Native LDL-induced oxidative stress in human proximal tubular cells: multiple players involved

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Abstract

Dyslipidemia is a well-established condition proved to accelerate the progression of chronic kidney disease leading to tubulo-interstitial injury. However, the molecular aspects of the dyslipidemia-induced renal damage have not been fully clarified and in particular the role played by low-density lipoproteins (LDLs). This study aimed to examine the effects of native non-oxidized LDL on cellular oxidative metabolism in cultured human proximal tubular cells. By means of confocal microscopy imaging combined to respirometric and enzymatic assays it is shown that purified native LDL caused a marked increase of cellular reactive oxygen species (ROS) production, which was mediated by activation of NADPH oxidase(s) and by mitochondrial dysfunction by means of a ROS-induced ROS release mechanism. The LDL-dependent mitochondrial alterations comprised inhibition of the respiratory chain activity, enhanced ROS production, uncoupling of the oxidative phosphorylation efficiency, collapse of the mtΔΨ, increased Ca2+ uptake and loss of cytochrome c. All the above LDL-induced effects were completely abrogated by chelating extracellular Ca2+ as well as by inhibition of the Ca2+-activated cytosolic phospholipase A2, NADPH oxidase and mitochondrial permeability transition. We propose a mechanistic model whereby the LDL-induced intracellular redox unbalance is triggered by a Ca2+ inward flux-dependent commencement of cPLA2 followed by activation of a lipid- and ROS-based cross-talking signalling pathway. This involves first oxidants production via the plasmamembrane NADPH oxidase and then propagates downstream to mitochondria eliciting redox- and Ca2+-dependent dysfunctions leading to cell-harming conditions. These findings may help to clarify the mechanism of dyslipidemia-induced renal damage and suggest new potential targets for specific therapeutic strategies to prevent oxidative stress implicated in kidney diseases.

Keywords: Low density lipoproteins • kidney proximal tubular cells • reactive oxygen species • mitochondria • NADPH oxidase • cytoplasmic phospholipase A2 • chronic kidney disease • redox signalling • lipid signalling • ROS-induced ROS release.

Introduction

Oxidized low-density lipoproteins (oxLDL) are proved to exert their role in the multi-steps atherogenetic process much more pronouncedly than native LDL (nLDL) [1, 2]. Although the chemical nature of the LDL oxidized products and their specific effects have not yet been identified, nevertheless the occurrence of different receptors for nLDL and oxLDL suggests a distinct mechanism of action [3]. Vascular endothelium is not the unique target of LDL pathogenetic properties. Indeed oxLDL have been recently shown to elicit inflammatory and fibrotic processes also in extra-vascular tissues such as renal mesangial and tubular epithelial cells [4, 5].

Under normal physiological conditions, the glomerular filtrate contains almost undetectable amount of lipoproteins. However, in chronic kidney disease (CKD), which is hallmarked by a progressive impairment of the glomerular barrier permeselectivity, the renal tubule can be exposed to high molecular-weight molecules [6]. Hyperlipidemia has been proved to accelerate the progression of the CKD inducing a tubulo-interstitial injury [7, 8]. Evidence of oxLDL-induced oxidative stress has been provided supporting the idea that alteration of the reactive oxygen species (ROS) homoeostasis is an early event in the oxLDL-related diseases priming the subsequent tissue/organ disfunction [3, 4, 9, 10].
The overwhelming body of evidence supporting the deleterious effects of oxLDL has weakened the interest on unmodified nLDLs, which, however, still deserve attention as their interaction with extra-vascular tissues has been poorly characterized. On these grounds the present study investigated the effect of nLDL exposure on an in vitro model of proximal tubular epithelium with specific focus on the cellular oxidative metabolism. We demonstrate for the first time that non-oxidized nLDL elicit dysregulation of the cellular oxidation state by activating a redox signalling between different ROS-producing compartments in the cell. The role of altered ROS homeostasis in the development of LDL-related kidney damage and possible therapeutic interventions are discussed.

Materials and methods

Cell culture

HK-2 cells (ATCC, Manassas, VA, USA), which are normal proximal renal tubular epithelial cells immortalized by transduction with the human papilloma virus 16 E6/E7 genes, were cultured in DMEM/F12 (Sigma-Aldrich, Milan, Italy) medium supplemented with penicillin (50 U/ml) and streptomycin (50 mg/ml) and with 10% heat-inactivated foetal calf serum (FCS) (Sigma). Cultured cells were grown in monolayers at 37 °C in a humidified atmosphere containing 5% CO₂.

Lipoprotein separation

LDL (d = 1.020–1.050 g/l) were separated from fasting normolipidemic plasma by sequential ultracentrifugation. The lipid (total and free cholesterol, triglycerides and phospholipid) content of lipoprotein fractions was measured by enzymatic techniques. The protein content was measured by the Lowry assay. Purified lipoprotein fractions were stored at 4 °C in sodium bromide with 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4 at 37 °C before use.

Lipoprotein fractions were washed in PBS, harvested by centrifugation and immediately assessed for O₂ consumption by a Clark-type electrode (Hansatech) in a humidified atmosphere containing 5% CO₂.

Measurement of cell respiration and NADPH oxidase activity

Cultured cells were gently detached from the dish by trypsinization, washed in PBS, harvested by centrifugation and immediately assessed for O₂ consumption by a Clark-type electrode (Hansatech) in a thermostated gas-tight chamber equipped with a stirring device. A total of 3 × 10⁶ viable cells/ml were assayed in 50 mM KPi, 10 mM Heps, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4 at 37 °C, after attainment of a stationary endogenous substrate-sustained respiratory rate, 2 μM/mi of oligomycin was added. The rates of O₂ consumption were corrected for 5 mM KCN-insensitive respiration. The respiratory control ratio (RCR) was obtained dividing the rates of the oxygen consumption achieved before and after the addition of oligomycin. NADPH oxidase activity was assessed by following the reduction of extracellular acetylated-cytochrome c (SIGMA). Briefly, 20 μM ferri-cytochrome c was directly added to the cell cultures 60 min before the end of the nLDL-incubation times. At the desired time-points 100 μl of the culturing medium was transferred to a microcuvette and the reduction level of ferrocyanochrome c evaluated by the absorbance in the triple-wavelength mode (A540–(A540–A550)) using a Δε = 19.1 mM⁻¹cm⁻¹. In a pilot testing with a 24 hrs-nLDL-treated cell sample the absorbance of ferrocyanochrome c increased linearly up to 90 min. The values attained were corrected for those obtained in parallel nLDL-treated cell samples but supplemented with superoxide dismutase (SOD) (500 U/ml) in the culturing medium.

Immunocytochemistry

HK-2 cells cultured at low density on fibronectin-coated 35 mm glass bottom dishes were fixed for 20 min at 37 °C with the following probes: 0.5 μM nonyl acridine orange (NAO) for the mitochondrial mass; 2 μM tetramethylrhodamine, ethyl ester (TMRE) for the mitochondrial membrane potential (mtr ΔΨ); 0.5 μM MitoSOX or 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCF-DA) for mitochondrial O₂⁻⁻ and cellular H₂O₂, respectively; 5 μM X-Rhod-1 AM for mitochondrial Ca²⁺. All the probes used were from Molecular Probes (Eugene, OR, USA). Stained cells were washed with PBS and examined by a Nikon TE 2000 microscope (images collected using a 60× objective (1.4 NA)) coupled to a Radiance 2100 dual laser (four-lines Argon–Krypton, single-line Helium–Neon) confocal laser scanning microscope system (Biorad). Confocal planes (18–20) of 0.2 μm in thickness were examined along the z-axes, going from the top to the bottom of the cells. Acquisition, storage and analysis of data were performed with LaserSharp and LaserPix software (Biorad) or ImageJ (NIH, Bethesda, MD, USA). Quantification of the emitted fluorescent signal was achieved by averaging the pixel intensity values within the outline of single cells, as a function of each focal plane. Correction was made for minimal background in cell-free fields. The integrated value of the xz profile was taken as a measure of the fluorescence intensity and quantified in arbitrary units. At least 20 cells were randomly selected in each of 8–10 different optical fields under the indicated conditions and statistically analysed.

Statistical analysis

Two tailed Student’s t-test was applied with a P < 0.05 to evaluate the statistical significance of differences measured throughout the data-sets reported.
Results

Native LDL cause enhanced ROS production in HK-2 cell line

LDLs isolated from healthy donors were tested for their oxidation state by electrophoretic mobility shift assay and UV spectrophotometry (see supplementary material and Fig. S1A,B). The results obtained indicated that the isolated LDLs did not show detectable evidence of oxidative modifications and remained as such under the experimental settings of the present study. Indeed, incubation of HK2 cell line with native LDL, for different intervals up to 24 hrs, did not modify significantly their oxidation state. Therefore the effects described hereafter are bona fide referred as elicited by non-oxidized native LDL. Twenty-four hours treatment of HK-2 cell line with nLDL (100 μg protein/ml) did not alter their viability (assessed by Trypan-Blue assay) neither caused marked morphological changes. Conversely similar treatment with in vitro-oxidised LDL caused profound alterations in the cultured cells diagnostic of induced-distress (supplementary material, Fig. S2A).

However when tested by the Annexin V/fluorescein diacetate assay the nLDL-treated HK2 cell line showed evidence of early signs of apoptosis as compared to untreated cells (supplementary material, Fig. S2B). As expected, incubation of HK2 with oxidised LDL revealed indication of late apoptosis/necrosis. The concentration of the nLDL used throughout this study (i.e. 100 μg/ml) was comparable with that used in studies aimed to assess the in vitro effect of oxLDL on endothelial cells and within the physio-pathological range in the glomerulo-filtrate of hyperlipidemic patients.

Figure 1A illustrates the effect of nLDL-treatment of HK-2 on ROS production as assessed by confocal microscopy performed with ROS-specific fluorescent probes. It is shown that 100 μg/ml of nLDL-treatment for 24 hrs caused a large increase of the dichlorofluorescein (DCF)-related fluorescence signal over the basal level, diagnostic of intracellular production of H2O2. This was fully prevented by N-acetyl cysteine (NAC) and not observed following treatment of HK-2 with 15 mg/ml of albumin. MitoSox, a probe sensing intramitochondrial O2•− production [11], failed to detect evidence of increased ROS-production. However, reassessment of the nLDL-induced DCF fluorescence under different instrumental settings resulted in brighter spotting in sub-cellular compartments clearly resembling the mitochondrial network morphology (Fig. 1B).

The LDL-induced ROS production was dose-dependent (Fig. 1C) with a 3.5 fold increase over the basal level already detectable at a concentration as low as 12.5 μg nLDL/ml, which levelled off at values of eight to ninefold at 25–100 μg LDL/ml. The nLDL-induced ROS overproduction was not due to down-regulation of the main anti-oxidant enzymes (SOD1, SOD2, catalase, GPX1, GPX4; supplementary material, Fig. S3).

Six and twelve hours of nLDL-incubation caused oxidative changes that did not restore after removal of nLDL from the culturing medium and persisted or even increased at 24 hrs. On the other hand, 3 hrs of exposition to nLDL did not cause apparently any actual or delayed effect on ROS production (Fig. 1D).

nLDL-Treatment of HK-2 cells caused mitochondrial dysfunction

Because MitoSox accumulates into the mitochondria by a trans membrane potential (mtΔΨ)-driven process [11], we tested the possibility that nLDL-treatment affected the mtΔΨ. To this aim we used tetramethylrhodamine ethyl ester (TMRE) a sensitive mtΔΨ-probe. Figure 2A shows that although the nLDL-treatment did not change significantly the mitochondrial mass and its overall morphology (assessed by the cardiolipin dye 10-N-NAO), nevertheless it caused a dramatic decrease of the mtΔΨ. This was further verified by high-resolution respirometry on intact cells. Figure 2B shows that nLDL-treatment resulted in a slight, although significant, decrease of the endogenous resting oxygen consumption rate. This, in the presence of oligomycin, a specific inhibitor of the mtΔΨ-driven H+–FoF1 ATP-synthase, was much higher in nLDL-treated than in untreated HK-2 cells. As a consequence a drop in the respiratory control coupling ratio (RCR) [12] was observed in treated cells.

The time-course of the effect of nLDL on the ΔΨ decline showed that it slightly progressed within 3–6 hrs to accelerate thereafter (Fig. 2C). Parallel detection of ROS formation displayed a significant DCF-fluorescence signal occurring after 6 hrs of nLDL-incubation. Importantly, when mitochondrial O2•− generation was assessed by MitoSox a clear spotted fluorescence signal was evident at 6 hrs which progressively disappeared at longer time of nLDL-treatment. This observation indicated that at relatively short time of nLDL-incubation the mtΔΨ, although reduced, still drove the electrophoretic MitoSox probe accumulation allowing to detect formation of mitochondrial O2•− . A severe collapse of the mtΔΨ, as that attained after 12–24 hrs of nLDL-treatment, impaired the mitochondrial accumulation of MitoSox. Mitochondrial ROS-generation was, however, still occurring as displayed by the mtΔΨ-independent DCF probe. Thus this result supported the direct involvement of mitochondria as source of superoxide and peroxide because the early steps of the nLDL-induced change in the cellular redox-state.

nLDL-Induced ROS production in HK-2 cells was prevented by inhibition of NAD(P)H oxidase

Besides mitochondria, the membrane-bound NADPH oxidase (NOX) constitutes an additional cellular source of ROS [13] and its activation has been extensively reported for endothelial cells upon exposure to oxLDL [14]. To ascertain the possible involvement of NOX to the observed nLDL-induced ROS production we tested the effect of pharmacological inhibitors. Figure 3A shows that co-incubation of nLDL with diphenyleneiodonium (DPI), a widely used pan-inhibitor of flavo-enzymes [15] or with apocynin, a more
Materials and Methods. The fluorescence intensities of nLDL-treated HK-2 cells (black squares) were normalized to that of untreated cells and represent the average ± standard error of means (S.E.M.) of three independent experiments (n = 3) together with statistical analysis. The effect of NAC coinubation with 100 μg/ml of nLDL is also shown as light-grey square. (

Effect of LDL wash-out on ROS production. HK-2 cells were treated with 100 μg/ml nLDL for 3, 6 and 12 hrs after that the cells were washed with a nLDL-free medium and maintained in culture for further 21, 18 and 12 hrs, respectively. Images of a representative experiment is presented showing the DCF-related fluorescence recorded before each nLDL wash-out and at 24 hrs from the beginning of the treatment. The histogram shows the quantitative analysis of the DCF fluorescence intensity. White bar, untreated cells; green bars, cell treated with nLDL for 3, 6 and 12 hrs; grey bars, cells incubated with nLDL for 3, 6 and 12 hrs, washed out and analysed after 24 hrs from the beginning of the treatment. Each bar is the average of three independent experiments ± S.E.M.; where indicated the statistical significance is shown. Bars inside all the micrographs: 30 μm.

Fig. 1 Treatment of HK-2 with nLDL results in unbalance of the cellular redox state. (A) LSCM for detection of H2O2 and mitochondrial O2− by the fluorescent probes DCF and MitoSox respectively. HK-2 cells were incubated for 24 hrs with 100 μg/ml nLDL alone or in the presence of 20 mM NAC or 15 mg/ml albumin. Untreated HK-2 cells were used as control. See Materials and Methods for details. Exciting Argon laser beam for DCF-related fluorescence was set at 5% of its maximal intensity and the PMT gain at 60%. Representative of at least four different preparations in each condition. (B) LSCM analysis of the DCF-related fluorescence and false-colours imaging. nLDL-treatment of HK-2 as in (A). The exciting Argon laser beam was set at 5% of its maximal intensity and the PMT gain at 30%. A false colours rendering of the enlarged detail was generated by ImageJ 1.38x (http://rsb.info.nih.gov/ij/). (C) Dose-dependence of DCF fluorescence. HK-2 cells were treated with the indicated concentrations of nLDL for 24 hrs and the DCF-related fluorescence recorded by LSCM as described in...
selective inhibitor of NOX [16] resulted in the complete abrogation of the ROS-linked DCF signal. Consistently, measurement of the SOD-inhibitable superoxide generation by the externally added cytochrome c reduction assay resulted in a progressive increase starting at 6 hrs of nLDL-incubation. Importantly, the extra-production of external superoxide over the basal level was either DPI- and apocyin-sensitive (Fig. 3B), thus supporting their attribution to NADPH oxidase activity.

NOX isoforms have a distinct cellular localization in the kidney [17]. RT-PCR analysis unveiled in HK-2 major expression of the NOX1 and NOX4 isoforms and differential absorbance spectrophotometry allowed to assess the presence of a catalytically active NOX-related b-type cytochrome (supplementary material, Fig. S4). Twenty-four hours treatment of HK-2 with nLDLs resulted neither in changes of the NOXs expression level nor in NOX-linked b-type cytochrome amount (data not shown).

LDL-linked mitochondrial ROS production in HK-2 cells is induced by NOX-related ROS signaling

Because NOXs release $O_2^{\cdot-}$ in the extracellular space and the probes used detected LDL-linked intracellular ROS production we pondered that ROS released by NOX acted as messengers triggering mitochondrial ROS generation. To test this hypothesis the HK-2 cells were co-incubated with nLDL and the membrane-impermeant ROS scavengers superoxide dismutase (SOD) and catalase (CAT). As shown in Figure 4A SOD and CAT prevented completely the nLDL-induced ROS generation when added together and largely when tested separately. This indicated that the effects of $O_2^{\cdot-}$ and of its dismutation product $H_2O_2$ were largely interchangeable.

As proof of principle we induced a mitochondrial oxidative stress by incubating HK-2 with myxothiazol plus oligomycin a condition forcing the respiratory chain to release ROS [18] and tested on it the effect of extracellular anti-oxidant scavengers. Figure 4B shows that under this condition SOD and CAT were unable to prevent the endogenous mitochondrial ROS production. Moreover, we tested the effect of externally added sub-cytotoxic concentration of $H_2O_2$ to untreated HK-2 on peroxide and mitochondrial superoxide production. As shown in Figure 4C, 6 hrs of incubation with 20 $\mu$M of the membrane permeant $H_2O_2$ resulted in enhanced generation of both peroxide and mitochondrial superoxide which was fully prevented by inhibition of the respiratory chain complexes. This shows that the DCF fluorescent signal was not trivially due to diffusion into the cell of the externally added $H_2O_2$ and that the mitochondrial respiratory chain was the main target and source of the observed $H_2O_2$-induced ROS release.

To further provide evidence that mitochondrial ROS production was triggered by NOX activation, HK-2 cells were incubated for 6 hrs with nLDL and the ensuing enhanced DCF staining was image processed. The intracellular fluorescence was thresholded in order to eliminate the low-intensity cellular signal. Following this procedure a networked high-intensity fluorescence signal emerged that could be clearly attributable to the mitochondrial subcellular compartment (Fig. 5A). The co-incubation of nLDL-treated HK-2 cells with either apocyin or SOD or the membrane-permeant anti-oxidant Tempol resulted in a practically complete prevention of the mitochondria-linked DCF fluorescence signal when the images were processed under identical conditions. Interestingly, co-incubation of HK-2 with nLDL and catalase alone resulted in residual mitochondrial DCF signal over the basal level. This suggests that the extracellular superoxide was somehow more efficient than peroxide in mediating the ROS-induced ROS production.

A recent report showed that $O_2^{\cdot-}$ can be transported by members of the chloride channel (ClC) [19]. As the renal proximal tubular cells express several members of the ClC family [20], we tested the effect of 4,4’-disothiocyanostilbene-2,2’-disulfonic acid (DIDS), a commonly used inhibitor of the ClCs [17]. Figure 5B shows that DIDS largely prevented the LDL-linked ROS production thus suggesting a role of ClCs in mediating the ROS-induced ROS release.
of the effect of nLDL on mtΔΨ, and H2O2 and mitochondrial O2•− production. HK-2 cells were treated with 100 μg/ml of nLDL for the indicated incubation times, then stained with the fluorescent probes and analysed by LSCM as described in Materials and Methods. An enlargement of the micrograph relative to the MitoSox-treated cells incubated for 6 hrs with nLDL is also shown. The lower graph shows the quantitative analysis of the fluorescence intensity for each probe as a function of the nLDL-time of incubation; the values are the averages ± S.E.M. (n = 3 for each condition). The statistical significance with respect to untreated HK-2 cells is also reported. Bars inside all the micrographs: 30 μm.
Fig. 2 Continued.
nLDL-Induced ROS production was dependent on extracellular Ca\(^{2+}\) and activation of cPLA2

Stimulation of the Ca\(^{2+}\)-dependent cytoplasmic phospholipase A2 (cPLA2) with release of arachidonic acid (AA) is a process known to activate NOX [13, 21]. Therefore, we tested the effect of arachidonyl trifluoromethyl ketone (AACOCF3), an inhibitor of cPLA2, on the nLDL-induced ROS overproduction. Figure 6A clearly shows that AACOCF3 abrogated completely the DCF-fluorescence in nLDL-treated HK-2. Moreover, either chelation of the external Ca\(^{2+}\) by EGTA and treatment with the Ca\(^{2+}\)-channel blocker verapamil caused, similarly, a depression of the nLDL-induced ROS production. Importantly AACOCF3 prevented the nLDL-dependent extra-cellular production of superoxide (Fig. 6B) supporting the occurrence of NOX activation. These results indicated that the pro-oxidant effect of nLDL on HK-2 was largely mediated by the reaction products of the cPLA2 whose activation was likely linked to stimulation of the inward current of external Ca\(^{2+}\) into the cell.

nLDL Caused intramitochondrial Ca\(^{2+}\) accumulation

Mitochondria are known to provide a buffering power toward intracellular calcium rise [22]. Thus, we monitored the effect of nLDL on the intramitochondrial calcium (mtCa\(^{2+}\)) level using the
specific probe Rhod-1 [23]. Figure 7A shows that nLDL-treatment caused a significant increase of the Rhod1-linked fluorescence signal. The mtCa\(^{2+}\) load was linked to the collapse of the mtΔΨ. Indeed, the mitochondrial calcium uniporter inhibitor ruthenium red (RR) abrogated the nLDL-induced mitochondrial Ca\(^{2+}\) uptake and prevented at the same time the nLDL-induced mtΔΨ collapse (Fig. 7A). Dantrolene (an inhibitor of the ryanodine calcium channel) was ineffective whereas EGTA prevented both the mtCa\(^{2+}\) load and the mtΔΨ decrease in nLDL-treated HK-2 (Fig. 7B). These observations confirmed that the mtCa\(^{2+}\) load was mostly linked to the intracellular entry of external Ca\(^{2+}\) with negligible if any involvement of the intracellular ER calcium stores. Noteworthy RR, although impairing the calcium entry in the mitochondria, had no effect on the nLDL-induced ROS production (Fig. 7A,B). Consistent with this result is the observation that the nLDL-induced decrease of mtΔΨ was not prevented by DPI or NAC (Fig. 7B). Conversely, both DPI and NAC suppressed the LDL-
induced \( \text{mtCa}^{2+} \) overload. Thus, the LDL-linked deregulations of the \( \text{mtCa}^{2+} \), ROS and \( \Delta \Psi \) homeostasis interplayed by a complex modality and partly occurred independently rather than sequentially. This conclusion was further supported by the time-course of the nLDL-linked \( \text{mtCa}^{2+} \) changes showing that the \( \text{mtCa}^{2+} \) overload was significantly delayed (Fig. 7C) with respect to the \( \Delta \Psi \) and ROS changes (Fig. 2C).

\( \text{mtCa}^{2+} \) overload is a condition known to open the mitochondrial permeability transition pore (MPTP) thereby collapsing \( \Delta \Psi \) and leading, in addition, to release of pro-apoptotic factors (such as cytochrome c) from the mitochondrial intermembrane space [22]. To test this possibility HK-2 were co-incubated with nLDL and cyclosporine A (CsA), an inhibitor of the MPTP. Figure 8 shows that CsA preserved efficiently the \( \Delta \Psi \) without significant effect on the nLDL-induced \( \text{mtCa}^{2+} \) load. As proof of principle the re-equilibration of calcium gradients by the \( \text{Ca}^{2+} \)-ionophore A23187 proved to prevent completely both the \( \text{mtCa}^{2+} \) and the \( \Delta \Psi \) LDL-induced changes. Further it was shown that whilst untreated HK-2 presented, as expected, a punctuate appearance of the intramitochondrial cytochrome c-linked immunofluorescence, treatment with nLDL resulted in blurring of the fluorescence suggesting dilution of cytochrome c in the cytoplasm. Both CsA and A23187 preserved the brilliant punctuate appearance of the signal.

Next we tested the effect of MPTP blocking on the nLDL-dependent ROS production. Figure 8 shows that CsA-treatment resulted in a large reduction of the nLDL-induced DCF fluorescence. Similar results were obtained by using the more specific MPTP-blocker Debio 025 (data not shown).

Taken together these observations support the conclusion that the nLDL-linked collapse of \( \Delta \Psi \) was due to calcium-induced MPTP opening.

**Inhibition of cPLA2 prevents nLDL-linked \( \text{mtCa}^{2+} \) and \( \Delta \Psi \) deregulation**

Figure 9 shows that treatment of HK-2 with AACOCF3 resulted in complete prevention of the nLDL-induced \( \Delta \Psi \) decrease and \( \text{mtCa}^{2+} \) load. In keeping with the reported effect of AACOCF3 in abrogating the nLDL-induced ROS formation (Fig. 6) this result suggested that the activation of cPLA2 and the formation of its reaction products were largely responsible of the observed mitochondrial dysfunction. However, the conflicting results of the effect of DPI- and NAC-treatment on the \( \Delta \Psi \) and \( \text{mtCa}^{2+} \), in spite of their ability to fully prevent the oxidative unbalance, would indicate that the cPLA2-products exert multiple independent actions.
Discussion

Our primary finding in this study shows nLDL causing unbalance of the oxidation state in human proximal tubular cells. Oxidative modification of LDL components has long been considered a main factor promoting in the vasculature development of atherosclerotic lesions [3]. This led investigators to focus on oxLDL effects exerted on extra vascular tissue neglecting those of nLDL so much as to consider the lack of significant effects of nLDL as a negative control to emphasize the alterations caused by oxLDL. However in most of these reports the timing of nLDL in vitro treatment is too short to let the effects being detectable. Our results show, indeed, that enhanced ROS formation became reliable after 6 hrs of exposition of HK-2 to nLDL. Interestingly, in a comparative study evaluating the influence of native and hypochlorite-modified LDL on gene expression in HK-2 cells...
nLDL up-regulated the transcription of genes involved in ROS metabolism and cellular stress so as hypochlorite-oxidized LDL but following longer incubations [25].

In the attempt to characterize the mechanism underlying the nLDL-induced changes in the HK-2 cell redox homeostasis, a number of interconnected signalling pathways were found to be involved. The scheme drawn in Figure 10 conveys the evidence presented in this study with others reported in literature and provides a verifiable mechanistic model of nLDL-induced cellular damage. The first step following the interaction of the nLDL with the HK-2 cells (Fig. 10, step 1) is the activation of an inward current of Ca$^{2+}$ (Fig. 10, step 2). This interaction is most likely mediated by binding of nLDL to its specific receptor (LDL-R). HK-2 express several members of the LDL receptor family [26] as well as Ca$^{2+}$ channel subtypes [27]. In addition to drive endocytic LDL transportation the LDL-R is known to trigger activation of a number of adaptive signalling pathways [28]. Whatever is the mechanism of action, the nLDL-induced increase of the Ca$^{2+}$ inward current is the earliest step required for all the subsequent events. Indeed chelation of external Ca$^{2+}$ or inhibition of the L-type Ca$^{2+}$ channel abolished completely the responsiveness of HK-2 to nLDL (Fig. 6A). Of note blockade of calcium influx through

Fig. 6 Extracellular Ca$^{2+}$ mediates the nLDL-induced cPLA2-linked ROS production. (A) HK-2 cells were incubated for 24 hrs with 100 μg/ml nLDL either alone or in the presence of 20 μM AACOCF3 or 0.5 mM EGTA or 30 μM verapamil and after that assessed for intracellular H$_2$O$_2$ production by DCF. Representative images of the LSCM analysis are show along with the statistical evaluation of the fluorescence intensity averaged ± S.E.M. from n = 3 for each condition. Bars inside all the micrographs: 30 μm. (B) Effect of AACOCF3 on the nLDL-mediated activation of the NADPH oxidase. The SOD inhibitable cytochrome c reduction values are averages ± S.E.M. from n = 3 for each condition; the statistical differences versus untreated HK-2 cells is also indicated when significant.
Fig. 7 Treatment of HK-2 with nLDL causes increase of mitochondrial Ca\(^{2+}\) uptake. (A) Effect of nLDL on the intramitochondrial level of Ca\(^{2+}\). HK-2 cells were treated for 24 hrs with 100 \(\mu\)g/ml of nLDL either alone or in combination with 5 \(\mu\)M ruthenium red (RR). After that the intramitochondrial Ca\(^{2+}\), m\(\Delta\psi\) and H\(_2\)O\(_2\) production were assessed by the specific probes Rhod-1, TMRE and DCF respectively as detailed under Materials and Methods. The panel shows a representative LSCM imaging. To note, in order to improve visually the data presented, the original red fluorescence of Rhod-1 was digitally rendered in yellow (by ImageJ 1.38x, NIH, USA, http://rsb.info.nih.gov/ij/) without altering the pixel intensity scale. Enlargements of single cell imaging for Rhod-1-probed of untreated and nLDL treated samples are also shown. (B) Quantitative analysis of the mtCa\(^{2+}\)-, m\(\Delta\psi\)- and H\(_2\)O\(_2\)-probe fluorescence intensities from LSCM imaging. In addition to the experimental setting shown in (A) the effect of coincubation of nLDL with 10 \(\mu\)M dantrolene, 0.5 mM EGTA, 10 mM DPI, 20 mM NAC is also reported for mtCa\(^{2+}\)- and m\(\Delta\psi\)-changes. Each bar represents the fluorescence intensity/cell averaged \(\pm\) S.E.M. from at least \(n = 3\). The statistical differences versus untreated HK-2 cells is also indicated when significant. (C) Time-dependence of the mtCa\(^{2+}\) changes as a function of the nLDL-incubation time. The Rhod-1-related fluorescence intensity for each time-point was measured as in (A, B) and refers to the average \(\pm\) S.E.M. of \(n = 3\). Bars inside all the micrographs: 30 \(\mu\)m.
L-type calcium channel proved to attenuate apoptogenic insults occurring in hypoxic renal tubular cells [29].

The enhanced nLDL-induced Ca\(^{2+}\) entry into the cell activates the cytoplasmic Ca\(^{2+}\)-sensitive isoform of the phospholipase cPLA2 (Fig. 10, step 3). The central role of cPLA2 in mediating the nLDL-linked cellular alterations is directly demonstrated by the preventing effect of its specific inhibitor AACOCF3 (Figs. 6 and 9). cPLA2 hydrolizes the C-2 ester bond of phospholipids with high affinity to phosphatidylycerine (PC) containing arachidonic acid (AA) bound to C-2 thereby releasing lipophosphatidylycerine (LPC) and the free fatty acid, both with a documented signal transducing activity [30]. AA is a powerful activator of the plasma membrane NOX(s) [31] and in this study we clearly show the involvement of NOX in the nLDL-linked oxidative imbalance (Fig. 10, step 4). Indeed, specific NOX inhibitors or externally added ROS-scavenging enzyme prevented completely the
ROS may stimulate further ROS production by mitochondria (Fig. 10, step 5). This is proved by the complete ablation of the intracellular mitochondrial ROS production following NOX-inhibition or scavenging extracellular ROS (Fig. 5A). It has been suggested that a cross-talk between NOX activation and the mitochondrial respiratory chain occurs whereby extracellular ROS can function as signalling molecules by a paracrine/autocrine mechanism (Fig. 4C). The effect of DIDS, an inhibitor of the plasma membrane chloride channel, would suggest that ROS-induced ROS production is partly mediated by transmembrane transport of the superoxide anion (Fig. 5B). Once into the cell, O$_2^-$ and H$_2$O$_2$ trigger ROS production by mitochondria. We cannot at the moment detail whether the activation is exerted directly by ROS or mediated by oxidized molecules acquiring signalling features neither the specific mitochondrial target. However, some clues are offered in the literature to get insights into the mechanism. Indeed reported propagation of an oxidative wave in the mitochondrial network was shown to be sensitive to inhibitors of either the MPTP or the inner mitochondrial anion channel [38- 40]. It is worth mentioning that evidence indicating the occurrence of a cross-talk between mitochondria and NOXs have been recently reported [41, 42]. However, in those studies mitochondria appear to be upstream independently of mt-Ca$^{2+}$ load but not the mtΔψ decrease whereas blockade of the mtCa$^{2+}$ uniporter by RR largely preserved the LDL-induced mtΔψ collapse but was ineffective on the ROS over-production (Fig. 7). These puzzling observations can be explained assuming the occurrence of distinct pathways converging on the same target. In particular, it is proposed that ROS can favour the mtCa$^{2+}$ overload by activating the Ca$^{2+}$ uniport (Fig. 10, step 6) as shown for other redox modulated transporting systems [43]. This would place the activation of the inward mtCa$^{2+}$ transport down-stream of the redox signalling pathway explaining the effects of anti-oxidants and RR. The nLDL-induced collapse of the mtΔψ seems to be dependent on both the mtCa$^{2+}$ level and on products of the cPLA2 catalysis. Indeed both the AAOCCF3-mediated inhibition of the cPLA2 and the RR-mediated suppression of the mitochondrial Ca$^{2+}$ uptake preserved the nLDL-linked decrease of the mtΔψ (Figs. 6, 9). The effect of CsA in preventing the nLDL-induced mtΔψ collapse as well as ROS production (Fig. 8) would indicate the causative involvement of the MPTP (Fig. 10, step 7).

In this study we observed that collapse of the mtΔψ although fully prevented by RR-treatment precedes mt-Ga$^{2+}$ accumulation (cf. Fig. 2C versus Fig. 7C) implying that the two events are apparently distinct and that mitochondrial depolarization may occur independently of mt-Ca$^{2+}$ uptake. Intramitochondrial Ca$^{2+}$ overload is a key inducer of MPTP opening leading to loss of mtΔψ [22]. However, it must be pointed out that activation of the MPTP by mt-Ga$^{2+}$ has been described as occurring first by enhancing the intrinsic flickering of the pore [44]. The transient opening of the pore causes a rapid ionic re-equilibration between the intra- and extramitochondrial compartments [45]. The transient opening of the pore initiates a myriad of redox-sensitive signalling pathways and their molecular determinants are being now partly disclosing [32, 33].

Besides NOXs, the mitochondrial respiratory chain constitutes the main source of ROS in the cell [18, 34]. An ever-growing mass of evidence highlights the involvement of dysfunctioning mitochondria in the onset/development of many human pathologies [35] including atherosclerosis [36]. Of interest, specific blockade of mitochondrial ATP production was reported to promote proximal tubular cholesterol loading [37].

nLDL-dependent cell redox alterations (Figs. 3, 4A). Isoforms of the macrophagic NOX, unrelated to the host defence, exert regulatory functions by production of ROS acting as signalling molecules [13, 17]. A number of cell functions turns out to be regulated by redox sensitive signalling pathways and their molecular determinants are being now partly disclosing [32, 33].

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extra-mitochondrial milieu leading to partial collapse of the mtΔΨ as well as to mt-Ca^{2+} exit. Therefore, mtΔΨ lowering would not necessarily require a high steady-state mt-Ca^{2+}. At high concentration of mt-Ca^{2+} the MPTP is permanently opened and the mtΔΨ fully released. Another possibility is that the mtΔΨ decrease is due to accumulation of the cPLA2 products. Indeed a large body of evidence reported in the literature shows that LPC and AA are all inducers of the MPTP opening [45–47]. In addition, AA has been reported to inhibit the OXPHOS acting as mild uncoupler as well as inhibitor of the ETC [48] (consistent with the respirometric results reported in Fig. 2B). Of course all the aforementioned mechanisms are not mutually exclusive but may act at once.

A further point emerged from this study is the apparent irreversibility of the nLDL induced oxidative unbalance. Indeed
removal of nLDL after 6–12 hrs of incubation (under our experimental protocol) did not result in resetting of the original cellular redox state but instead induced persistent alterations. This observation would suggest the occurrence of a forward feed back mechanism enabling self-maintenance of the oxidative insult once a critical threshold of effectors has been reached. The ROS-induced ROS production here proposed may well account for such a positive loop. Indeed release of ROS, in the form of the freely diffusible H$_2$O$_2$, from a mitochondrial subset could solicit ROS generation in other mitochondrial clusters thus propagating and self-maintaining the oxidative insult. It is worth mentioning that ROS signalling has been implicated in the activation of the plasma membrane L-type Ca$^{2+}$ channels, the cPLA2 and the MPTP [38, 49–51]. On the other hand, mTCa$^{2+}$ enhancement, release of lyso-PC and AA, opening of the MPTP are all conditions proved to cause mitochondrial ROS generation [22, 52, 53]. In addition, lyso-PC and AA were shown to activate directly the L-type Ca$^{2+}$ channel [54, 55]. All these observations are fully consistent with a self-fuelling mechanism.

The here-reported nLDL-induced alterations in HK-2 were largely reproducible in a different cell phenotype (i.e. podocytes; C. Picoli et al., unpublished data). This observation, in addition to its clinical relevance with respect to the role of hyperlipidemia in the progression of the CKD [7], would argue that the here-reported nLDL-induced alteration might be of more general interest. Progressive chronic renal disease of all types is characterized by tubular (atrophy, hypertrophy, hyperplasia) and interstitial (inflammatory cell infiltration, fibrosis) pathological changes, the severity of which correlates well with the degree of proteinuria and the decline in glomerular filtration. In response to proteinuria and additional factors [4–10], tubular cells are activated to produce a large number of chemoattractants, proinflammatory and profibrotic cytokines and matrix proteins, which cause, at least in part, interstitial inflammation and fibrosis [8–10]. The clinical relevance of our observations results evident in keeping with the potential role of redox signalling in activating pro-inflammatory and fibrogenic pathways (Fig. 10, step 8) [8–10]. Dyslipidemia is a well-established factor contributing to exacerbate chronic renal disease independently of the priming noxious glomerular events [4–7]. This notion has led to the successful utilization of statin-based therapy to slow down or even halt the progression of CKD [56]. It is worth noting that statins, in addition to their hypcholesterolemic capacity, exhibit additional pleiotropic effects including modulation of inflammatory processes. The anti-inflammatory actions of statins likely arise from their ability to prevent the synthesis of isoprenoid intermediates that are responsible for the post-translational-isoprenylation on the C-terminus of a variety of proteins including small GTPases such as Rac [57]. Because Rac-1/2 are critically involved in the activation of NOXs [14], it is tempting to speculate that the efficacy of statins may partly result from impairment of plasma membrane-translocation of Rac-1 necessary for NOX activation.

In conclusion the interplay between changes in the intracellular redox ‘tone’ and the proinflammatory and profibrogenic cytokines- and growth factors-mediated stimuli offers a mechanistic basis to understand the role and effect of dyslipidemia in the renal chronic disease. The nLDL-related activation of the ROS generating network disclosed in this study suggests novel pharmacological targets to be potentially exploited in the development of combined therapeutic strategies.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

LDLs were isolated from healthy donors and tested by the oxidation state-dependent electrophoretic mobility shift assay. *In vitro* Cu$^{2+}$-treated LDLs were used as control sample (oxLDL) and resulted in a complete downward electrophoretic mobility shift of the single band present in the isolated LDLs. Staining of the gel with either Coomassie Blue or Oil O Red gave comparable results. However, Cu$^{2+}$-treatment performed under similar condition but in the culturing medium (DMEM) did not cause any change in the electrophoretic LDL-mobility. A similar result was obtained assaying LDLs recovered from the medium of cultured human proximal tubule cells (HK-2) after 24 hrs of incubation (Fig. S1A).

Oxidation of polyunsaturated lipids results in formation of diene-derivatives with well-defined UV-absorbance spectral features. It was shown that although Cu$^{2+}$-treatment of LDL resulted, as expected, in a spectral signature diagnostic of oxidation-linked dienes formation, incubation of LDL with HK-2 for 24 hrs did not reveal any spectral evidence of oxidation (Fig. S1B). In addition, time-resolved UV spectral analysis of LDL in the early phase of Cu$^{2+}$-treatment confirmed the oxidation-preventing properties of DMEM likely due to the presence of antioxidant compounds in its composition [1] (data not shown). Taken together, these results indicated that the isolated LDLs did not show detectable evidence of oxidative modifications and remained as such under the experimental settings of the present study.

Twenty-four hours-treatment of HK-2 cell line with nLDL (100 µg protein/ml) did not alter their viability (assessed by Trypan-Blue assay) neither caused marked morphological changes. Conversely similar treatment with *in vitro*-oxidised LDL caused profound alterations in the cultured cells diagnostic of induced-distress (Fig. S2A). However, when the cells were tested for apoptosis (by the Annexin V assay) the occurrence of significant marks of early apoptosis was found in the 24 hrs-treated HK-2 cells as compared with untreated cells (Fig. S2B). Ox-LDL-treatment resulted, on the
other hand, in clear features of advanced cell death (late apoptosis/necrosis).

**Supplementary Material**

**Characterization of isolated LDL.** The oxidative state of isolated LDLs was assessed by gel electrophoretic shift assay [2]. Briefly, 5 µg LDL was loaded on 1% agarose gel and run for 2 hrs at 75 mA in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3. The gel was fixed in methanol, acetic acid and water 4:1:5 for 25 min and stained with either Brilliant Blu G (by standard protocols) or Oil-O-red. Before oil-O-red staining (0.2% in 60% ethanol, overnight) the gel was dried at 80 °C for 1 hr. After staining, the gel was washed by bleaching solution (ethanol:water, 6:4). Oxidized LDL was obtained by treating the isolated LDL with 5 µM Cu²⁺ (SO₄)₂⁻ for 3 hrs and then dialyzed against phosphate buffer saline (PBS) solution. The spectral diene-linked features of the oxidized LDLs were evaluated by second derivative analysis of differential UV spectra [3].

**Cell viability assay.** HK-2 cells were tested with the Annexin V-Cy3/6-carboxyfluorescein diacetate apoptosis detection kit (Sigma-Aldrich) following the instruction of the manufacturer. The combination of the two probes enables to distinguish different grade/type of cell death as follow: viable cells (AV negative/CF positive), apoptotic cells (AV positive/CF positive), late apoptotic or necrotic cells (AV positive/CF negative).

**Reverse Transcription-Polymerase Chain Reaction.** For non-quantitative RT-PCR total cellular RNA isolated by Trizol reagent was reverse transcribed to cDNA with specific antisense primers following the SuperScript Reverse Transcriptase II protocol. Samples of 5 µL of RT reaction were PCR-amplified in a total volume of 50 µL with 50 pmols each of forward and reverse primers. The primer sequences used were: NOX1, for-5'-TTAACAAGACGCTGATCTCGT-3', rev-5' - GCTGGAGAGAATGGAGGCAAG-3' (Tₐ₉₉ = 58 °C); NOX2, for-5'- ACTCTTGGTGTACGACTG-3', rev-5'- AGGAGGACAGCAGATTGCG-3' (Tₐ₉₉ = 58 °C); NOX2s, for-5'- CTTTCTCTGGGGGCAAGC-3', rev-5'- TAACGGGTAAAGACTTTG-3' (Tₐ₉₉ = 60 °C); NOX4, for-5'- CTCTCGGCACT-3', rev-5'-TTGGGTCACACAGACACG-3' (Tₐ₉₉ = 58 °C); NOX5, for-5'-TTATGGGCTACTTTGGTAC-3', rev-5'-GGGGTTGGCCTTGGTT-3' (Tₐ₉₉ = 58 °C); SOD1: for-5'- GATCCTGCTACATGATTCC-3', rev-5'-GGGGTTGGCCTTGGTT-3' (Tₐ₉₉ = 58 °C); SOD2: for-5'- CAAAATGCACCGCACTGAC-3', rev-5'-GAAATTCTGGCCG-3' (Tₐ₉₉ = 58 °C); GPRX1: for-5'- AGGTAGTAAGCGTGCTCCCACACAT-3', rev-5'-TTGGGTCACACAGACACG-3' (Tₐ₉₉ = 58 °C); GPRX4: for-5'- CCAGAGTAAACTACACTAGCTGTC-3', rev-5'-TTGATCTTCTGTGACTCCCCTGGC-3', β-actin: for-5'- TGGACATCCGCAAAGACCTG-3', rev-5'- GCCAGTCCACACGGAAGTACTT-3' and the following cycling parameters: initial denaturation for 10 min. at 94 °C, followed by 45 cycles with 15 s at 94 °C, 30 s at 64 °C (for GPX4) or 60 °C (for all other transcripts) and 15 s at 72 °C and 10 min terminal elongation at 72 °C. Melting curve analysis and agarose gel electrophoresis were performed to confirm the specificity of the amplification products. The efficiency of amplification, determined by serial dilutions of templates, was close to 100% for all transcripts and the linear regression coefficients were >0.998.

**Spectrophotometric Analysis.** A total of 5 x 10⁵ HK-2 cells in 100 µL of 100 mM Tris, pH 7.4, were lysed with 2% Triton X-100 in the presence of a protease inhibitor mixture. Optical spectra from 400 to 500 nm of the oxidized (by 10 mM ferricyanide) and reduced (by a few grains of dithionite) samples were recorded in a microvolume (50 µL) cuvette. The baseline drifts because of the residual turbidity of the suspension was largely removed by differential analysis (reduced minus oxidized) and further corrected for a polynomial baseline passing throughout the cytochromic isosbestic points. The NOX-cytochrome b-related spectral shift was induced by 20 mM tert-butyl isocyanide (t-BICN) on the reduced absorbance spectra of the HK-2 cell lysate [4]; a ΔA₂₅₀₋₆₆₀ = 126 M⁻¹ cm⁻¹ from the reduced minus reduced + t-BICN differential spectra was used for estimation of the b type cytochrome.

**Fig. S1 Characterization of the oxidation state of isolated LDL.** (A) Electrophoretic shift assay. A comparative electrophoretic mobility of native LDL, Cu²⁺-oxidized LDL and following exposure to HK-2 cells is shown. Paired gels were stained to detect either proteins (Bluant Blu G) or lipids (Oil-O-Red) as described in the supplementary material. (B) Differential UV spectra of HK-2 exposed LDL. Isolated LDLs were suspended in DMEM at the concentration of 100 µg/ml and added either to an empty culturing dish or to a dish plated with HK-2 cell. After 24 hrs of incubation equal volumes from the culturing media were withdrawn from the two dishes and spectrally scanned. The spectra shown was obtained subtracting the absorbance of the LDL suspended in the sole medium from that of the LDL which were exposed to the HK-2 cells. Identical results were obtained at intermediate interval of incubation (3, 6, 12 h; not shown). For comparison the spectra of Cu²⁺-oxidized LDL is also shown with its second derivative.
spectra indicating the spectral features of the peroxidation-linked dienes formed.

**Fig. S2** Effect of nLDL and oxLDL treatment on HK-2 cell viability. (A) Phase contrast micrographs of HK-2 treated for 24 hrs with 100 μg/ml of either nLDL or oxLDL. (B) Cell-death assay. HK-2 cells treated with 100 μg/ml of either nLDL (24 hrs) or oxLDL (6 h) were tested by the Annexin V/carboxy-fluorescein assay as indicated in the supplementary material. The upper panel shows a representative confocal microscopy imaging with the red and green fluorescence indicating membrane binding of Annexin V and cell loading/maintenance of carboxy-fluorescein respectively. As compared to control HK-2 cells (CTRL) nLDL-treated cells resulted to be all CF positive but with an enhanced number of Annexin V-positive cells (pinpointed by arrows) indicative of late apoptosis. OxLDL-treatment resulted in CF negative and Annexin V-positive cells indicative of late apoptosis or necrosis. The histogram shows the quantitative analysis expressed as fluorescence ratio of the two probes of at least 100 cells for each conditions selected from three separate experiments. The bars indicate the mean values (±SEM); *P < 0.01 versus CTRL, **P < 0.001 versus CTRL, ***P < 0.01 versus nLDL-treated. All the bars inside the micrographs are 30 μm.

**Fig. S3** Quantitative RT-PCR analysis of antioxidant enzymes. The quantification of transcripts abundance in treated HK-2 cells (100 μg/ml of LDL for 24 hrs) was determined performing with the ΔΔCt method, the untreated HK-2 cells being the reference sample and β-actin the internal control. SOD1 and SOD2, superoxide dismutase isofrom 1 and 2, respectively; CAT, catalase; GPX1 and GPX2, glutathione peroxidase isofroms 1 and 4, respectively. The bars are means ± S.E.M. of three independent measurements. See supplementary material for primers used, PCR conditions and further details.

**Fig. S4** NOX expression in HK-2. (A) RT-PCR analysis of NOX isoform expression. The agarose gel shows the amplicon level of the catalytic subunits of the NADPH oxidase isoforms NOX 1, 2, 4, 5 together with the splicing variant NOX 2s. MK, markers. See supplementary material for primers used, PCR conditions and further details. (B) Differential spectrophotometric analysis of NOX-related cytochrome b. The spectrum of Na2S2O4-treated minus untreated cell lysates was first recorded by a split beam spectrophotometer, then 20 mM of t-BICN was added to the reduced sample (measure cuvette) and a second spectra recorded. The resulting ΔAbs spectrum, diagnostic for the presence of NOX-linked cytochrome b, and the calculated amount of NOX is shown on the right. See supplementary material for further details.

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