Biochemistry and Biophysics Reports 34 (2023) 101441

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

New perspective for an old drug: Can naloxone be considered an antioxidant agent?

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ARTICLE INFO

Keywords:
Naloxone
Platinum-based sensors
PC12cells
Hydrogen peroxide
Oxidative stress

ABSTRACT

Background: Experimental evidence indicates that Naloxone (NLX) holds antioxidant properties. The present study aims at verifying the hypothesis that NLX could prevent oxidative stress induced by hydrogen peroxide (H_2O_2) in PC12 cells.

Methods: To investigate the antioxidant effect of NLX, initially, we performed electrochemical experiments by means of platinum-based sensors in a cell-free system. Subsequently, NLX was tested in PC12 cells on H_2O_2-induced overproduction of intracellular levels of reactive-oxygen-species (ROS), apoptosis, modification of cells' cycle distribution and damage of cells' plasma membrane.

Results: This study reveals that NLX counteracts intracellular ROS production, reduces H_2O_2-induced apoptosis levels, and prevents the oxidative damage-dependent increases of the percentage of cells in G2/M phase. Likewise, NLX protects PC12 cells from H_2O_2- induced oxidative damage, by preventing the lactate dehydrogenase (LDH) release. Moreover, electrochemical experiments confirmed the antioxidant properties of NLX.

Conclusion: Overall, these findings provide a starting point for studying further the protective effects of NLX on oxidative stress.

1. Introduction

Naloxone (NLX) is an opioid receptor antagonist clinically used for reversal of opioid overdose. Studies conducted *in vitro* have demonstrated that NLX can exert lipid antioxidant activities [1]. Additionally, several studies have showed that NLX reduces oxidative stress in motor neurons [2–4]as well as in microglial cells and can attenuate the degeneration of dopaminergic neurons by inhibiting superoxide production in microglia [5,6]. Moreover, NLX has demonstrated efficacy in different experimental *in vivo* models of stroke, brain injury, myocardial ischemia, and spinal cord injuries [3,4], in which oxidative stress also plays a significant role [7].

Oxidative stress is defined as an imbalance between reactive-oxygen-species (ROS) levels and endogenous antioxidants [8]. ROS are intermediates produced by normal oxygen metabolism and their increase above physiological/tolerable concentrations in neurons may contribute to and/or cause the development of oxidative damage [9,10].

In this study, we firstly explored the antioxidant activity of NLX in a cells-free system by means of electrochemical techniques using platinum-based sensors, appropriately modified in accordance with a commonly used method for antioxidant proprieties determination of several compounds [11,12]. Electrochemical techniques represent sensitive, fast, and simple methods to highlight the scavenging proprieties towards oxidizing compounds. When antioxidant molecules are in solutions, they can act as reduction agents or simply be oxidised on the surface of inert electrodes [12]. Subsequently, we evaluated the activity of NLX treatment on H_2O_2-dependent oxidative damage in PC12 cells by assessing (a) intracellular ROS levels, (b) apoptosis, (c) cell cycle distribution and (d) membrane damage by lactate dehydrogenase (LDH) release assay.

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https://doi.org/10.1016/j.bbrep.2023.101441
Received 2 December 2022; Received in revised form 30 January 2023; Accepted 13 February 2023

The purpose of the current work is to explore the hypothesis that NLX can counteract the oxidative stress induced by H_2O_2 in rat pheochromocytoma PC12 cells. These cells originate from the adrenal medulla and are capable of synthesizing, metabolizing and secreting catecholamines. Moreover, PC12 cells are more sensitive to oxidants than others and, consequently, are widely used for studying apoptosis and antioxidant mechanisms [13,14].

2. Materials and methods

2.1. Electrochemical experiments

Electrochemical experiments were performed as previously described [15,16] in a classical three-electrode cell comprising of 20 ml of PBS (pH = 7.4). At room temperature, all electrochemical processes were performed with an anodic potential of +700 mV vs Ag/AgCl (3 M) (Bioanalytical system Inc, West Lafayette, IN 4796 USA) Working electrodes were developed, as previously reported [17,18] (Fig. 1). H_2O_2 (catalog. number: H1009-100 ML, Sigma) was employed as an oxidant agent and ascorbic acid (AA) as a positive control in order to demonstrate the antioxidant activity of NLX (catalog. number: N-7758, Sigma). Previously, it was demonstrated that H_2O_2 current monitored at the electrode surface could be depleted by a simultaneous homogeneous redox reaction due to the presence of reducing compounds [19]. Consequently, the electrodeposition of PPD polymer [20] on sensors was needed to avoid the electrochemical monitoring of other electroactive compounds except H_2O_2. Then, after having verified the shielding capabilities of the polymer against NLX, antioxidant activity of NLX, as well as that of AA, was assessed against a fixed concentration of H_2O_2 (250 μM) by adding increasing concentrations of the compound ranging between 25 and 100 μM, while AA ranged from 50 to 500 μM of concentration. The antioxidant activity of the compounds will be evaluated on the basis of the decrease that the current generated by the fixed HP concentration will undergo, to which 100% will be attributed. Variations with respect to this value will be expressed as a percentage decrease.

All the chemicals used for amperometric experiments were from Sigma Aldrich (Milano, Italy).

2.2. Cell culture

PC12 cells derived from a pheochromocytoma cell line (ATCCCRL-1721) (passages 12–25) of the rat adrenal medulla were maintained at 37 °C in a 95% humidified atmosphere and 5% CO_2 in 100 mm plastic culture petri in Dulbecco's-modified eagle medium, DMEM/F-12, HEPES, no phenol red, Gibco (catalog. number: 11039021, Life Technologies Italia) supplemented with 10% horse serum, HS New Zealand origin, Gibco (catalog. number: 16050122, Life Technologies Italia), 5% foetal bovine serum, FBS South America origin, Biowest (catalog. number S1810-500,WWR) and 1% of penicillin/streptomycin 10,000 U/mL, Gibco (catalog. number: 15140122, Life Technologies Italia) [21].

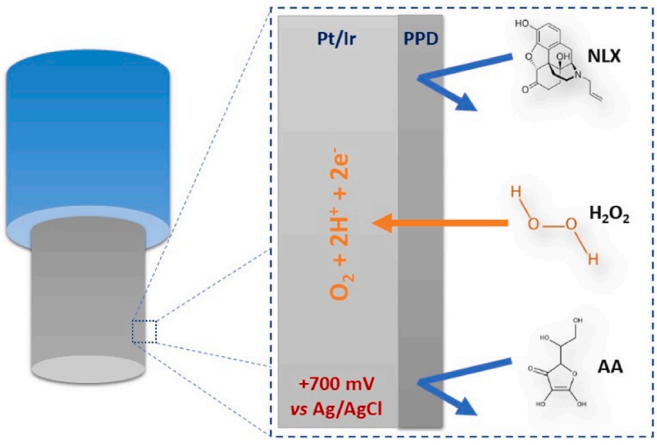

Fig. 1. Schematic representation of electrochemical assessments. Pt/Ir working electrode (cylinder) with an active surface of 1 mm and 125 μm Ø. Pt/Ir: Platinum/Iridium wire (90:10); PPD: polyorthophenylenediamine polymer.

2.3. Drug treatments

PC12 cells were treated for 24 h with NLX hydrochloride (25 and 50 μg/ml) (catalog. number: N-7758, Sigma); these concentrations were chosen based on literature data [2,3,22]. All experiments were performed exposing, for 24 h, PC12 cells to NLX (25 or 50 μg/ml) alone or to NLX (25 or 50 μg/ml) and H_2O_2, (100 μM) (catalog. number: H1009-100 ML, Sigma), applied 30 min after NLX (Fig. 2). All experiments were performed in triplicate.

2.4. ROS detection

The intracellular ROS content was assessed using Total ROS Detection Kit (ROS-ID® Total ROS detection kit; catalog number: ENZ-51011, Euroclone). PC12 cells were plated in 6-well plates at a density of 1.5×10^6 cells/well and exposed to NLX treatments at concentrations of 25 and 50 μg/ml [22] alone or with H_2O_2 100 μM. Cells were collected 24 h after treatment by adding 400 μl of 0.25% Trypsin-EDTA, phenol red Gibco (catalog. number: 25200056, Life Technologies, Italia) and centrifuged for 10 min at 1200 rpm. The pellets were re-suspended in PBS and stained for 30 min in the dark with ROS Detection Reagent. The FACSCANTO cytometer (Becton & Dickinson, USA) was used to detect intracellular ROS production after incubation [23]. The data were analysed with FACSDiva 2.2. All experiments were performed in triplicate.

2.5. Apoptosis analyses

PC12 cells, seeded in 6-well plate at 1.5×10^6 cells/well concentration, were exposed to NLX alone or with H_2O_2, as reported above. After 24 h of treatment, the apoptosis was evaluated using Annexin V-FITC and propidium iodide (eBioscience Annexin V-FITC Apoptosis Detection Kit, catalog. number: BMS500FI-20, Thermo Fisher Scientific, Life Technologies Italia). Annexin-V binds to phosphatidylserine at the surfaces of apoptotic cells as a marker for apoptosis. Annexin-V expression was detected with a flow cytometer in FITC and PI channels, showing early and late apoptosis, respectively [24]. In detail, cells were incubated with Binding Buffer 1X containing Annexin V-FITC and PI for 15 min in dark. Flow cytometry was performed by FACSCANTO (Becton & Dickinson, USA) [25] and the data were analysed with FACSDiva 2.2. All experiments were performed in triplicate.

2.6. Cell cycle distribution

After 24 h of treatment with NLX, alone or with H_2O_2, PC12 cells (seeded in 6-well plates at 1.5×10^6 concentration) were washed two times with PBS and centrifuged at 1200 rpm for 10 min. The pellets were fixed in 70% cold ethanol and then stained with propidium iodide (eBioscience Propidium Iodide Staining Solution, catalog. number: 00-6990-50, Invitrogen, Life Technologies Italia) for 15 min. Subsequently, for each sample, 30000 total events were acquired by flow cytometer FACSCANTO (Becton & Dickinson, USA) [23]. The percentage of cells in each phase of the cell cycle, G1, S and G2/M, was analysed with ModFit LT 5.0 software [26]. All experiments were performed in triplicate.

2.7. LDH assay

This test is based on the measurement of LDH release, a stable

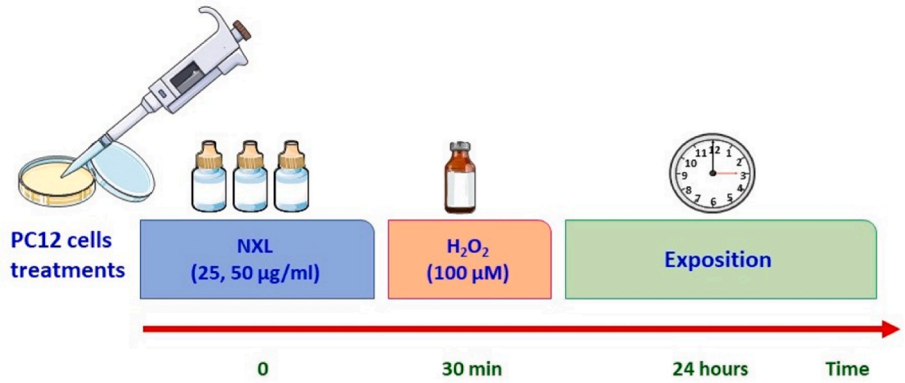

Fig. 2. Timeline of the experimentation. NLX treatment (time 0) was performed 30min before H_2O_2. The cells were processed after 24 h of exposure.

enzyme that is normally present in the cytoplasm of all cells but is rapidly released into the supernatant in response to plasma membrane damage. According to the instructions in the user's manual, CytoTox 96® Non-Radioactive Cytotoxicity Assay (catalog. number: G1780), Promega Corporation, USA) was used to assess cellular LDH release [27]. All experiments were performed in triplicate.

2.8. Statistical analysis

Data were reported as the mean ± standard deviations (SD) of triplicates. In the presence of overall significant main effects and interactions (p values < 0.05) following One-Way-analysis of variance (ANOVA), Bonferroni multiple comparisons as post hoc, was performed. In electrochemical experiments data were expressed as a percentage change in the H_2O_2 (250 µM) current. All data were evaluated by using Graph-Pad Prism 8.2.1 software (Inc. USA).

3. Results

3.1. Antioxidant activity of naloxone against H_2O_2 in electrochemical experiments

As previously reported [19], as a homogeneous reaction can occur in the electrochemical cell when H_2O_2 and a reducing compound are present, the electrodes were suitably modified through the electrodeposition of a PPD polymer, to be able to detect on the surface of the electrode only the currents derived from the oxidation of H_2O_2. The monitoring of the H_2O_2 current alone allowed us to evaluate the modifications in the presence of increasing concentrations of reducing species (and therefore with antioxidant activity). NLX determined a decrease in H_2O_2 currents of about 0.179% for each micromole/liter added in the electrochemical chamber, while ascorbic acid reduced H_2O_2 current by only 0.043% for each micromole/liter added (Fig. 3). As shown in panel a, NLX decreased the current of H_2O_2 (250 µM) in a dose-dependent manner, until the concentration of 100 µM, where the decrease was of about 80%. The use of the well-known antioxidant ascorbic acid (panel b) as positive control reduced the fixed concentration of H_2O_2 (250 µM) dose-dependently. In particular, the addition of increasing concentrations of ascorbic acid resulted in a proportional decrease in H_2O_2 current of approximately 80% at the concentration of 500 µM. Therefore, the antioxidant potency of NLX resulted five times greater than that of ascorbic acid (panel b).

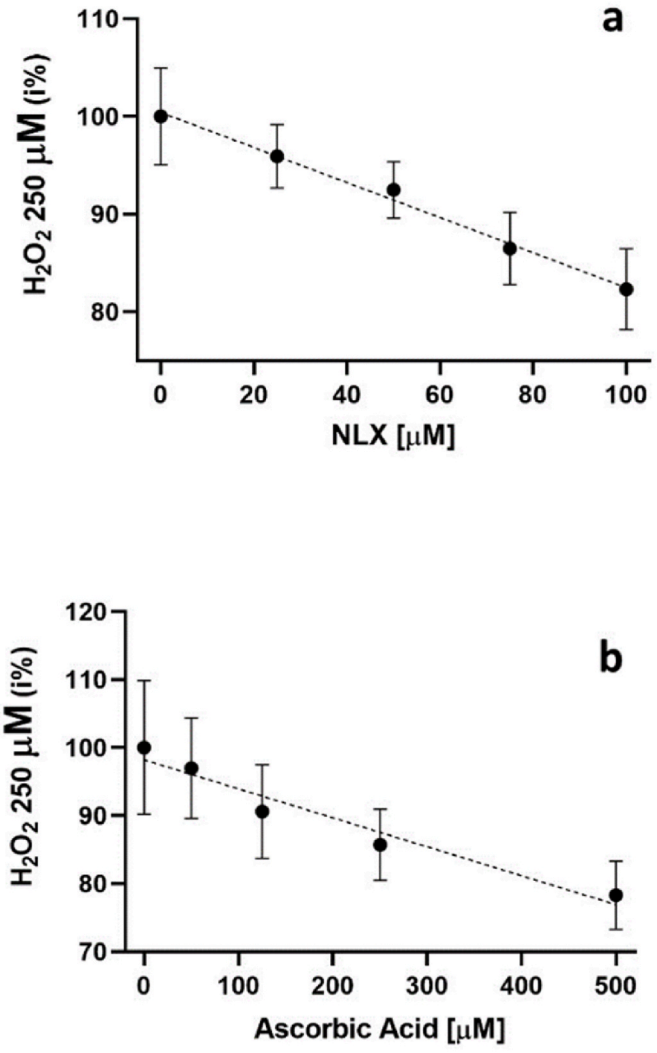

Fig. 3. Antioxidant activity by electrochemical assessments exerted on a fixed concentration of H_2O_2 (250 µM) by NLX (0–100 µM) (panel a) or by ascorbic acid (AA, panel b) (0–500 µM).

3.2. Effects of naloxone on ROS production

Data from ROS detection experiments were achieved to evaluate the antioxidant properties of NLX, alone or in the presence of H_2O_2. In Fig. 4, panel a shows that ROS levels in cells incubated with NLX alone did not significantly differ in comparison to the control group ($p > 0.05$); however, as shown in panel b, H_2O_2 significantly increased (68%, $p < 0.05$) ROS production in comparison with the control (48%). NLX induced a significant reduction ($p < 0.05$) up to 27% (NLX 25 µg/ml) and 28% (NLX 50 µg/ml), of ROS levels both when compared to H_2O_2 (68%) and to the control group (40%).

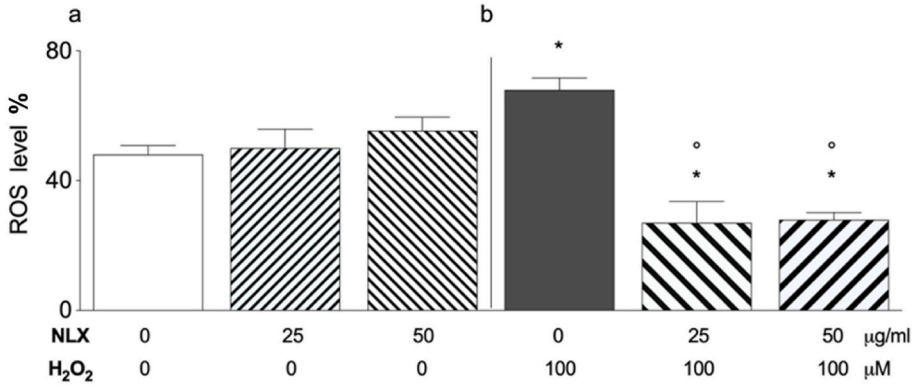

Fig. 4. Evaluation of effect of NLX (25 and 50 µg/ml) on the ROS production in PC12 cells, without (panel a) or with H_2O_2 (100 µM) (panel b) at 24 h of exposure. Bonferroni multiple comparisons shows a significant difference for $p < 0.05$ *versus* untreated cells (control): * and *versus* H_2O_2: °.

Moreover, in physiological conditions, cells have basal levels of ROS that remained unaffected by the 24 h application of NLX (either 25 and 50 µg/ml) and, in fact, NLX significantly reduced ROS overproduction, due to H_2O_2-mediated oxidative damage, to levels significantly lower than controls ($p < 0.05$).

3.3. Effects of naloxone on apoptosis

The apoptosis studies (Fig. 5) revealed that, under the present experimental conditions, NLX at the concentration of 25 µg/ml, but not of 50 µg/ml, significantly ($p < 0.05$) rises apoptotic cells (29%) in comparison with the control group (11%) (panel a). As shown in panel b, H_2O_2 significantly ($p < 0.05$) increases apoptotic cells (68%) with respect to the control group (11%). NLX, in presence of H_2O_2, significantly ($p < 0.05$) decreases, in a concentration-dependent manner, apoptotic cells from 68% (H_2O_2) to 55% (25 µg/ml) and 20% (50 µg/ml).

3.4. Naloxone effect on cell cycle analysis

The cell cycle analysis has been performed to evaluate, by flow cytometry, the effects of NLX on PC12 cells' cycle distribution phases with or without H_2O_2 exposure (Fig. 6). Generally, both NLX concentrations (25 and 50 µg/ml) prompt a similar ($p > 0.05$) cell cycle distribution phase (panel a), if compared with the control group. On the other hand, under H_2O_2, a higher number of PC12 cells remained stagnate in the G2/M phase if compared with the control group ($p < 0.05$) (panel b). The amount of the stagnation in the G2/M phase was

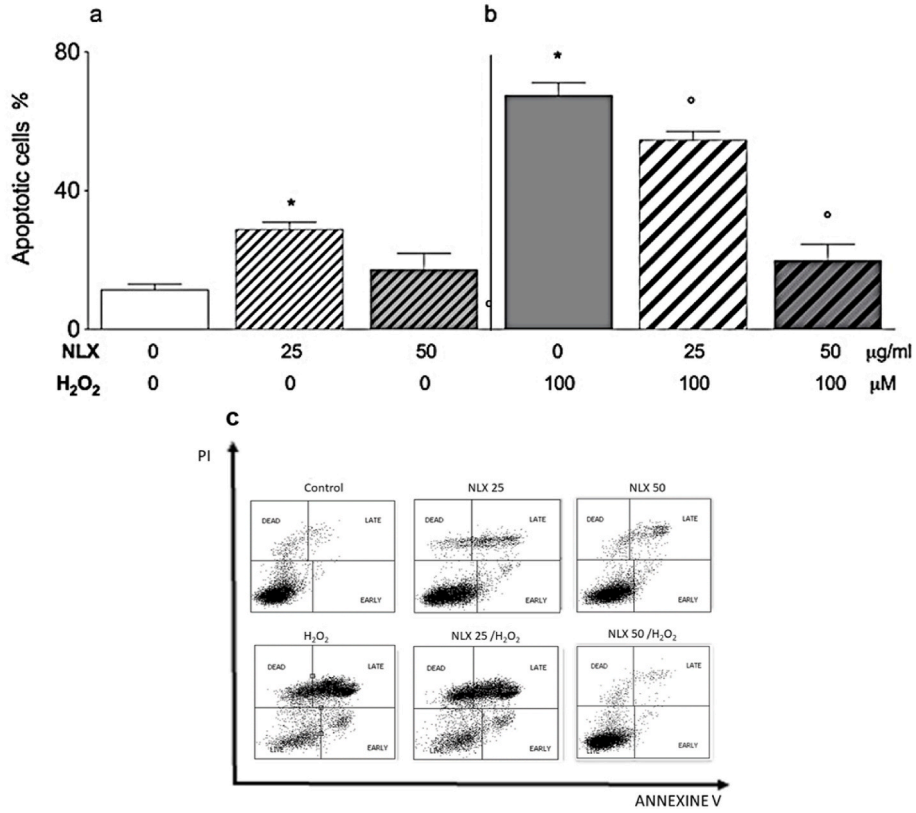

Fig. 5. Evaluation of effect of NLX (25 and 50 µg/ml) on the percentage of total apoptosis of PC12 cells, without (panel a) or with H_2O_2 (100 µM) (panel b) at 24 h of exposure. Dot spot graph showed the rates of PC12 at various stages of apoptosis and represents one out of three independent experiments (panel c). Bonferroni multiple comparisons shows a significant difference for $p < 0.05$ versus untreated cells (control): * and versus H_2O_2: °.

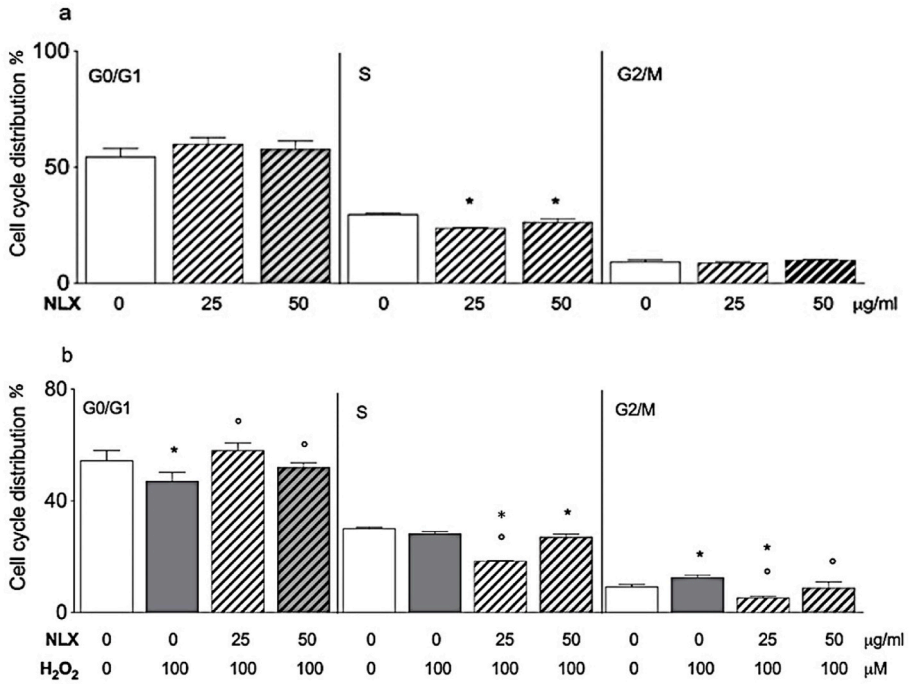

Fig. 6. Distribution of cell cycle after NLX (25 and 50 µg/ml) incubation. Panels a and b show the effects of NLX without or with H_2O_2 (100 µM) in PC12 cells at 24 h of exposure. Bonferroni multiple comparisons shows a significant difference for $p < 0.05$ versus untreated cells (control): * and versus H_2O_2: °.

lower in presence of both NLX concentrations ($p < 0.05$), compared in the H_2O_2 group. NLX treatment on oxidative damage, at the dose of 25 µg/ml, carried the cells to a lower value than the control, suggesting that NLX incubation (25 and 50 µg/ml) significantly ($p < 0.05$) reduces the number of cells induced into quiescence by H_2O_2 in the G2/M phase. Likewise, treatment with NLX also appeared to improve (G0/G1 phase) of the cell cycle of PC12 cells subjected to oxidative stress (panel b).

3.5. Effects of naloxone on LDH release

LDH is an enzyme released upon damage of plasma membrane. The LDH assay was performed to evaluate NLX's effect under H_2O_2, after 24 h of cells exposure (Fig. 7). NLX (25 and 50 µg/ml) significantly ($p < 0.05$) reduced the levels of LDH release (6%, 10%) when compared with control PC12 cells (20%), as can be seen in panel a. In presence of H_2O_2-induced LDH release, incubation with NLX (25 and 50 µg/ml) showed a significant ($p < 0.05$) reduction of oxidative injury (10%, 13%) when compared with H_2O_2 group (29%), panel b.

4. Discussion

NLX is an antagonist of the opioid receptors, widely expressed on cells of the central and peripheral nervous systems [28,29]. Polyphenols are the largest family of antioxidants. NLX contains an aromatic hydroxyl group at the C3 position of the structure that allows to consider it as a complex phenolic compound [1,30]. The main structural feature responsible for the antioxidative and free radical scavenging activity is the phenolic hydroxyl group since phenols can donate the hydrogen atom of the phenolic hydroxyl to the free radicals, thus stopping the propagation chain during the oxidation process [30]. This aromatic structure led us to focus our attention on possible antioxidant properties and activities of NLX.

The aim of our study was to investigate the effects of NLX on H_2O_2-induced oxidative stress in PC12 cells [31,32]. Using platinum-based sensors for electrochemical tests, we firstly explored the NLX's antioxidant properties in an *in vitro* cell-free model. Then we extended our study assessing intracellular ROS levels, apoptosis, cell cycle

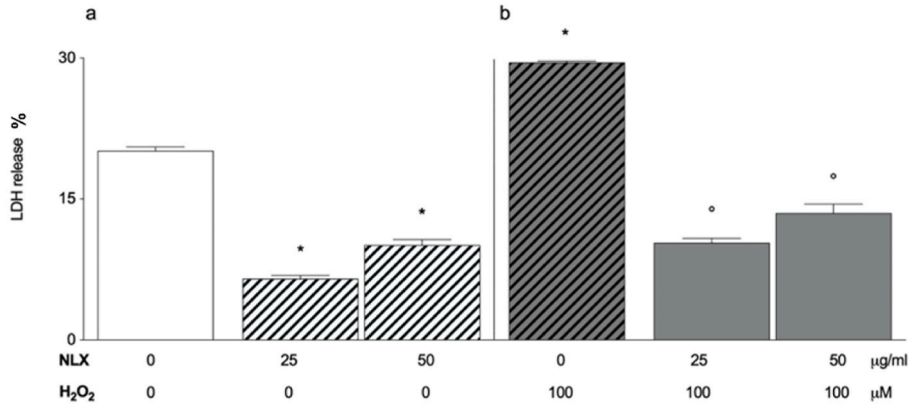

Fig. 7. Evaluation of LDH release after NLX (25 and 50 µg/ml) incubation. Panels a and b show the effects of NLX without or with H_2O_2 (100 µM) in PC12 cells at 24 h of exposure. Bonferroni multiple comparisons shows a significant difference for $p < 0.05$ *versus* untreated cells (control): * and *versus* H_2O_2: °.

distribution, and plasma membrane damage by measuring LDH release in an *in vitro* model using a PC12 cell culture.

Our current findings obtained from "cell-free" electrochemical tests using a platinum sensor, demonstrated that NLX can have antioxidant activities. At increasing concentrations (25–100 μM), NLX induced a decrease of H_2O_2 currents; this impact was 5 times higher than that of ascorbic acid, a well-known antioxidant, used as reference.

By using convergent and complementary assays our study provided proof that NLX has an interesting and significant antioxidant activity since overall protects PC12 cells against H_2O_2-induced damage. In fact, NLX firstly, decreases ROS; secondly, NLX decreases apoptotic cell death; thirdly, the results of the cell cycle analysis reveal that NLX reduces the percentage of cells in G2/M phase increased by oxidative stress. Lastly, NLX, alone or in combination with H_2O_2, reduces LDH release.

Previous extensive literature indicates that the cells are constantly exposed to ROS whose increase above a given level results in oxidative stress [33]. Exposure to H_2O_2 triggers a rise of ROS above basal levels that causes damage in cells [21,23]. In our experimental conditions, NLX prevents the intracellular ROS increase, a finding partially in agreement with those of the letter of Mikawa and colleagues [34] in which NLX was reported to counteract ROS overproduction from neutrophils isolated from healthy adult volunteers.

As it is well known, oxidative stress is involved in the induction of apoptosis [35] and several studies have reported that the overproduction of ROS causes apoptosis [33,35,36]. Notably, NLX in our study reduced apoptotic cells at both of the concentrations examined in the presence of H_2O_2 exposure. According to the information presented above, this result is consistent with the hypothesis that NLX might determine a dose-dependent reduction of the number of H_2O_2-induced apoptotic cells by reducing ROS levels.

Our results showed that, at both concentrations, NLX had no influence on cell cycle distribution compared to untreated cells, which is partially in agreement with a previous report from Chen [36]. However, our results from the cell cycle analysis also showed that NLX, in cells exposed to H_2O_2, lowered the percentage of cells stagnant in G2/M phase increased by H_2O_2-induced oxidative damage. To further sustain the protective profile of NLX on oxidative injury, LDH release was detected. These tests revealed that NLX (at both concentrations examined) not only reduced the release of LDH, providing protection on its own, but also prevented LDH release upon cells' exposure to H_2O_2. This result agrees with its reported ability to reduce LDH release [37] following oxygen and glucose deprivation in PC12 cells, another type of oxidative damage. A most likely explanation about the effect of NLX on LDH release could be also attributed to a regulation of different signalling pathways (c-fos, c-jun and nur77) induced by oxygen and glucose deprivation [37].

Overall, considering these results, our future experiments will focus on studying drug delivery systems, as reported for quercetin [38,39] or, as for genistein by our research group [23,40], that can improve the efficacy of NLX.

It would also be interesting to study the effect of NLX against other agents that can induce oxidative stress, as reported by other researchers [38,41].

In conclusion, we have highlighted the antioxidant capacity of naloxone by means of different experiments, both in a cell-free system/chemical-based and *in-vitro* experiments. This aspect of naloxone, still little explored, interested us and led us to study the complex mechanism of action of this well-known drug from another point of view.

Author contributions

All the authors wrote and approved the final version of this manuscript.

Declaration of competing interest

The authors have no conflict(s) of interest to declare.

Acknowledgements

The authors acknowledge Sassari University for research funding (FAR 2019 and 2020) to Dr Rossana Migheli and Prof Alessandra Tiziana Peana. Fondi M.I.U.R, Attività base di ricerca di cui all'art. 1, commi 295 e seguenti della L. 11 dicembre 2016 n. 232 (FFABR) 2017–2021.

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