO + Sil animals. Values shown as mean ± SEM, $^*p < 0.05$ *vs.* C57 group, N = 6.

Lipids (mg/dL)	C57	apoE-KO	apoE-KO + sil
Triglycerides	69 ± 7.7	219 ± 14*	236 ± 18*
Total plasma cholesterol	87 ± 5.2	1235 ± 332*	1367 ± 172*
Low-density lipoprotein	32 ± 3.6	178 ± 6,2*	182 ± 2.7*
High-density lipoprotein	40 ± 4.1	19.50 ± 1.0*	20 ± 3.2*
Very low-density lipoprotein	31 ± 6.2	1090 ± 63*	1174 ± 172*

Values shown as mean ± SEM, $^*p < 0.05$ *vs.* C57 group, N = 6.

In order to perform the comparative proteomic analysis, ten 2-DE gels (2 – C57, 4 – apoE-KO and 4 from apoE-KO + Sil – supplementary Fig. S2), were obtained. Within each experimental group, the protein profiles obtained were highly reproducible regarding the total number of protein spots, their intensities and relative positions (correlation coefficient > 0.90).

The factor analysis revealed changes between the C57 and apoE-KO groups regarding the profile of spots distribution. The gels from apoE-KO + Sil group exhibited a profile closer to that of C57 than of apoE-KO group, indicating similarities between apoE-KO mice treated with sildenafil and healthy animals (supplementary Fig. S3). A representative 2-DE gel image of mice liver mitochondrial proteins is shown in Fig. 3B.

A total of 30 spots with different intensities were detected by Image Master 2D Platinum and analyzed by mass spectrometry. The MALDI-TOF/TOF analysis successfully identified 28 spots with a single protein, revealing 12 distinct proteins (supplementary Table 1). In two cases, more than one protein was identified in the same spot and those were excluded from the analysis. After considering the sum of all spots' volumes attributed to the same protein in each experimental group, statistical differences were found in 8 proteins and the relative changes are shown in Fig. 4.

ApoE-KO mice presented higher spots volumes corresponding to catalase (+339%), transferrin (+914%), albumin (+220%), vitamin D binding protein (VBDP, +47%), heat shock protein A5 (HSP A5, +39%) arginase 1 (+208%) and a calreticulin reduction (-83%) when compared to C57 mice. After treatment with sildenafil, robust changes were found in at least three key proteins spots related to oxidative stress: urate oxidase (Uox, +120%), transferrin (-71%) and catalase (-23%), when compared to apoE-KO mice (Fig. 5, Table 2).

Discussion

Over the last decade, our group has extensively employed apoE-KO mice as an oxidative stress research tool. The present study shows for the first time that the antioxidant effects of sildenafil, which can lead to hepatoprotection in this animal model of hyperlipidemia, occur *via* differential expression of liver mitochondrial proteins. Here, we described the effects of this drug on the ROS levels and apoptotic liver cells of apoE-KO mice. In addition, through 2DE/MS—MS analysis, we succeeded in identifying important mitochondrial proteins that are likely to be involved in redox homeostasis.

In a previous work [9], the authors observed high levels of DNA damage in liver cells of apoE-KO mice. Considering that this process is often related to oxidative stress [24], we expected to detect high levels of ROS in liver cells of apoE-KO mice. Accordingly, our results from flow cytometry analysis revealed that apoE-KO liver cells are exposed to a greater redox imbalance, characterized by high levels of global oxidative stress, superoxide anion and peroxynitrite/hydroxyl radical, when compared to C57 mice (Fig. 1), indicating that hyperlipidemia *per se* was able to produce high levels of ROS. Moreover, we found higher numbers of early apoptotic liver cells in hyperlipidemic mice when compared to their healthy counterparts (Fig. 2), suggesting a possible relationship between high levels of ROS and the arising of apoptotic liver cells.

Apoptosis plays a crucial role in the removal of unwanted or transformed cells, and hyperlipidemia is one of the several known factors capable of inducing apoptosis mediated by ROS production [24]. It has been shown that ROS (particularly hydrogen peroxide) are able to oxidize B-cell lymphoma-2 (Bcl-2) – a key regulator of intrinsic apoptosis pathway – consequently inducing apoptosis [25,26]. Our results are in accordance with the aforementioned studies, as the hyperlipidemic mice presented high levels of global oxidative stress (which includes hydrogen peroxide) as well as high

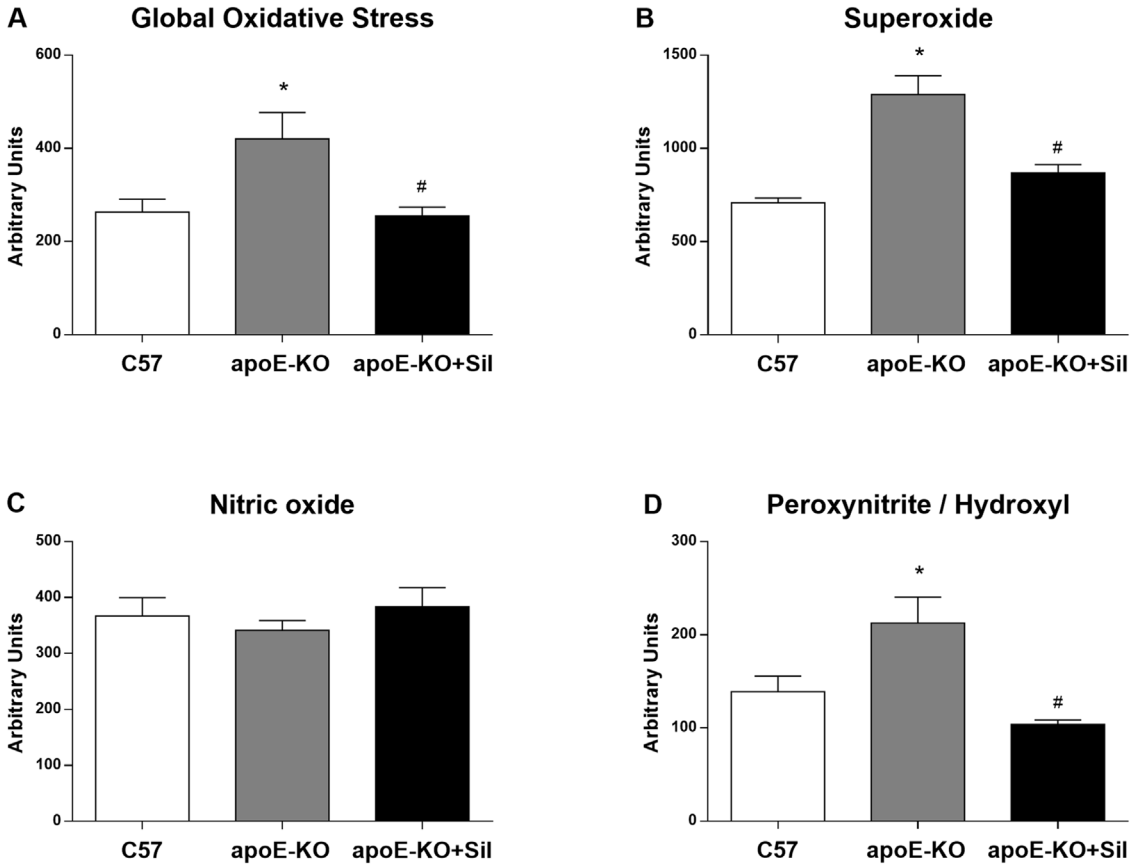

Fig. 1. Sildenafil reduces ROS levels in mice liver cells. Bars represent arbitrary units after flow cytometric analysis of ROS levels: (A) Global oxidative stress, (B) Superoxide anion, (C) nitric oxide and (D) peroxinitrite. ROS were detected using DHE, DCF, DAF and HPF, respectively. Data are presented as means ± SEM; $^{*}p < 0.05$ *vs.* C57, $^{\#}p < 0.05$ *vs.* apoE-KO. C57: control group; apoE-KO: hyperlipidemic mice and apoE-KO + sildenafil: treated animals.

numbers of apoptotic cells. Taken together, our results bring new evidence that the exposure of liver cells to high levels of serum lipids may be harmful to the organ's homeostasis.

We also showed that apoE-KO mice treated with sildenafil presented lower ROS levels in liver cells, as well as lower levels of hepatic apoptosis, when compared to non-treated animals. Similar findings have also been observed in several other tissues after treatment with sildenafil: apoE-KO mice mononuclear cells [9], aorta [11] and bone marrow cells [17]; mouse stenotic kidney cells [16]; and rat kidney cells in a contrast-induced nephropathy model [18].

It is worthy of note that the ROS reduction observed after treatment with sildenafil resembles what is reported for well-known antioxidants, including N-acetylcysteine, reduced glutathione and catalase [25]. These data support our hypothesis that sildenafil has a protective effect against apoptosis in liver cells during oxidative stress.

As mentioned before, our main goal here was to describe the effects of sildenafil on the ROS levels of apoE-KO liver cells in a more comprehensive way. For this purpose, we performed a comparative proteomic approach to study the influence of sildenafil in the liver mitochondrial proteome. In a previous study [27], authors described that changes in liver mitochondrial protein profile – mitoproteome – of apoE-KO mice were accompanied by typical organ damage evidenced by histological alterations. In the present work we did confirm liver oxidative stress in apoE-KO mice and the benefits of sildenafil, but, the main novelty revealed here by mitoproteomic analysis was the modulation of proteins related to redox homeostasis (Fig. 5, Table 2).

Protein spots corresponding to catalase were overexpressed in the apoE-KO group (4.4-fold higher) when compared to C57 group. This enzyme plays a crucial role in redox homeostasis, as it promotes hydrogen peroxide degradation. The high levels of catalase found in apoE-KO group are consistent with elevated circulating lipids levels, which should lead to H_2O_2 production from organelles that oxidize fatty acids, such as mitochondria. In addition, our results are in agreement with a previous report describing an increase in both activity and expression of cardiomyocytes mitochondrial catalase from high-fat feeding mice [28], being therefore considered an antioxidant response.

We also identified overexpression of transferrin spots volumes (10.3-fold higher) in apoE-KO group compared to C57. This glycoprotein is expressed by the liver and secreted in the plasma, driving iron into the cells to participate in several physiological processes [29]. However, the reduced form of iron (Fe^{2+}) catalyzes the conversion of hydrogen peroxide into the highly reactive hydroxyl radical that is capable of damaging several cellular structures [28].

We were indeed surprised to detect transferrin in a mitochondrial enrichment sample. This result may be explained by a recently detailed mechanism of iron delivery, named kiss-and-run [30]. In this process, an endosome is formed after transferrin-iron binding to its cognate membrane receptor (Tf-R), resulting in iron release through a direct interaction with mitochondria.

Thus, the presumable increase in the iron supply to the mitochondria could be related to the high levels of ROS and elevated numbers of apoptotic cells observed in the apoE-KO group. These results are in accordance with a previous study showing that iron

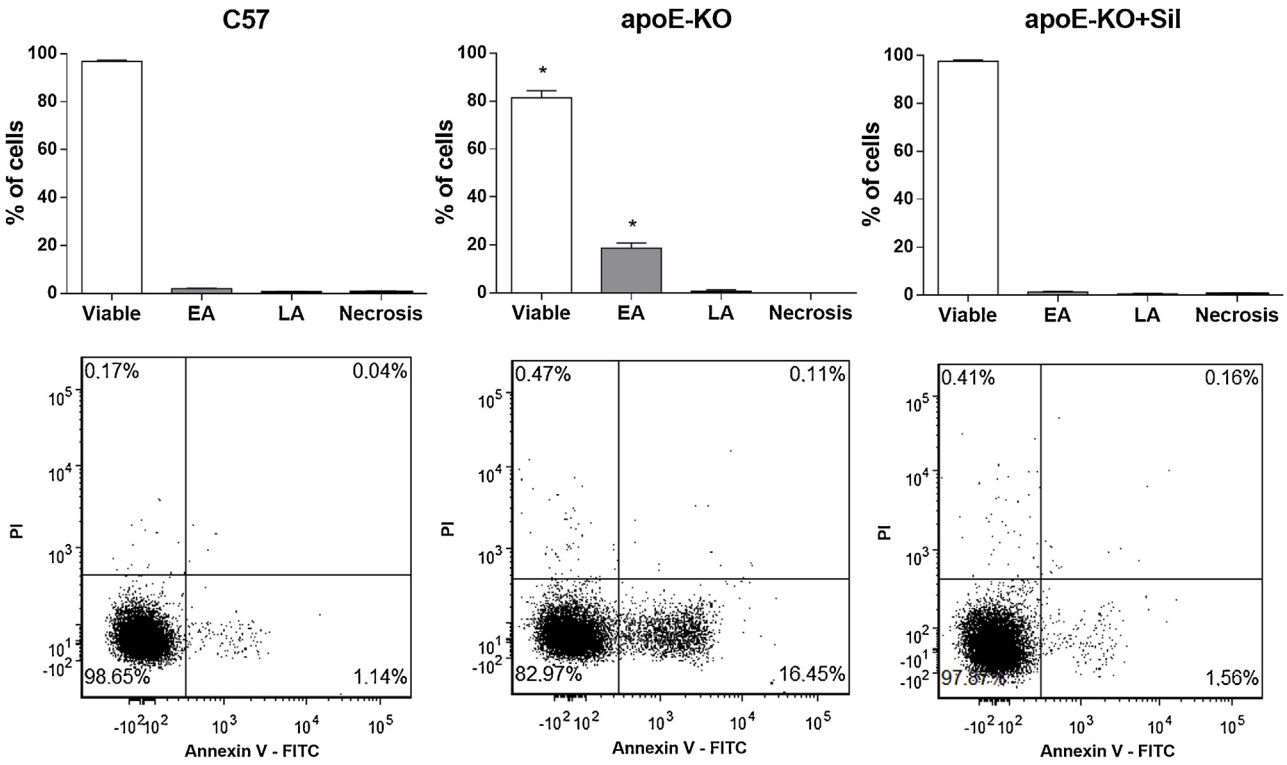

Fig. 2. Apoptosis pattern in mice liver cells. Bars show the average percentage of mice liver cells from four stages: Viable, early apoptosis (EA), late apoptosis (LA) and necrosis detected by annexin V - FITC and PI staining. Below each graph are depicted the respective representative dot plot panels. Values are presented as mean ± SEM; $^{*}p < 0.05$ *vs*. C57.

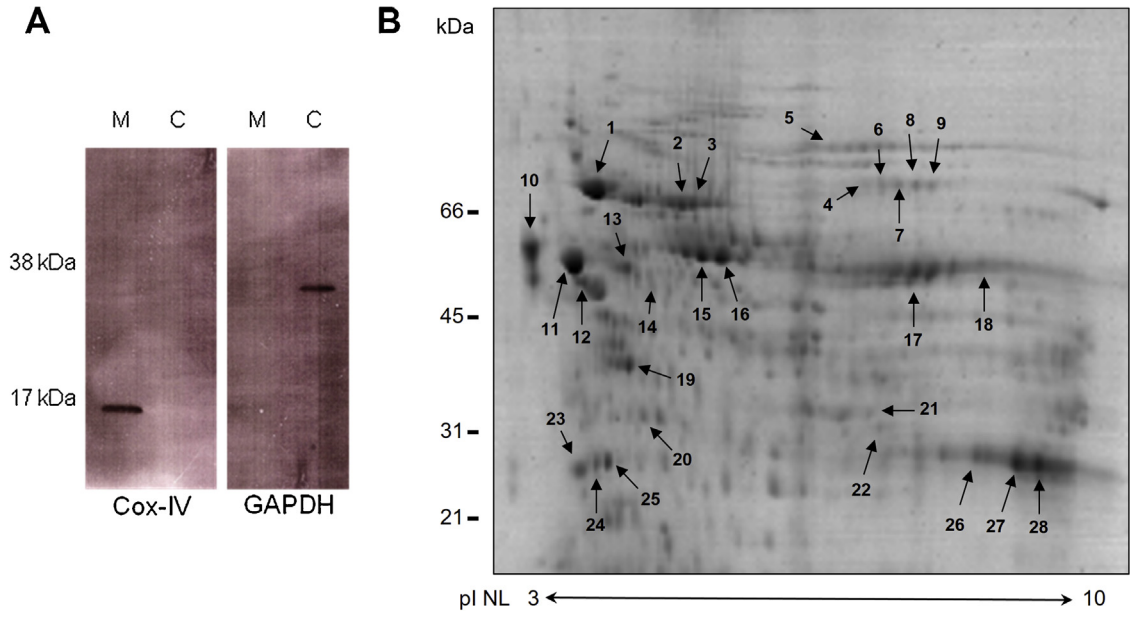

Fig. 3. Mitochondrial proteins separation. (A) Purity of mitochondrial fraction assessed by Western Blot of Cox-IV and GAPDH proteins. M and C columns: mitochondrial and cytosolic fractions respectively. (B) Representative 2-DE map of liver mitochondrial proteins of C57 mice.

overload promotes apoptosis in erythroid cells by inducing the expression of apoptotic protease-activating factor 1 [31].

After treatment with sildenafil, we observed mild reduction of the catalase spots volume (22%) and a drastic reduction of transferrin spots volume (70%). These results could be associated with the reduction of liver oxidative stress observed in apoE-KO + Sil group, corroborating the protective effect proposed for sildenafil, besides suggesting that transferrin plays a key role in ROS production.

It has been described that under pro-inflammatory conditions, such as hyperlipidemia and oxidative stress, some transcription factors are activated in order to synthesize xantine oxidase (XO) [32]. This enzyme converts hypoxanthine to xanthine and xanthine to uric acid, the final product of purine catabolism. In addition, it has been

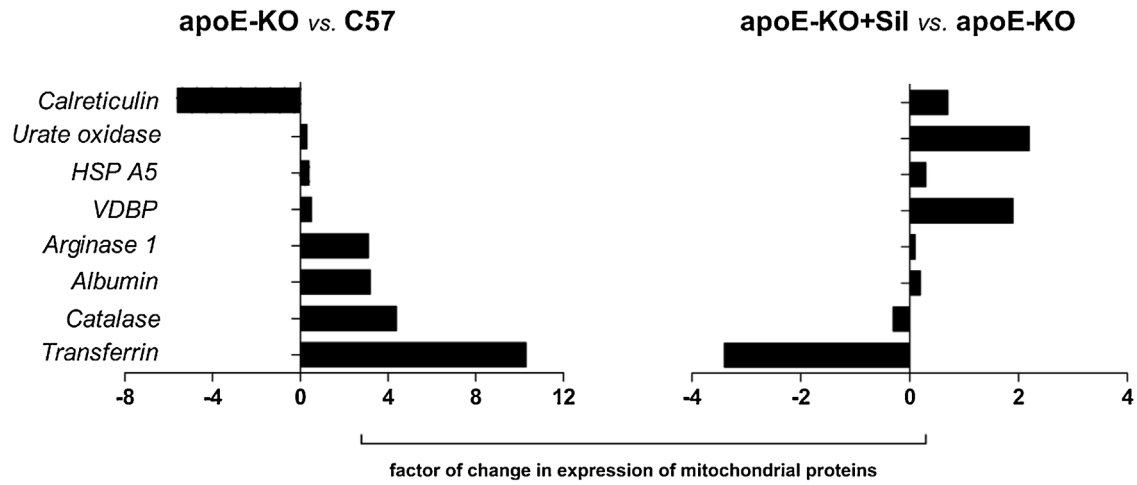

Fig. 4. Relative changes in liver mitochondrial protein spots volume. Left panel: differences between healthy (C57) and hyperlipidemic (apoE-KO) mice; right panel: protein spots volume pattern after treatment with sildenafil (apoE-KO + sil).

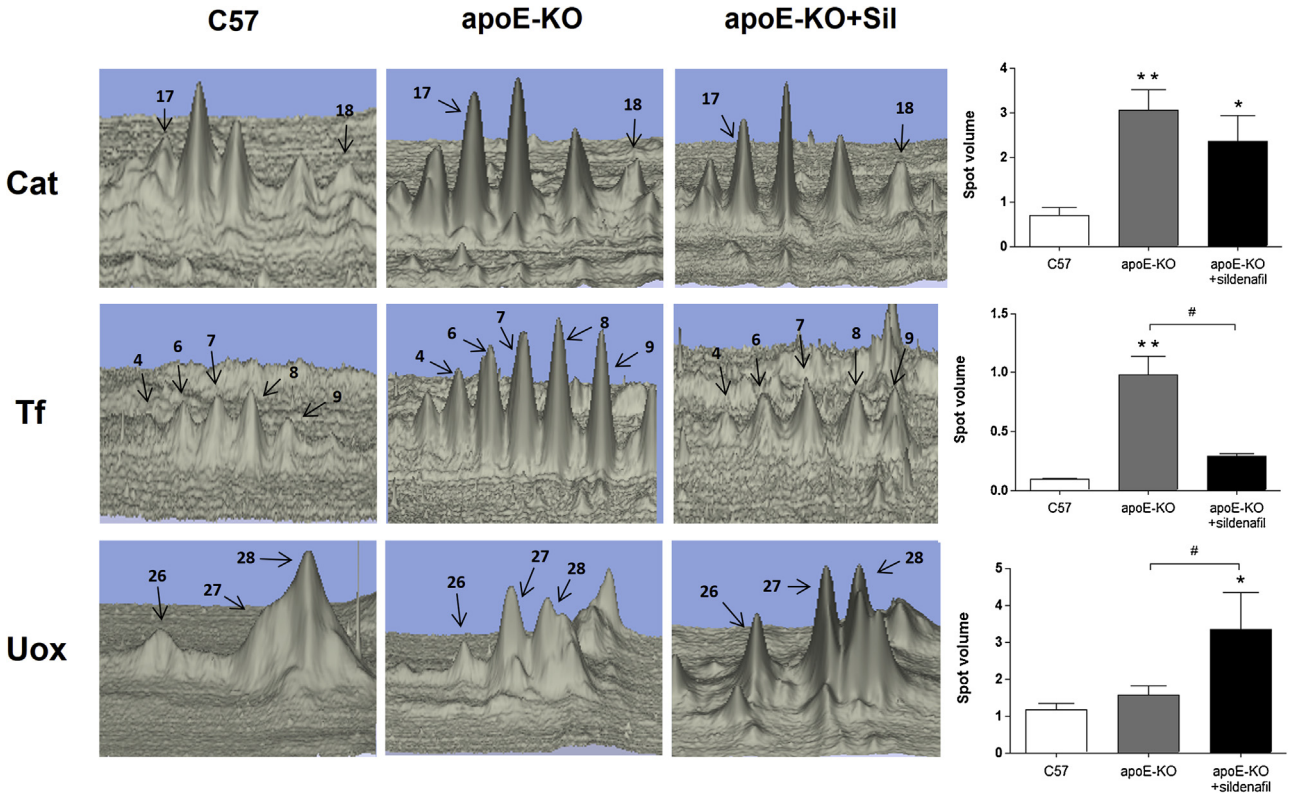

Fig. 5. Effects of sildenafil treatment on liver mitochondrial protein expression. 3D views of spots corresponding to Catalase (Cat), Transferrin (Tf) and Urate oxidase (Uox). Bars show the average of spot volumes. Values are presented as mean ± SEM; $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* C57, $^{\#}p < 0.05$ indicates differences between apoE-KO and apoE-KO+ sildenafil groups.

Table 2
Protein spots identified from mice liver mitochondria related to oxidative stress. pI = Isoelectric point, GO = Gene Ontology.

Spot No.	Protein (UniProtKB accession number)	Molecular Mass (kDa)	pI	Protein Coverage (%)	Protein Score	GO - Biological Process
4, 6, 7, 8, 9	Transferrin (**AAH08559.1**)	70.9	6.3	8	332	Ferrous iron import across plasma membrane
17, 18	Catalase (**NP_033934.2**)	60.1	8.1	24	918	Aging; Hydrogen peroxide catabolic process; Cellular response to ROS
26, 27, 28	Urate Oxidase (**EDL11965.1**)	23.5	8.7	17	289	Purine nucleobase metabolic process

pI = Isoelectric point, GO = Gene Ontology.

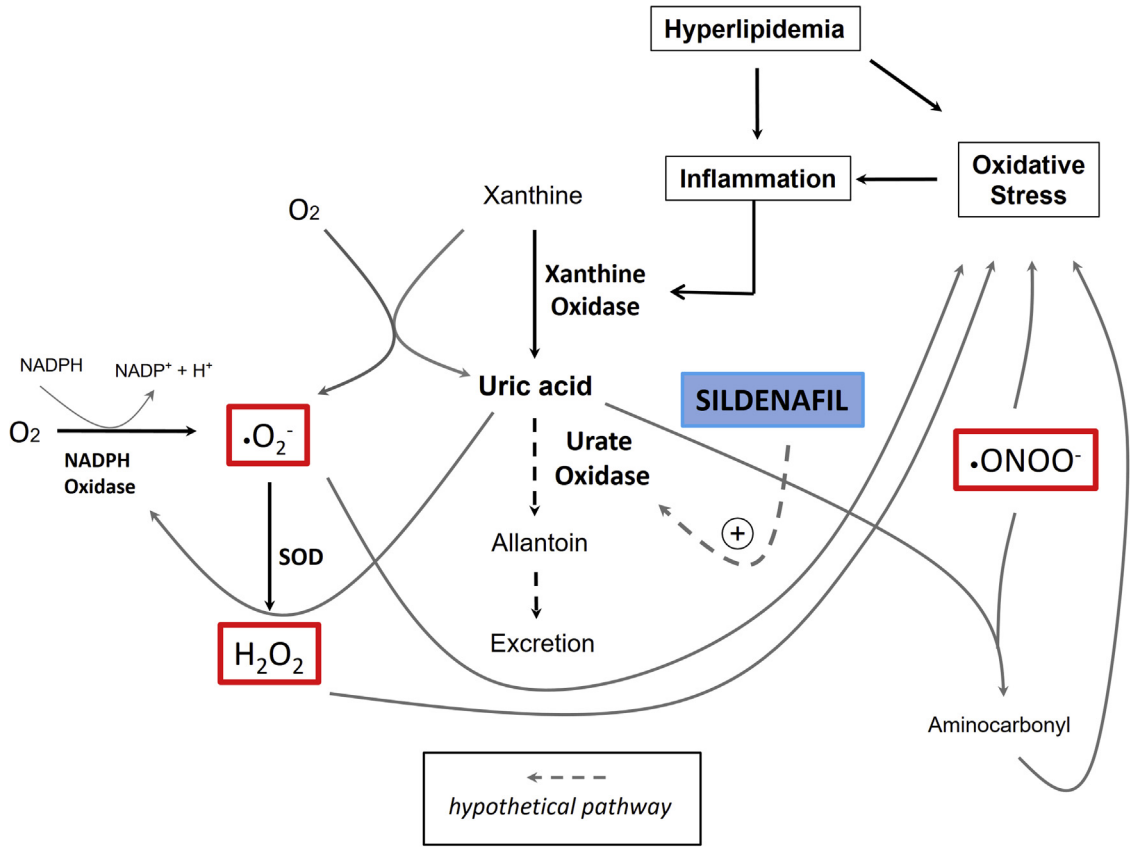

Fig. 6. Proposed model for the mechanism of sildenafil antioxidant action. Uric acid plays a pivotal role on ROS formation. Mice treated with sildenafil displayed over-expression of urate oxidase, an enzyme that degrades uric acid forming allantoin, a low reactivity molecule. SOD: superoxide dismutase.

also demonstrated that high levels of uric acid are strongly associated with several metabolic diseases [33], and, although their pathogenesis is extremely complex and incompletely understood, it is well established that oxidative stress is common in all of them [34,35].

Uric acid plays a pivotal role in free radical supply in a variety of radical-forming systems. Aminocarbonyl radical is one of the most reactive uric acid-derived radicals identified so far, being formed after breakdown of the uric acid structure due to the peroxinitrite nucleophilic attack [36]. Further evidence indicate that uric acid is capable of increasing the enzymatic activity of NADPH oxidase [33], which is well known as an important source of superoxide anion, therefore supporting the pro-oxidative effect of uric acid.

In vertebrates – except humans – uric acid is further degraded by urate oxidase (Uox) into allantoin, which is excreted in the urine and presents low reactivity [36]. Therefore, Uox prevents build-up of uric acid and its toxic effects, being considered an antioxidant enzyme. Interestingly, we also observed a large increase in the urate oxidase spots volume in treated animals, 2.2- and 2.9-fold compared to hyperlipidemic and healthy mice, respectively. This up-regulation of Uox – and the likely reduction of uric acid levels – may orchestrate a set of protective events against oxidative stress: the reduction of uric acid bioavailability may be associated to the reduction of NADPH oxidase activity, leading to lower levels of superoxide anion production, as well as lower production of the uric acid byproduct, aminocarbonyl, after reaction with peroxinitrite.

Thus, based on the aforementioned considerations, we propose that the high levels of Uox observed in apoE-KO + Sil could be associated to redox homeostasis, since Uox can prevent the toxic effects attributed to uric acid. Fig.6 summarizes our hypothesis.

In conclusion, the present study indicates that the antioxidant effect of sildenafil involves the degradation of uric acid, besides the well-known NO-cGMP pathway. We have also shown that this drug affects the expression of catalase and transferrin in the liver of apoE-KO mice, which are also related to the redox homeostasis in the mitochondria. In addition, we add to the growing body of evidence placing sildenafil as an antioxidant drug, as it markedly decreased the production of ROS in liver cells of apoE-KO mice, interfering also with the apoptotic process observed in these cells. We believe our results contribute a great deal to the better understanding of the antioxidant effect of sildenafil, potentially aiding also in the search for novel therapeutic strategies against pathophysiological processes elicited by hyperlipidemia.

Conflict of interest

None.

Contribution statement

TNM carried out experimental analysis, acquisition, analysis and interpretation of the data and drafted the manuscript. GBN, ABM, MAL, MLP, ATF and JP participated in data acquisition, analysis and interpretation. SSM, ECV and SGF contributed to the conception, design and supervision of the study and interpretation of data. All authors read and approved the final version of the manuscript.

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reagents. Both institutions have no involvement in study design, analysis and interpretation of data and/or in the writing of the manuscript.

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