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Parkinsonian toxin-induced oxidative stress inhibits basal autophagy in astrocytes via NQO2/quinone oxidoreductase 2: Implications for neuroprotection

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Abbreviations: Ab, antibody; AVs, autophagic vacuoles; BNAH, benzylhydri nicotinamide riboside; CQ, chloroquine; CA-DCF-DA, 5(6)-carboxy-2',7' dichlorofluorescein diacetate; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; FACS, flow cytometry; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; K3, menadione; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; MitoSOX, 3,8-phenanthridinediarnine, 5-(6’-tri phenylphosphoniumhexyl)-5,6 dihydro-6-phenyl; MPTP, 1-methyl 4-phenyl 1,2,3,6-tetrahydropiridine; NMDPEF, N-[2-(2-methoxy-6H-dipyrido [2,3-a:3,2-e]pyrrolizin-11-yl)ethyl]-2-furamide; NFE2L2, nuclear factor, erythroid 2-like 2; OS, oxidative stress; PBS, phosphate-buffered saline; p-, phosphorylated; PQ, parquat; ROS, reactive oxygen species; RT, room temperature; shRNA, short hairpin ribonucleic acid; siRNA, small interfering ribonucleic acid; SN, substantia nigra; TTBS, Tween-Tris buffered saline; WB, western blotting.

Oxidative stress (OS) stimulates autophagy in different cellular systems, but it remains controversial if this rule can be generalized. We have analyzed the effect of chronic OS induced by the parkinsonian toxin parquat (PQ) on autophagy in astrocytoma cells and primary astrocytes, which represent the first cellular target of neurotoxins in the brain. PQ decreased the basal levels of LC3-II and LC3-positive vesicles, and its colocalization with lysosomal markers, both in the absence and presence of chloroquine. This was paralleled by increased number and size of SQSTM1/p62 aggregates. Downregulation of autophagy was also observed in cells chronically exposed to hydrogen peroxide or nonlethal concentrations of PQ, and it was associated with a reduced astrocyte capability to protect dopaminergic cells from OS in co-cultures. Surprisingly, PQ treatment led to inhibition of MTOR, activation of MAPK8/JNK1 and MAPK1/ERK2-MAPK3/ERK1 and upregulation of BECN1/Beclin 1 expression, all signals typically correlating with induction of autophagy. Reduction of OS by NMDPEF, a specific NQO2 inhibitor, but not by N-acetylcysteine, abrogated the inhibitory effect of PQ and restored autophagic flux. Activation of NQO2 by PQ or menadione and genetic manipulation of its expression confirmed the role of this enzyme in the inhibitory action of PQ on autophagy. PQ did not induce NFE2L2/NRF2, but when it was co-administered with NMDPEF NFE2L2 activity was enhanced in a SQSTM1-independent fashion. Thus, a prolonged OS in astrocytes inhibits LC3 lipidation and impairs autophagosome formation and autophagic flux, in spite of concomitant activation of several pro-autophagic signals. These findings outline an unanticipated neuroprotective role of astrocyte autophagy and identify in NQO2 a novel pharmacological target for its positive modulation.

Introduction

Parkinson disease (PD) is a common neurodegenerative disorder that is primarily characterized by progressive loss of dopaminergic neurons in substantia nigra (SN) pars compacta and consequent reduced dopamine production. The glial cells (astrocytes and microglia) play a complex role in dopaminergic neurotoxicity.1-5 Both microglia and astrocytes can degrade...
dopamine with consequent generation of oxidative stress (OS) and detrimental effects on dopaminergic neurons.\(^6\,7\) While parkinsonian neurotoxins activate microglial cells\(^8\) leading to neuro-inflammation in nigrostriatal area, the protective effects of astrocytes in development and regeneration of the nigrostriatal dopamine system largely outweigh the pro-inflammatory role of the glia.\(^3\,8\,9\) Astrocytes contribute to the blood-brain barrier\(^8\) and protect neurons from OS induced by hydrogen peroxide (H\(_2\)O\(_2\)), toxic metabolites and neurotransmitters, including dopamine, 6-hydroxydopamine, and glutamate.\(^{10\,\text{-}\,12}\) This property has been associated with a basally high expression of NFE2L2 (nuclear factor, erythroid 2-like 2) that leads to increased synthesis and secretion of glutathione.\(^13\)

Autophagy, a lysosome-dependent degradation pathway of self-destructive, normally runs at low basal level to ensure cell homeostasis and is activated in response to starvation and metabolic stress to prevent accumulation of damaged organelles and protein aggregates.\(^{14\,\text{-}\,16}\) Defects in the autophagy machinery have been implicated in several neurodegenerative disorders, including PD.\(^{17\,\text{-}\,18}\) The progressive accumulation of protein inclusions containing SQSTM1/p62, ubiquitin and/or SNCA/PD,\(^{19\,\text{-}\,21}\) Lewy bodies disease, and related disorders\(^{21\,\text{-}\,23}\) is observed not only in neurons, but also in astrocytes, suggesting that defective autophagy is common to both cell subsets present in affected areas of the brain. The vast majority of studies that address dysregulation of autophagy by toxins mimicking PD are limited to dopaminergic cell lines and primary neurons as cellular models of PD.\(^{24\,\text{-}\,25}\) However, under physiological conditions, when the blood-brain barrier functions correctly, dopaminergic neurons of the SN are not directly exposed to parkinsonian toxins, which are instead absorbed and metabolized by astrocytes.\(^6\,\,8\)

In fact, astrocytes are the first target of neurotoxins and thanks to their powerful antioxidant machinery they represent the first line of brain defense against such toxins.\(^{15\,\text{-}\,26\,\text{-}\,28}\)

Parkinsonian toxins applied to rodents or primates reproduce only certain features of PD.\(^{25\,\text{-}\,29}\) Among them, SNCA accumulation and Levy bodies-like protein inclusions are effectively induced only by PQ\(^{30}\) and rotenone.\(^{31}\) All parkinsonian toxins generate reactive oxygen species (ROS) and/or reactive nitrogen species by disruption of the mitochondrial electron transport chain and/or production of toxic metabolites leading to altered energy metabolism.\(^{32}\) In addition, PQ-induced cellular toxicity depends on the presence of oxidoreductases able to reduce PQ by one electron transfer,\(^{33}\) such as plasma membrane NOX 1 (NADPH oxidase 1)\(^{34}\) or mitochondrial COX/ubiquinol: cytochrome c oxidase.\(^{35}\) We have recently identified a cytoplasmic flavoenzyme, NQO2/quinone oxidoreductase 2 (NAD[P][H dehydrogenase, quinone 2), as another critical mediator of PQ-induced OS and toxicity.\(^{36}\) Silencing of NQO2 conferred a partial or full protection in astroglial cells exposed to intermediate concentrations of PQ, and inhibition of NQO2 activity by its specific inhibitor prevented PQ-induced OS, cell death in vitro and animal mortality in models of acute PQ-induced systemic- and SN-toxicity.\(^{36}\) These findings provided further evidence that NQO2 may play a toxifying role in biological systems in the presence of certain xenobiotics.\(^{37}\)

ROS generated by H\(_2\)O\(_2\) or MPP\(^+\) (the active MPTP metabolite) induce a strong autophagic response in dopaminergic cell lines,\(^{38\,\text{-}\,39}\) and primary cortical and nigrostriatal neurons,\(^{40\,\text{-}\,41}\) leading to the activation of a cell death program. Nevertheless, it is unclear if the rule “OS causes autophagy” can be applied to all cell systems and different types of OS. Indeed, several lines of evidence suggest that OS may also have a negative impact on the autophagy machinery, as recently reviewed by us.\(^{17\,\text{-}\,25}\)

Here, we demonstrate that chronic exposure to pro-oxidants, such as PQ and H\(_2\)O\(_2\), reduces basal autophagy in astrocytes. This effect is dose-dependent and occurs also at nonlethal concentrations of PQ. Coculture experiments showed that the impairment of autophagy limited the capacity of astrocytes to protect dopaminergic neuroblastoma cells from a lethal OS. Intriguingly, in spite of a marked decrease in LC3 lipidation, PQ induced a series of proautophagic signals including BECN1 upregulation, and MAPK8 and MAPK1/3 activation. Furthermore, we identify NQO2 as a mediator of the negative effects of PQ on autophagy. Thus, PQ-induced OS generates both positive and negative signals affecting the autophagy machinery and antioxidant capacity of astrocytes, and the balance between these signals can be modulated pharmacologically by NQO2 inhibition. The present findings have important pathophysiological implications: first, they uncover a neuroprotective role of autophagy in astrocytes that outlines the active involvement of these cells in the pathogenesis of OS-related neurodegenerative diseases; second, the fact that inhibition of NQO2 activity can restore protective autophagy in OS astrocytes could be clinically exploited for the treatment of OS-associated neurodegeneration.

**Results**

**Parquat negatively affects basal autophagy in astrocytoma cells**

We previously reported that astrocytoma U373 cells exposed to PQ-induced OS undergo a delayed, nonapoptotic cell death.\(^{36}\) Whether autophagy was involved in either promoting or counteracting this phenomenon remained to be elucidated. The western blotting analysis of U373 cell lysates revealed a time- and dose-dependent decrease of lipidated LC3 (LC3-II), a fundamental component of phagophore and autophagosomal membranes (Fig. 1A and B). Next, we analyzed the levels of SQSTM1, a ubiquitin-binding protein that functions as a receptor for autophagic substrates\(^{32\,\text{-}\,34}\) and is an abundant component of protein inclusions associated with neurodegenerative disorders.\(^{21\,\text{-}\,23}\) SQSTM1 levels were dose-dependently increased 24 h post-treatment (Fig. 1C and D), suggesting a reduced formation or turnover of autophagosomes. The lack of significant increase in SQSTM1 levels at 48 h was possibly due to SQSTM1 precipitation into insoluble aggregates, considering the presence of high amounts of SQSTM1 in the cell lysate fraction insoluble to 1% Igepal (Fig. 1C). The above observations were confirmed by immunofluorescence staining for autophagic structures (Fig. 2A). PQ (100 µM, 24 h) caused a more than 3-fold decrease in LC3-positive autophagic vacuoles (AVs) (Fig. 2B).
and a strong reduction in AV-positive cells (Fig. 2C) compared to untreated cells. This was accompanied by a mild increase in the number of SQSTM1 granules (Fig. 2A and D) and a strong increase in the SQSTM1 staining intensity (Fig. 2A and E). In addition, PQ strongly impaired the colocalization of SQSTM1 and LC3 (Fig. 2A and F), suggesting that many SQSTM1-targeted aggregates were not engulfed within autophagosomes.

Paraoquat inhibits the formation of autophagosomes

Autophagy is a dynamic process and an apparent LC3-II downregulation may be due to scarce production or prompt consumption of autophagosomes. The treatment with drugs that abrogate vacuolar acidification, such as chloroquine (CQ), helps to discriminate between these 2 possibilities. To determine if the decrease of LC3 dots was the result of reduced formation or of accelerated consumption of autophagosomes, we added CQ to U373 cells during the last 4 h of PQ treatment (Fig. 3A). In cultures not exposed to PQ, CQ greatly increased the number of LC3-positive vesicles, while in PQ-treated cells this increase was modest (Fig. 3A and B). Accordingly, the levels of LC3-II protein in cells treated with 10 or 100 μM PQ along with CQ were lower than in CQ-only treated cells (Fig. 3C). We further confirmed that any treatment with CQ, ranging from 1 up to 18 h before cell lysis, resulted in a lower LC3-II accumulation in PQ-treated cells than in control cells (Figs. 4–8 and data not shown).

The inhibitory effect of PQ was not limited to basal autophagy. In fact, the herbicide also inhibited rapamycin-induced autophagy in U373 cells, as the induction of LC3-II levels by rapamycin was potently blocked by PQ, both in the presence and absence of CQ (Fig. 3D). These data consistently demonstrate that in this cell system PQ blocks autophagy before or at the step of autophagosome formation.

Inhibition of autophagy limits the anti-oxidant and neuroprotective capabilities of astrocytes

Chronic exposure to very low concentrations of PQ likely contributes to PD pathogenesis. To mimic this condition in vitro, we chronically applied nonlethal concentrations of PQ for several days. 1 μM PQ is not lethal for at least 9 d to U373 astrocytes, if cells are maintained in daily-changed culture medium containing 4.5% glucose (data not shown). 1 μM PQ applied for 7 d (long term, LT-PQ) led to a significant decrease in LC3-II, more evident in the presence of CQ, and caused a concomitant SQSTM1 accumulation (Fig. 4A and B) and aggregation (Fig. 4C). Next, we asked if the impairment of autophagy in astrocytes could affect their viability and antioxidant capacity. After 24-h exposure to H2O2, LT-PQ cultures contained significantly less viable cells than control U373 cultures, yet LT-PQ U373 cells were more resistant to H2O2 than dopaminergic neuroblastoma SH-SYSY cells (Fig. 4D). We have previously reported that SH-SYSY cells subjected to similar concentrations of H2O2 rapidly activate autophagy and eventually undergo autophagy-dependent cell death. The important role of astrocytes, among others, is to protect dopaminergic neurons from oxidative insults. This antioxidant capacity of astrocytes can be assessed in vitro in coculture with neuronal cells. We established mixed cell cultures and found that dopaminergic neuroblastoma SH-SYSY cells were significantly protected from H2O2 if cultured in the presence of control U373 cells, but not if cocultured with LT-PQ U373 (Fig. 4E and F). In the latter case, a decreased ratio of viable GFAP-negative cells (SH-SYSY) compared to GFAP-positive U373 cells was observed (Fig. 4E and F). These data indicate that a chronic exposure to low concentration of PQ negatively affects autophagy and concomitantly diminishes the antioxidant and neuroprotective capacities of astrocytes.

Downregulation of LC3-II is a response to chronic, but not transient, OS in astrocytes

Astrocytes are highly resistant to OS. The lack of autophagic response to H2O2 in primary mouse astrocytes has been previously attributed to no ROS generation. To exclude
such a possibility in U373 cells, we measured ROS levels in response to the herbicide and H$_2$O$_2$ by using the fluorescent probes CA-DCF-DA and MitoSox, which preferentially detect hydroxyl radical (OH$^\cdot$) and mitochondrial and cytoplasmic superoxide (O$_2$$^\cdot$)), respectively. PQ turned out to be a poor inducer of OH$^\cdot$ compared to H$_2$O$_2$ (Fig. 5A), but both PQ and H$_2$O$_2$ caused a significant increase of O$_2$$^\cdot$), although the kinetics and the extent of its induction were different for PQ and H$_2$O$_2$ (Fig. 5B). In addition, the rapid induction of O$_2$$^\cdot$) in U373 cells by H$_2$O$_2$ temporally correlated with a dose-dependent decrease of LC3-II levels at 4 h and 24 h post-treatment, in the presence or absence of CQ (Fig. 5C and D). H$_2$O$_2$ also induced a dose-dependent formation of insoluble SQSTM1 aggregates (Fig. 5C and D), which, however, did not accumulate with time as cell death rapidly occurred (data not shown).

We further addressed if the negative effect of PQ and H$_2$O$_2$ on basal autophagy in U373 cells was an unusual feature of this cell line or a common response to prolonged OS in astrocytes. Primary mouse astrocytes were expanded for 10 d after isolation before exposing the cells to OS. 100 $\mu$M PQ and 1 mM H$_2$O$_2$ applied for 16 h had little effect on LC3 lipidation, but a longer exposure (48 h) to pro-oxidants led to a significant decrease in LC3-II levels (Fig. 5E) and in LC3 dots (Fig. 5F). This effect occurred days before any signs of cell death, as primary astrocytes showed very low mortality until d 4 and d 3 post-treatment in response to 100 $\mu$M PQ (Fig. 7H) and 1 mM H$_2$O$_2$ (data not shown), respectively. In addition, primary astrocytes exposed for 7 d to a low concentration of PQ (3 $\mu$M) also accumulated SQSTM1 aggregates in contrast to astrocytes cultured under the same conditions, but without PQ (Fig. 5G).

**PQ triggers upstream pro-autophagic signals**

To find the mechanism underlying the PQ-induced block of basal autophagy, we measured the levels of BECN1 upon PQ treatment, as downregulation of this protein inhibits basal and starvation-induced autophagy. Unexpectedly, we found a dose-dependent induction of BECN1 in cells exposed to different concentrations of PQ (Fig. 6A). Interestingly, BECN1 accumulated in the insoluble fraction of lysates at the 48 h time point (Fig. 6B), suggesting that it was part of a membrane-associated oligomeric complex. This interpretation was supported by immunofluorescence staining for BECN1, which showed increased intensity and colocalized with PIK3C3/VPS34 upon 16 h treatment with PQ (Fig. 6C).

We investigated other possible signaling pathways regulating autophagy in response to PQ. MTOR activation leads to autophagy suppression in the majority of cell systems. However, 100 $\mu$M PQ did not induce significant activation of MTOR at early time points, whereas it strongly inhibited MTOR after 24 h (Fig. 6D), when autophagy was clearly suppressed in our system. Accordingly, RPS6KB/p70S6 kinase phosphorylation, which reflects MTOR activity, was inhibited or unchanged up to 6 h after PQ treatment and strongly suppressed after 24 h (Fig. 6E). These data suggest that the negative regulation of autophagy by PQ does not involve MTOR. Starvation-induced autophagy and MPP$^+$-induced autophagy are mediated by the activation of MAPK8/JNK1 (mitogen-activated protein kinase 8). Similarly, a transient increase in MAPK1/ERK2-MAPK3/ERK1 activity correlates with autophagy induction and is required for MPP$^+$ and zVAD-induced autophagic cell death. PQ led to a significant elevation of MAPK8 phosphorylation within 1 h, followed by a very slow decrease back to control levels (Fig. 6F). Phosphorylation of MAPK1/3 was similarly modulated by PQ-induced stress, but a significant increase in p-MAPK1/3 was still detectable 24 h post-treatment (Fig. 6G). Sustained MAPK1/3 activation blocks the maturation of autophagosomes in cancer cell lines exposed to the pesticide Lindane. Remarkably, the MEK1 inhibitor U0126 had little
effect on basal LC3-II levels in control cells and further reduced LC3-II in PQ-treated cells (Fig. 6H), although it strongly decreased MAPK1/3 phosphorylation in PQ-treated and control cells (Fig. 6I). These data indicate that MAPK1/3 activation is not an inhibitory signal elicited by PQ.

**Inhibition of OS by a NQO2 inhibitor, but not by N-acetylcysteine, enhances autophagy in U373 cells and in primary astrocytes exposed to PQ**

To examine the causal role of OS in PQ-induced downregulation of basal autophagy we used N-acetylcysteine (NAC), a thiol antioxidant, and NMDPFEF (N-2-[2-(2-methoxy-6H-dipyrido[2,3-a;3,2-c]pyrrolizin-11-yl)ethyl]-2-furamide), a pharmacological inhibitor of NQO2 activity and a powerful PQ-specific antioxidant in U373 cells. According to our previous observations, 10 μM NMDPEF strongly reduced PQ-induced ROS, as detected by MitoSox (Fig. 7A). 5 mM NAC showed comparable effects on mitochondrial ROS, but the effects of the NQO2 inhibitor were more stable over time compared to NAC (Fig. 7A). Remarkably, the addition of NMDPFEF to U373 cells for 24 h potently enhanced LC3-II processing (Fig. 7B) and increased the number of AV dots both in PQ-treated and untreated cells (Fig. 7C and D). In contrast, cotreatment with NAC did not restore LC3-II levels in PQ-treated astrocytoma cells, but it dose-dependently decreased LC3-II in control cells (Fig. 7D). Of note, NMDPFEF not only increased the number of LC3-positive dots, but it restored the normal percentage of LC3-LAMP1 double-positive autolysosomes, which were markedly reduced in PQ-treated cells compared to control cells (Fig. 7C and E), suggesting that NQO2 inhibition stimulates autophagy flux. This observation was confirmed by acridine orange staining and flow cytometry used to assess quantitatively acidic vesicles such as autolysosomes and lysosomes. NMDPFEF increased, whereas NAC reduced, the number of acidic vesicles, in control and PQ-treated cells (Fig. 7F).

The pro-autophagic effect of the NQO2 inhibitor was further confirmed in primary mouse astrocytes. NMDPFEF restored LC3-II levels in primary cultures treated with PQ (Fig. 7G and H), which correlated with a significant protective effect of this agent against PQ-induced cell death (Fig. 7I), in accordance to what we previously observed in U373 cells. Thus, a NQO2-specific antioxidant, but not a generic inhibitor of PQ-induced OS, can prevent deleterious effects of PQ on LC3 lipidation and autolysosome formation.

**NQO2 mediates the negative regulation of LC3-lipidation by PQ**

We further investigated the active involvement of NQO2 in the negative regulation of basal autophagy induced by PQ. NMDPFEF dose-dependently and time-dependently induced autophagy, as shown by the increase in LC3 lipidation and BECN1 (Fig. 8A). This effect was not due to inhibition of the autophagic flux, since the upregulation of LC3-II in the presence of NMDPFEF and CQ was much higher than in vehicle-treated cells in the presence of CQ (Fig. 7B). NQO2 is well expressed in primary astrocytes and U373 cells, although very low levels of NQO2 can be detected in mouse brain total lysates, suggesting an astrocyte-specific expression of this enzyme (Fig. 8B). Since NQO2 activity contributes to OS in astrocytes, it may play a role in the negative regulation of autophagy by chronic OS. To test this hypothesis, we stimulated NQO2 activity in U373 cells with the NQO2 substrate menadione (K3). Stimulation of NQO2 activity rapidly inhibited LC3 lipidation at 1 and 6 h, although at later time points LC3-II accumulated most likely due to inhibition of the autophagic flux (Fig. 8C). This effect correlated with NQO2 activity. 10 μM K3 led to 3- to 4-fold activation of NQO2 at 1 and 6 h, respectively, while PQ stimulated

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*Figure 3. Reduction in the number of LC3 dots by PQ is not due to accelerated autophagic flux. (A) U373 cells were treated or untreated with 100 μM PQ for 24 h and with or w/o chloroquine (CQ, 25 μM) for 4 h before fixation with paraformaldehyde. A representative confocal image (extended focus of 5-μm deep stack) for each treatment and staining is shown. Bar: 25 μm. (B) Mean number of LC3 vesicles per cell +/- SEM, calculated on n = 6 images per treatment, from 3 independent experiments described in (A). Statistical analysis was performed by Mann-Whitney test, n = 6, *P < 0.05, **P < 0.01. (C) WB analysis of LC3-II and SQSTM1 in U373 cells treated as in (A) and exposed to 10 and 100 μM PQ, +/- CQ (25 μM) added 6 h before lysis. (D) PQ blocks rapamycin-induced autophagy in U373 cells. 5 μM rapamycin (Rap) +/- PQ was applied for 24 h. The autophagy flux was blocked by 25 μM CQ applied 3 h before lysis and LC3 levels were analyzed in CQ-treated and untreated cells.*
2-fold NQO2 activation within 20 h (Fig. 8D). Next, we addressed the role of NQO2 in autophagy by genetic modulation of its expression. NQO2 was silenced by transient transfection of different NQO2-specific siRNAs or overexpressed by a stable expression of the human NQO2-coding lentiviral construct (Fig. 8E). NQO2 knockdown prevented PQ-induced reduction in LC3 lipidation and partially reduced PQ-dependent accumulation of SQSTM1 (Fig. 8F and G). In addition, NQO2 silencing markedly reduced the LC3 response to NMDPEF alone, but augmented the response to NMDPEF in the PQ-treated cells (Fig. 8F and G). We further analyzed LC3 processing in U373 cells stably overexpressing NQO2. NQO2 overexpression reproducibly led to a stronger reduction of LC3 lipidation than in control vector-expressing cells in response to PQ (Fig. 8H and I). Strikingly, this correlated with a significantly higher sensitivity of NQO2-transfected cells to PQ-induced toxicity, since these cells massively underwent cell death 48 h after the treatment with PQ in contrast to control vector-transfected cells, which were still alive at this time point (Fig. 8J). The increased mortality was mediated by an increased NQO2 activity in NQO2-overexpressing U373 cells, since its inhibition by the NQO2 inhibitor NMDPEF efficiently protected these cells against cell death. In addition, cotreatment with the autophagy inhibitor 3-methyladenine and PQ increased the percentage of dead cells in NQO2-overexpressing and in control cells (Fig. 8J). Thus, the residual autophagy in PQ-treated cells has a protective function in astroglial cells, and its stimulation with the NQO2 inhibitor protects from PQ-induced cell death.

PQ and NMDPF cotreatment, but not PQ alone, stimulates NFE2L2 activity independently from SQSTM1

Finally, we asked whether NFE2L2 was involved in PQ-dependent inhibition of autophagy. NFE2L2 is a ubiquitously expressed transcription factor that regulates the expression of genes bearing an antioxidant response element (ARE) in the promoter region, including NQO2. Importantly, autophagic activity inversely correlates with NFE2L2-mediated antioxidant responses, due to SQSTM1-dependent disruption of KEAP1-NFE2L2 binding on
Figure 5. Inhibition of autophagy in astroglial U373 cells and in primary astrocytes by PQ is mediated by oxidative stress. (A) ROS induction by PQ and H\textsubscript{2}O\textsubscript{2} as detected by CA-DCF-DA in U373 cells. Live fluorescence of cell monolayers was analyzed by fluorometry 1 h after staining with CA-DCF-DA. (B) Mitochondrial ROS induction by PQ and H\textsubscript{2}O\textsubscript{2} as detected by MitoSox staining and flow cytometry in U373 cells. Statistical analysis for (A) and (B): one-way ANOVA followed by Dunnett’s post-test, \( n = 6 \) samples for each CA-DCF-DA group, 6 groups; *\( P < 0.05 \), **\( P < 0.01 \). (C) H\textsubscript{2}O\textsubscript{2} inhibits basal autophagy in U373 cells. The cells were treated with H\textsubscript{2}O\textsubscript{2} (0.2 or 1 mM) for the indicated times, +/- CQ (50 \( \mu \)M, added 1 h before the lysis). LC3 and SQSTM1 expression was analyzed by WB after 15% and 8% SDS-PAGE, respectively. (D) Densitometric analysis of LC3-II (+CQ) and SQSTM1 bands (no CQ) from (C). Mean +/- SEM from 3 independent experiments. Statistical analysis: 2-way ANOVA followed by Bonferroni post-test, time vs concentration, \( n = 3 \) samples for each group, 5 groups for each OD analysis; *\( P < 0.05 \), **\( P < 0.01 \), compared to vehicle-treated cells. (E) Inhibition of autophagy in primary astrocytes in response to prolonged oxidative stress. Primary cultures of astrocytes isolated from mouse cortex were treated or untreated with PQ (100 \( \mu \)M) or H\textsubscript{2}O\textsubscript{2} (1 mM) +/- CQ (50 \( \mu \)M, 3 h before lysis) and analyzed for markers of autophagy as described in (C). (F) 48 h before the treatment described in (E) the astrocytes were transfected with GFP-LC3 construct and analyzed for the presence of green dots. Representative, extended focus confocal images of GFP-LC3-expressing cells, pretreated for 3 h with CQ (50 \( \mu \)M), are shown. Bar: 10 \( \mu \)m. (G) Upregulation of SQSTM1 aggregates in primary mouse astrocytes exposed to prolonged treatment with PQ. Eight d after isolation primary cells were split and then cultured in the presence (LT-PQ) or absence (Control) of 3 \( \mu \)M PQ for 7 d. Primary cultures were then stained for GFAP and SQSTM1 and analyzed by confocal microscopy. Bar: 20 \( \mu \)m.
Figure 6. PQ activates pro-autophagic signaling pathways in spite of its negative effect on LC3 lipidation. (A) Samples from experiments described in Fig. 1 (6 and 24 h PQ treatment) were analyzed for BECN1 expression by 8% SDS-PAGE and WB. Densitometric analysis of data (in lower panel) was performed as described in Fig. 1B (TUBA/α-tubulin was used as loading control). (B) PQ causes accumulation of insoluble BECN1 in cells exposed for 48 h to PQ. Samples of insoluble proteins in lysates were prepared and analyzed as described in Figure 1C. Statistical analysis for (A) and (B): one-way ANOVA followed by Dunnett’s post-test; n indicates number of independent experiments; 4 groups, 3 – 6 OD values for each group; *P < 0.05, ***P < 0.001. (C) U373 cells were seeded on coverslips and treated or untreated with 100 μM PQ for 6 h, fixed and stained for BECN1 (green) and PIK3C3 (red). Representative confocal images of middle sections of stained cells are shown. Bar: 25 μm. (D–G) U373 cells were treated for the indicated time with 100 μM PQ and cell lysates were analyzed by NuPage 4–12% gradient gel electrophoresis followed by WB. MTOR (D), RPS6KB/p70S6 kinase) (E), MAPK8 (F) and MAPK1/3 (p44/p42) (G) phosphorylation were detected by phospho-specific antibodies. Densitometric analysis of n = 3 to 5 independent experiments was performed. Statistical analysis: one-way ANOVA for (D and E, 7 groups each experiment) and Mann-Whitney test for (F and G, 5 – 9 OD values for each control group, 3 – 5 OD values for other groups); *P < 0.05. (H) UO126 MEK1 inhibitor (0.2 μM) has little effect on basal autophagy and does not restore autophagy in PQ-treated cells. (I) Effects of UO126 used in (I) on MAPK1/3 phosphorylation in U373 cells.
the one hand and due to NFE2L2-dependent upregulation of SQSTM1 on the other. First, we assessed the expression at protein level of NFE2L2, NQO2, and SQSTM1 in U373 cells exposed to PQ, NMDPF, or PQ+NMDPF for up to 24 h (Fig. 9A). PQ induced the accumulation of SQSTM1 at 24 h, as expected, but did not alter NFE2L2 levels. Though not statistically significant, a transient upregulation of NQO2 protein level was observed in PQ-treated cells. NMDPF alone had no effects on the expression of these 3 proteins. However, when the cells were cotreated with PQ and NMDPF, NFE2L2 levels significantly increased at 24 h (Fig. 9A). Next, NFE2L2 transcriptional activity was determined by luciferase reporter assay monitoring ARE induction in control, PQ, NMDPF, and PQ+NMDPF treatment conditions. The cotreatment with PQ and NMDPF robustly induced ARE-dependent transcription (Fig. 9B), which is consistent with the upregulation of NFE2L2 under these conditions (Fig. 9A). Finally, we asked whether SQSTM1 could be involved in NFE2L2 expression and activity. To this end, we repeated the experiments in siRNA-SQSTM1-silenced cells. Data shown in Figs 9C and D confirm that PQ alone does not induce NFE2L2 and also demonstrate that transcriptional activation of NFE2L2 (as probed by luciferase activity) in PQ+NMDPF-treated cells occurred independently from SQSTM1.

**Discussion**

The data presented in this paper led to several original and rather unexpected conclusions. We showed for the first time that prolonged OS reduces the basal autophagy rate in primary astrocytes and U373 astrocytoma cells exposed to PQ or H₂O₂, as evidenced by decreased LC3 lipidation correlating with impaired autophagosome formation and concomitant accumulation of SQSTM1 aggregates. Paradoxically, this occurs in spite of an apparent activation of the majority of pro-autophagic pathways by PQ. This inhibitory effect on autophagy is mediated by the quinone reductase NQO2 and can be prevented by pharmacological and genetic modulation of its activity. Prolonged inhibition of basal autophagy by low-doses of PQ has functional consequences on the antioxidant capacity of
astrocytes and negatively affects their neuroprotective capability toward dopaminergic cells exposed to OS.

These findings are unexpected for several reasons. First, numerous previous reports documented the causal role of OS induced by H$_2$O$_2$ in the activation of autophagy in different cell systems, implicating O$_2$•$^-$ as the main messenger of this response. Second, in neuroblastoma cell lines and in Drosophila synapse development OS induced by PQ was reported to induce autophagy. Third, MPPT/MPP$^+$, which is structurally related to PQ, excessively stimulates an autophagic response, leading to cell death in different neuronal cells. Accordingly, we also observed such a negative effect of NAC in our system (Fig. 7B). In contrast, the specific NQO2 inhibitor NMDPEF, that apparently shows a similar antioxidant activity, elicited an increase of the autophagic activity in response to OS (Fig. 7). This discrepancy suggests that the effect of an antioxidant on cell physiology depends on its target(s) and its way of action. Similarly, the source, type and time of action of OS may have opposite effects on the autophagy machinery, as also emerges from some recent findings and from more careful analysis of the literature data.

ROS generated in response to different stimuli seem to both induce and inhibit upstream signaling pathways regulating autophagy and may also negatively influence lysosomal function. For example, it has been reported that rotenone inhibits autophagic flux via inhibition of lysosomal degradation, as substantiated by increased levels of SQSTM1 and SNCA in dopaminergic SH-SY5Y cells. The suppression of autophagic flux was also observed in hepatoma cells in response to H$_2$O$_2$ and TNF/TNFα and in PRNP (prion protein)-deficient hippocampal primary neurons, but not in normal hippocampal neurons exposed to H$_2$O$_2$, suggesting a possible role of a functional PRNP in regulation of the autophagic response to OS.

In our experimental conditions, PQ-induced OS exerted a dual effect on the autophagy machinery: on the one hand, it activated autophagy-inducing signals including MAPK1/3 and MAPK8 pathways, inhibited MTOR and upregulated BECN1 (Fig. 6) and, on the other hand, it impaired LC3 processing, AV formation (Figs. 1–3) and led to a reduced number of autolysosomes (Fig. 7C and E). The negative regulation of basal autophagy by PQ (as well as by H$_2$O$_2$) was time- and dose-dependent. The effect was hardly detectable after short exposure or with low concentration (1 μM) of PQ (Fig. 1A). However, the prolonged exposure to low concentrations or the acute exposure to higher doses of PQ invariably led to the impairment of basal autophagy.

At low concentrations of PQ positive and negative signals are balanced and no detectable response in terms of autophagy induction is observed, in spite of a toxic accumulation of oxidative products. In the long run, such a situation potentially leads to astroglial degeneration. Indeed, astroglial cells survived up to 1072 Volume 11 Issue 7

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7 d under nonlethal concentrations of PQ (1 μM), but eventually showed a significant reduction in LC3-II levels associated with a significant accumulation of insoluble SQSTM1 aggregates (Fig. 4A–C), with a concurrent reduced resistance to OS and neuroprotective capacity toward dopaminergic cells (Fig. 4D–F). This observation can explain why PQ chronically administered to rodents causes accumulation of protein inclusions, similar to Lewy bodies in SN and other brain regions, before any loss of dopaminergic neurons becomes apparent. Accordingly, a recent report addressing the autophagic response to PQ in vivo reported appreciable modulation of autophagic markers in different regions of the brain, suggesting a possible inhibition of Figure 8. For figure legend, see page 1074.
Autophagy in vivo by PQ, but not by maneb, another herbicide implicated in PD etiology. However, the interpretation of the results obtained in rodent models is hampered by the difficulty in assessing the rate of autophagic flux in vivo.

Our in vitro system made it possible to show for the first time an inhibitory effect of PQ on the autophagy machinery in astrocytes. In addition, we could demonstrate that such a negative effect is mediated by the oxidoreductase NQO2. Several data support this conclusion: (i) pharmacological inhibition of NQO2 by NMDPEF potently upregulates LC3-II levels and autophagosome formation, as well as autophagosome-lysosome fusion and cell survival as well as PQ; (ii) NQO2 activation by menadione in astroglial cells leads to a rapid decrease in LC3-II levels; (iii) silencing of NQO2 expression by siRNA or shRNA reduces, whereas NQO2 overexpression (iv) augments, the inhibitory effect of PQ on LC3 processing. Induction of NQO2 activity and SQSTM1 accumulation by PQ did not correlate with NFE2L2 activation. Intriguingly, in PQ-NMDPEF cotreated cells NFE2L2 expression and activity were upregulated.

Astrocytes have been reported to possess high basal expression of NFE2L2, and apparently PQ cannot further induce its expression, unless NQO2 is inhibited. This complex regulation definitely deserves a more in depth investigation. However, considering the lack of correlation between NFE2L2 and SQSTM1 levels (Fig. 9A) and no effect of SQSTM1 silencing on NFE2L2 activity and levels (Fig. 9C and D), we can conclude that PQ+NMDPEF-induced NFE2L2 upregulation is SQSTM1 independent in U373 astroglial cells.

Another interesting finding presented here is that primary astrocytes and astroglial cell lines express much higher NQO2 protein levels compared to other brain cells (Fig. 8B). Consistently, we found that PQ exerted its inhibitory effect on autophagy mainly in astrocytes and to a lesser extent in other cell types (Janda et al., unpublished observations).

We have recently demonstrated that NQO2 clearly contributes to OS induced by PQ likely because its catalytic activity is intrinsically linked to ROS generation. Here, we confirm these findings and show that overexpression of NQO2 further sensitizes astroglial cells to PQ-induced cell death (Fig. 8J), an effect associated with impairment of autophagy. Most importantly, we show that NQO2-mediated inhibition of autophagy in astroglial cells has pathophysiological consequences since it limits the intrinsic ability of these cells to protect dopaminergic cells from OS.

The mechanism involved in free radical production by NQO2 is unknown and difficult to envisage in consideration of the fact that PQ2 catalyzes 2-electron reductions thought to be free of ROS byproducts. In addition, the toxifying effects of NQO2 activity are difficult to reconcile with a proposed detoxifying function of NQO2 in oxidative dopamine metabolism. Nevertheless, since NQO2 causes toxic activation of certain substrates (menadione, mitomycin) and contributes to ROS production by PQ, it cannot be excluded that similar ROS generating events accompany dopamine quinone reactions mediated by NQO2.

Thus, under certain conditions, NQO2 can lead to chronic OS and autophagy impairment, which may be prevented by pharmacological inhibition of its activity. The upregulation of basal autophagy associated with the inhibition of NQO2 activity in the absence of exogenous OS (Fig. 8A) suggests that ROS-independent mechanisms are also involved. The exact molecular mechanism of NQO2-mediated inhibition of basal autophagy is presently under investigation. Based on the differential effects exerted by the antioxidants NAC (more general) and NMDPEF (specific to NQO2-related ROS), we envisage a role for the localized NQO2-dependent production of O2•−, which might in turn affect the machinery for LC3 processing.

Regardless of the molecular mechanisms implicated in NQO2-dependent regulation of autophagy, the identification of this novel regulator of autophagy in oxidatively stressed...
cells has important practical implications. NQO2 can be easily modulated pharmacologically by several natural and synthetic inhibitors, including NMDPEF and certain flavonoids such as quercetin and resveratrol. These findings will foster future studies to define if pharmacological targeting of NQO2 in vivo can prevent SQSTM1-positive protein inclusions and pathological changes present in PD and other neurodegenerative disorders.

Material and Methods

Cell culture
Astrocytoma U373 cells (kind gift of Michela Pollicita, University Tor Vergata, Rome, Italy) were cultured as previously described. After thawing, cells were expanded in DMEM 4.5% glucose (LONZA, 12-604) and then routinely cultured in standard medium: DMEM 1% glucose (LONZA, 12-707),
supplemented with 2 mM t-glutamine (PAA Laboratories, M11-004), 10% fetal calf serum, 1 mM sodium pyruvate (Gibco, 11360-070), 100 units/ml penicillin, 100 μg/ml streptomycin (Pen/Strep, Gibco, 15070-063), in standard 5% CO₂ conditions. The medium was regularly changed every 48 h.

Isolation of primary astrocytes

Brains of 5 newborn pups (mouse strain Bl6/129, postnatal d 0–1) were isolated immediately after decapitation as previously described. Briefly, the brains were placed on plastic dishes containing sterile phosphate-buffered saline (PBS; Lonza, 4984) plus Pen/Strep (1:100). Cortices were dissected, meninges carefully removed and the tissue was cut into small pieces with scissors. All the cortex tissue was then collected, resuspended in 10 ml of DMEM, containing trypsin and DNAase (Sigma-Aldrich, T4799, P4513) and digested for 30 min at 37°C. The digested cortex was washed and plated on 5 culture dishes (10 cm) in standard medium. The next day the medium was changed and then renewed twice a wk. At confluence (usually at d 7–9 after plating) the culture was washed 3 times with PBS, trypsinized in 0.05% trypsin for 5–8 min and plated 1:3. When confluent, the primary cells were split again and plated for required experiments. All experiments were performed before 4 weeks after isolation. The percentage of astrocytes in primary culture was assessed on the basis of GFAP positivity upon staining with rabbit anti-GFAP (Thermo Scientific, OPA-06100), followed by anti-rabbit Alexa Cy5 (Molecular Probes, A-21070). The animal care and procedure for sacrifice complied with the recommendation of the local ethics committee.

Reagents and antibodies

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich: paraquat (M2254; 100 mM stock in PBS), chloroquine (C6628; 25 mM stock in PBS), 3-methyladenine (M9281; 10 mg/ml, 95% ethanol), DMSO (D4540), NAC (A9165; 1 M stock in PBS), were kept in aliquots at −80°C and thawed shortly before each treatment. Rapamycin (37094) was kept at 5 mM stock at −20°C. H₂O₂ (95321) was diluted from a 33% stock into 1 M aliquots and stored at −20°C. NMDPEF (a kind gift of Philippe Delagrange, Servier, France) was stored as 5 mM stock at 80°C.

The following antibodies (Abs) used for western blotting (WB) were purchased from MBL International Corporation: rabbit anti-LC3 (PM036; used 1:4000 and occasionally 1:1000), rabbit anti-BECN1 (PD017; 1:2000), anti-SQSTM1/p62, mouse (M162-3; 1:1000) or rabbit (PM045; 1:2000). In some experiments, the following Santa Cruz Biotechnology Abs were used: rabbit anti-BECN1 (sc-11427; 1:200), goat anti-NQO2 (sc-18574; 1:200), rabbit anti-NFE2L2/NRF2 (sc-722, C-20; 1:1000), anti-TUBA/α-tubulin (sc-23948; 1:800) and rabbit anti-GAPDH (clone F6235, sc-25778; 1:1000). Other Abs used were from Sigma-Aldrich: rabbit anti-NQO2 (HPA332; 1:1000), anti-ACTB/β-actin (clone A40; 1:5000) and mouse anti-GFAP (G4546; 1:1000). The following Abs were from Cell Signaling Technology (used at 1:2000 or 1:1000): rabbit anti-LC3B (used only for lysates from primary astrocytes; 2775), anti-p-MTOR (Ser2448; 2971) and anti-MTOR (clone 7C10; 2983S), rabbit anti-p-RPS6KB/p70S6 kinase (Thr389; 9205S) and anti-total RPS6KB/p70S6 kinase (2708S), anti-p-MAPK1/3 (P-p44/42, T202/Y204, clone 197G2, 4377S) and anti-MAPK1/3 (p44/42, clone 3A7, 9107), anti-RPS6KB (p-SAPK/JNK, T183/Y185, 4668). The Abs used for confocal immunofluorescence analysis were: rabbit anti-PI3KC3/VPS34 (RayBio, RB-08-0037; 1:200), mouse anti-BECN1 (MLB, PM015; 1:100), mouse anti-LAM1 (BD Biosciences, 611042; 1:100). Secondary antibodies anti-rabbit (Alexa Fluor 568, A-11011 and Alexa Fluor 488, A21206) and anti-mouse (Alexa Fluor 633, A-21052) were used at 1:800 dilution and were purchased from Molecular Probes. Fluorescein (FITC), Alexa Fluor 405 (Molecular Probes, F432; 1:400) was used to detect actin.

Treatments and experimental setup for western blotting analysis

U373 cells were seeded on plastic dishes 24 h before treatment at the density 2 × 10⁴ cells/cm² or 1 × 10⁴ cells/cm² and lysed 24 or 48 h after initial treatment, respectively. All treatments were performed in freshly renewed medium and the compound (PQ, NMDPEF, H₂O₂, NAC) was added for the indicated time (usually 6, 24, 48 h) before the end of the experiment, so that control and treated cells were all lysed at the same time. CQ (25 μM or 50 μM) was added 1 to 6 h before the end of the experiment, as specified; the time of exposure to CQ was identical for each sample in the same experiment. For prolonged incubation, U373 cells were seeded at low density (5 × 10⁴/cm²) and cultured in 4.5% glucose DMEM for 7 d. A half volume of the culture medium was renewed each day. Cells were exposed to 1 μM PQ for 7, 6, 4 and 2 d before the lysis and lysed at the same time on day 7. CQ (25 μM) was added 1 h before lysis. For signaling experiments the cells were treated for 15 and 30 min, and 1, 3, 6 and 24 h.

Western blotting

Cell monolayers were washed once with PBS and scraped in cold Lysis buffer 1 (LB1) containing 50 mM Tris-HCl, pH 7.3, 150 mM NaCl and the following reagents from Sigma-Aldrich: 1% Igepal (I7771), 1 mM EDTA (E5134), 1 mM phenylmethylsulfonyl fluoride (P7626), phosphatase inhibitors (1mM NaF [S1504], 0.01 mM sodium orthovanadate [S6508]) and protease inhibitor cocktail (Complete Mini, Roche Diagnostic, 11836153001). The lysate was placed on ice for 10 min and then centrifuged at 4°C, 15000 g for 10 min. The supernatant fraction was then mixed (3:1) with Laemmli Sample buffer (4: 100 mM Tris-HCl, pH 6.8, 4% SDS [Sigma-Aldrich, L4390], 16% glycerol, 20% 2-mercaptoethanol [Carlo Erba Reagents, 460691]), boiled 15 min at 90°C and then stored at −80°C until analysis. Samples were boiled again for 3 min at 90°C before loading onto 15% or 8% polyacrylamide SDS (SDS-PAGE) gels. Protein concentration was quantified by BCA protein assay (Thermo Scientific, 23227). Alternatively, for analysis of detergent-resistant fractions of the lysates, cell monolayers were lysed in LB1 as above. After centrifugation (4°C, 15000 g for 10 min), the pellets were washed once in 200 μl cold LB1
buffer and then lysed in 30 µl Laemmli Sample buffer (4x) by heating 10 min at 90°C. Next LB1 buffer (30 µl) was added and samples were heated again 10 min at 90°C. For loading on gels, it was assumed that protein concentration in the pellet fraction was proportional to the protein concentration in the supernatant fraction.

For analysis of serine-threonine phosphorylations (p-MTOR, p-RPS6KB, p-MAPK1/3, p-MAPK8/JNK) cell monolayers were washed in PBS containing NaF (1 mM) and lysed as described previously. In general, 10–20 µg of protein lysate was loaded on 15% or 8% SDS-PAGE gels or 4–12% Bis-Tris gradient NuPAGE gels (Invitrogen, NP0323). For NQO2 expression analysis, 50 µg of protein lysate was loaded on 10% gels. After electrophoresis, polypeptides were electrophoretically transferred to PVDF membranes (Bio-Rad, 162-0177), which were thereafter saturated with 5% milk (Panrea AppliChem, A0830) in TTBS for 1 h at room temperature (RT). The primary antibody was usually incubated overnight, followed by 2-h incubation with secondary antibody. Blots were developed with the reagents (ImmunoStarTM, Bio-Rad, 170–5070 or Santa Cruz Biotechnology). For NQO2 overexpression was verified and NQO2-overexpressing and control vector cells were used for experiments.

Immunofluorescence staining

For immunofluorescence staining, U373 cells were seeded (20–30 × 10^3 cells/cm^2) on cover slips and the next day treated as indicated, washed in PBS, fixed in 4% paraformaldehyde (Sigma-Aldrich, 158127) in PBS (pH 7.4) for 5 min and then blocked for 30 min at RT with PBS (pH 7.4) containing 1% fetal bovine serum, 0.1% Tween 20 (Sigma-Aldrich, P-1379) (blocking buffer). For immunodetection of antigens, the cells were incubated for 1 h at RT with the primary antibody; afterward the coverslips were washed 3 × in blocking buffer and then incubated for 45 min with the secondary antibody. Antibody dilutions were made in blocking buffer. After washing once in blocking buffer and 2 times in PBS, the cover slips were finally mounted on glass slides with 4 µl Slow-fade Gold mounting reagent (Life Technologies, S36936). A negative control was performed by omitting the primary antibody.

Confocal microscopy and analysis of images

Intracellular localization of LC3-positive vesicles, SQSTM1 dots and other markers of interest was assessed by confocal microscopy. Images were collected on a Leica TCS sp5X confocal microscope (LAS AF V2.2 DMI 6000 Argon Me Ne 543, Me Ne 633) with a 40 × ApoPLA oil immersion objective, magnification 5 ×, and 60 µm aperture (Leica Biosystems Inc., Buffalo Grove, IL, USA). Z stacks of images were collected as described previously. Extended focus projection were used to generate the images used for counting of total number of LC3 vesicles and SQSTM1 dots per cell. The colocalization analysis of LAMP1 and LC3 or SQSTM1 and LC3 was performed on a single central plane confocal image showing 2 to 5 cells. Counting of dots was performed manually and by ImageJ software. The parameters for ImageJ analysis were adjusted suitably to obtain similar results to manual counting. Several independent images with at least 50 cells were collected for each experiment. The mean of dots per cell was first calculated for single image and then the mean for the entire experiment was calculated by adding the single image means divided by the total number of images.

Live imaging of GFP-LC3 positive vesicles

Primary mouse astrocytes (12 d after isolation) were plated at the density 2 × 10^4 × cm^-2 on coverslips in 12-well plates and the next day transfected with 0.75 µg of GFP-LC3 plasmid (kind gift of Dr E. Bampton, Leicesteher, UK) per well with 1.5 µl Lipofectamine 2000 (Invitrogen, 11668027) according to the manufacturer’s instructions. 48 h after transfection, cells were treated as required and 24 h later live cell images were recorded on an A1r Nikon inverted confocal microscope equipped with resonant scanner for fast imaging and motorized XY stage by using a 63X CFI Plan APO VC 1.4 DT:0.13mm immersion oil objective (Nikon AG Egg/ZH, Switzerland). For
confocal z-axis stacks, 25 images separated by 0.2 μm along the
z-axis were acquired.

Detection of acidic vesicles by flow cytometry
U373 cells were seeded and cultured on 24-well plates as for
WB experiments, but at the end of the 24-h treatment the cells
were incubated for 15 min with 10 μg/ml acridine orange
(Sigma-Aldrich, 318337). Next, the cells were washed once in
PBS, trypsinized for 3 min, collected with 1 ml 10% fetal calf
serum culture medium in conical tubes (for flow cytometry anal-
ysis), centrifuged at 800 g for 5 min and suspended in 0.4 ml
PBS containing 1% BSA (Santa Cruz Biotechnology, 9048-46-
8) and 0.1% EDTA (Sigma-Aldrich, E5134). Cells were acquired
in the far red (>650 nm, FL3) channel and in the green channel
(510–530 nm, FL1) by FACSCanto II (BD Biosciences, Eren-
bodgem, Belgium). Mean fluorescence intensity (MFI) for FL1
and FL3 channels was determined on the entire viable cell pop-
ulation by BD FACSDiva software. The ratio of MFI (FL3)/MFI
(FL1) was calculated for each sample and data analyzed by
Microsoft Excel.

Mitochondrial ROS detection by MitoSox
To detect ROS in mitochondria U373 cells were treated or
untreated with PQ (200 μM) or H2O2 (200 μM) before analy-
sis. MitoSox (Molecular Probes, M36008) staining and flow
cytometry were performed as previously described.36

NQO2 activity assay
NQO2 activity in U373 cells was measured as previously
described40 with a few modifications. Briefly, U373 cells plated
and cultured under standard conditions were treated with
10 μM menadione (K3, Supelco, 4-7775; 10 mM stock in etha-
nol), or PQ and then homogenized on ice with a glass tissue
grinder (Wheaton, 357538) in reaction buffer (50 mM Tris-
HCl, pH 7.5, 1 mM n-octyl-β-D-glucopyranoside [Sigma-
Aldrich, O8001-100 MG]). 5 μg of freshly prepared cell homo-
genates were added to a reaction mixture containing 150 μl
of reaction buffer and 10 μM BNAH (Santa Cruz Biotech-
nology, 208609) and no K3. The decrease in BNAH fluores-
cence was monitored for 30 min at RT and normalized to a
spontaneous decay in BNAH fluorescence in the absence of
cell homogenate.

ROS detection by a DCF (dichlorofluorescein) derivative
U373 cells were plated at standard conditions (2 × 104/cm2)
in 96-well plates. PQ (100 μM) or H2O2 (200 μM) was added
at 24, 6 or 3 h or 60, 30 and 15 min before analysis (all treat-
ments were performed 6 times). Cell staining with cytoplasmic
ROS fluorescent indicator 5(6)-carboxy-2',7'·dichlorofluorescein
diacetate (CA-DCF-DA; Sigma-Aldrich, 21884) was performed
1.5 h before analysis. For this purpose, the conditioned medium
+/− PQ or H2O2 was temporarily removed from cells (kept in
the incubator in the meantime) and the cells were incubated with
prewarmed basal DMEM containing 20 μM CA-DCF-DA or
vehicle (DMSO) for 30 min. Stained cells were gently rinsed in
warm basal DMEM and incubated again for 1 h with
conditioned medium. Where indicated, PQ or H2O2 was added
for short-term treatments. Fluorescent signal detection of live cell
monolayers was performed on multilabel fluorimeter Victor 2
(Perkin-Elmer, Singapore) with fluorescein filter settings. Back-
ground readings (cells stained with DMSO) were subtracted for
analysis of fluorescence of DCF-stained cells.

Transfection of siRNA and cDNA and luciferase reporter
assay
Transfection of reporter plasmids and siRNA was performed
with Lipofectamine 2000 (Invitrogen, 11668027), which was
used according to the manufacturer’s instructions. SQSTM1
siRNA (EHU027651) and control siRNA (EHUEGFP) were
purchased from Sigma-Aldrich. Reporter assay plasmids (ARE–
firefly luciferase and thymidine kinase (TK)-Renilla luciferase
(internal control) were purchased as Cignal reporter kit (Qiagen,
CCS-5020L) and used according to the manufacturer’s instruc-
tions. In particular, 1.5 to 2 μg of reporter DNA and 1 μg of
siRNA per 35-mm plate were used for the transfection of subcon-
fluent U373 cells. Luciferase activities were measured by using
the Dual-Luciferase Reporter Assay system (Promega, E1910) in
96-well plates on a Multilabel luminometer/fluorimeter Victor 2
(Perkin-Elmer, Singapore). The experiment was run in triplicate
and each measurement was repeated 3 times.

Data analysis and statistical procedures
Statistical analysis of all the experiments was performed using
GraphPad Prism software (version 3.0). The data were pre-
sent as means ±SEM (95% confidence interval) of 3 or more
independent experiments, unless otherwise specified. The statisti-
cal significance of the differences between treatments was
evaluated by analysis of variance test ANOVA, one-way or
2-way depending on the number of variable parameters, fol-
lowed by Dunnett’s or Bonferroni multiple comparison tests,
respectively. Mann-Whitey or unpaired 2-tailed t test test were
used for 2 small-size (n < 10) or 2 middle-size (n ≥ 10) group
comparisons.

Optical density of western blots was assessed by Quantity
One image software (Bio-Rad) for each individual blot and
the respective loading control. The main OD values were
divided by loading control OD and normalized to OD values
in untreated cells. The latter were assigned an arbitrary value
of “1” or 100%. All experiments were replicated at least 3
times.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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