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The Importance of Mitophagy in Maintaining Mitochondrial Function in U373MG Cells. Bafilomycin A1 Restores Aminochrome-Induced Mitochondrial Damage

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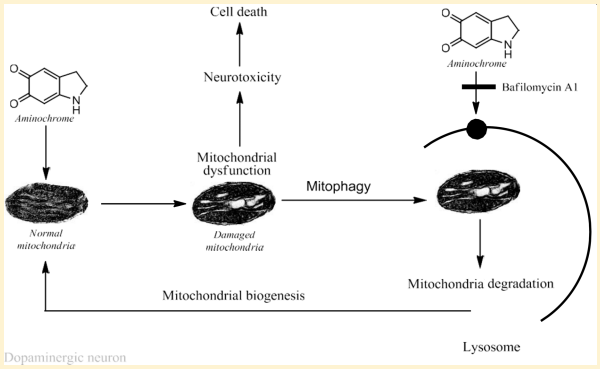
ABSTRACT: Aminochrome, an orthoquinone formed during the dopamine oxidation of neuromelanin, is neurotoxic because it induces mitochondria dysfunction, protein degradation dysfunction (both autophagy and proteasomal systems), α-synuclein aggregation to neurotoxic oligomers, neuroinflammation, and oxidative and endoplasmic reticulum stress. In this study, we investigated the relationship between aminochrome-induced autophagy/lysosome dysfunction and mitochondrial dysfunction in U373MGsiGST6 cells. Aminochrome (75 μM) induces mitochondrial dysfunction as determined by (i) a significant decrease in ATP levels (70%; $P < 0.001$) and (ii) a significant decrease in mitochondrial membrane potential ($P < 0.001$). Interestingly, the pretreatment of U373MGsiGST6 cells with 100 nM bafilomycin-A1, an inhibitor of lysosomal vacuolar-type H^+-ATPase, restores ATP levels, mitochondrial membrane potential, and mitophagy, and decreases cell death. These results reveal (i) the importance of macroautophagy/the lysosomal degradation system for the normal functioning of mitochondria and for cell survival, and (ii) aminochrome-induced lysosomal dysfunction depends on the aminochrome-dependent inactivation of the vacuolar-type H^+-ATPase, which pumps protons into the lysosomes. This study also supports the proposed protective role of glutathione transferase mu2−2 (GSTM2) in astrocytes against aminochrome toxicity, mediated by mitochondrial and lysosomal dysfunction.



KEYWORDS: *Glutathione transferase, Astrocytes, Dopamine, Mitochondria, Mitophagy, Aminochrome, Lysosome dysfunction*

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INTRODUCTION

The role of mitochondrial dysfunction in the neurodegenerative process of Parkinson's disease has been supported by the finding that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induces severe symptoms of Parkinsonism in humans, accumulates in the mitochondria and inhibits complex I.[1−6] A 20 to 30% decrease in complex I was reported in the substantia nigra pars compacta.[5−7] A 20% decrease in complex I activity was also observed in cybrid cells expressing mitochondrial DNA isolated from a Parkinson's disease sufferer.[8] A decrease in complex I activity and oxygen consumption was measured by using postmortem frontal cortexes from Parkinson's disease patients.[9]

The oxidation of dopamine to neuromelanin generates *o*-quinones such as aminochrome.[10−13] Aminochrome induces protein degradation dysfunction of both lysosomal and proteasomal systems by preventing the fusion of autophagosomes and lysosomes and increasing lysosome pH when GSTM2 is silenced.[14] Aminochrome also induces mitochondrial dysfunction in neuronal cell lines by inactivating complex I, decreasing the mitochondrial membrane potential, and inhibiting ATP production.[15−23] Mitochondria dysfunction induces mitophagy to degrade damaged mitochondria. However, the mechanism and its relationship with aminochrome in mitochondrial and lysosomal dysfunction is not clear yet. Therefore, the aim of this study was to study the role of the lysosomal/autophagy system in the degradation of mitochondria in aminochrome-induced mitochondrial dysfunction.

RESULTS AND DISCUSSION

Aminochrome Induces Mitochondrial Dysfunction. We have investigated the possible relationship between the aminochrome-induced dysfunction of the autophagy/lysosomal systems and mitochondrial dysfunction. It has been reported that the impairment of the mitochondrial membrane potential resulted in increased mitophagy promoted by PARK2 and PINK1.[24−27] Normal mitochondrial function requires a well-functioning macroautophagy/lysosomal system in order to recycle damaged mitochondria and promote mitochondrial biogenesis. The relationship between aminochrome-induced lysosomal/autophagy dysfunction and mitochondrial function was studied by using U373MG, U373MGsiGST6 (U373MG cells expressing a siRNA against GSTM2 with only 26% of GSTM2 expression), and U373MGpSR cells, expressing the pSuper.retro.puro plasmid alone.[14] We compared the levels of

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ATP in the U373MG, U373MGpSR, and U373MGsiGST6 cells incubated with a cell-culture medium alone or when aminochrome was added to the cell-culture medium. ATP levels were similar: there was 154 ± 9, 155 ± 8, and 155 ± 6 nmol of ATP/mg of protein in each of the cell lines when they were incubated with cell-culture medium, respectively. However, aminochrome induced a significant decrease in ATP levels in all of the cell lines treated with 30, 50, or 75 μM aminochrome, but this decrease was more pronounced in the U373MGsiGST6 cells (47 ± 1 nmol ATP/mg protein; $P < 0.001$); this was only 30% of the untreated cells (Figure 1A).

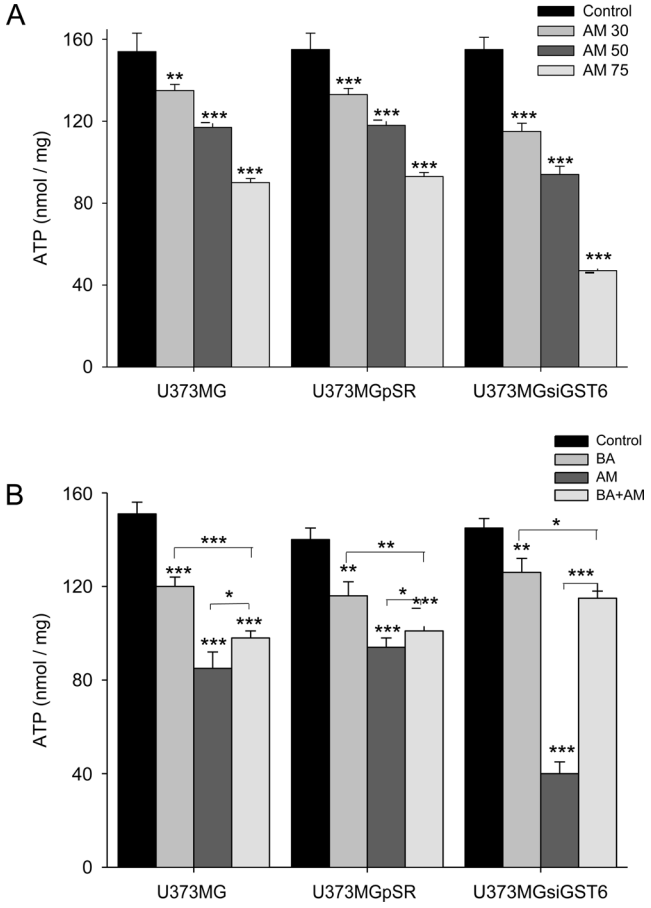

Figure 1. effect of aminochrome on cellular ATP levels in the absence and presence of bafilomycin A1. (A) A concentration-dependent decrease in the ATP level was observed in the presence of 30, 50, and 75 μM aminochrome incubated 24 h in U373MG, U373MGpSR, and U373MGsiGST6 cells. (B) The preincubation of the cells with 100 nM bafilomycin A1 induces a significant recovery in ATP level in U373MGsiGST6 cells. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's *t* test. (**$P < 0.01$; ***$P < 0.001$).

These results suggest that the integrity of the mitochondrial membrane potential ($\Delta\Psi$m) must be affected by aminochrome. Following from this, we therefore measured the mitochondrial membrane potential by using the JC-1 kit. A significant decrease in mitochondrial membrane potential was observed in U373MG (0.60 ± 0.02 red/green ratio; $P < 0.001$) and U373MGpSR (0.60 ± 0.02; $P < 0.01$) cells treated with 75 μM aminochrome. However, aminochrome's effect on the mitochondrial membrane potential was even stronger in the treated U373MGsiGST6 (0.44 ± 0.06; $P < 0.001$) cells in comparison with U373MG, U373MGpSR, and U373MGsiGST6 cells incubated with cell-culture medium (1.0 ± 0.08, 0.95 ± 0.03, 0.99 ± 0.07, respectively; Figure 2A–F).

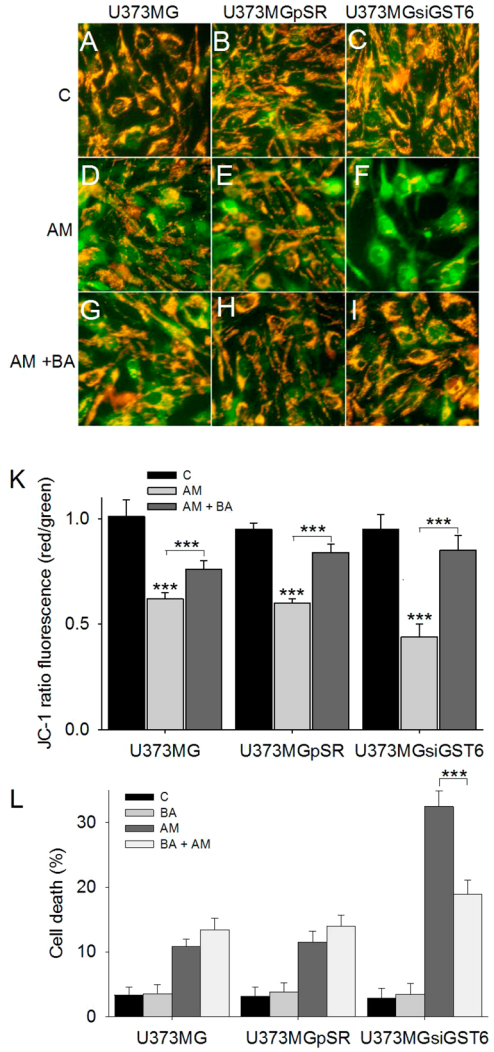

Figure 2. Effect of bafilomycin A1 on aminochrome-induced mitochondria membrane-potential impairment and cell death. (G–I) The pre-incubation with 100 nM bafilomycin A1 for 2 h of U373MG, U373MGpSR, and U373MGsiGST6 cells inhibited the significant decrease usually caused by the (D–F) treatment with 75 μM aminochrome for 24 h. As a control, (A–C) U373MG, U373MGpSR, and U373MGsiGST6 cells were incubated with cell-culture medium. The results are the mean ± SD ($n = 3$), expressed as the ratio between red/green fluorescence and quantified by the software ImageJ. The statistical significance was assessed by using analysis of variance (ANOVA) for multiple comparisons (***$P < 0.001$).

These results demonstrated that aminochrome impairs mitochondrial membrane potential and decreases cellular ATP levels. The impairment of the mitochondrial membrane potential promotes mitophagy to degrade damaged mitochondria.[24−27] Therefore, it should be expected that aminochrome-induced mitochondrial dysfunction will lead to increased mitophagy.

Role of Mitophagy in Mitochondrial Function. These results suggest that the aminochrome-damaged mitochondria require degradation by the autophagy/lysosomal systems.

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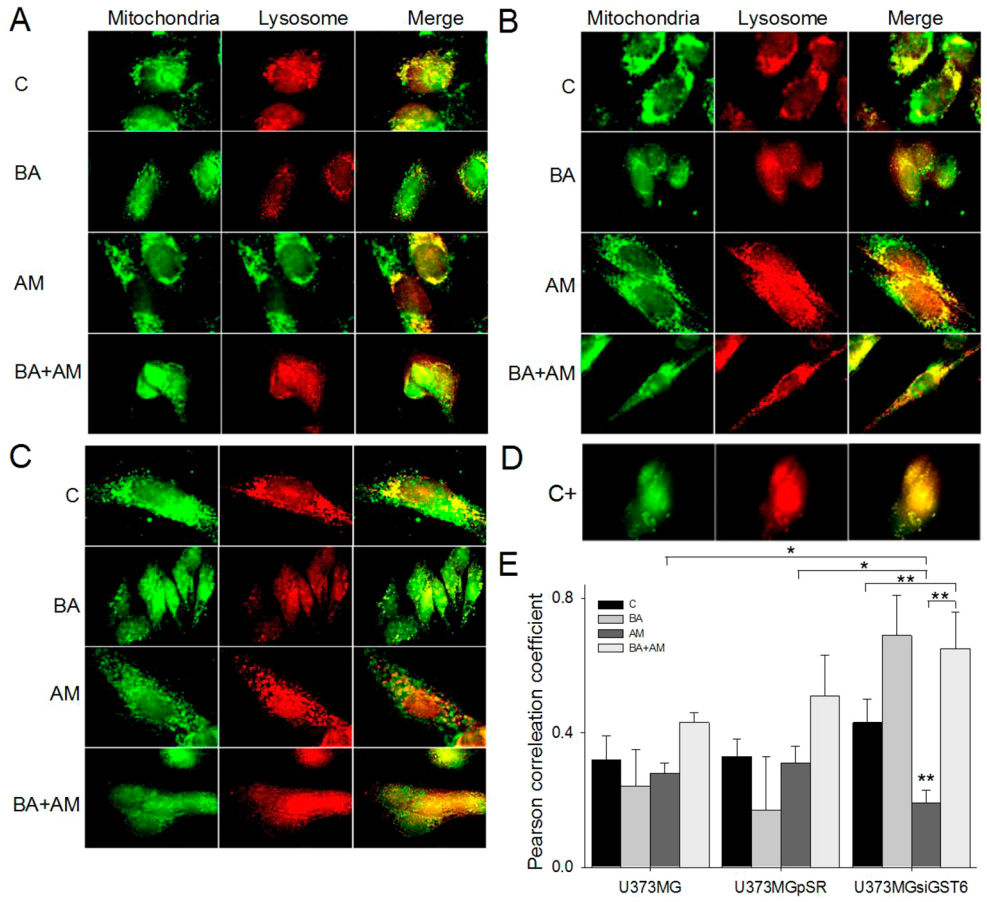

Figure 3. Effect of bafilomycin A1 on colocalization between mitochondria and lysosomes. The colocalization of mitochondria and lysosomes was measured in (A) U373MG, (B) U373pSR, and (C) U373MGsiGST6 cells incubated with cell-culture medium. (D) Positive control of mitophagy 1 μM of valinomycin. The Pearson correlation coefficient was plotted in (E), and the statistical significance was assessed by using analysis of variance (ANOVA) for multiple comparisons. BA: 100 nM bafilomycin A1, AM: 75 μM aminochrome, and BA+AM: 100 nM bafilomycin A1 and aminochrome; (B) U373pSR cells and (C) U373MGsiGST6 cells were incubated under the same conditions as in (A) for 24 h.

However, it has been reported that aminochrome induces autophagy and lysosomal dysfunction.[14,28] Therefore, we analyzed the colocalization of mitochondria with lysosomes by using immunofluorescence, since under conditions of functional mitophagy, mitochondria will colocalize with lysosomes. The U373MGsiGST6 cells treated with 75 μM aminochrome showed a significant decrease in the colocalization of mitochondria and lysosomes with respect to the untreated U373MGsiGST6 cells (2.3-fold decrease; $P < 0.01$;Figure 3C,E). No significant difference between the control and aminochrome-treated cells were observed in the U373MG and U373MGpSR cells (Figure 3A,B,E). A decrease in the colocalization between mitochondria and lysosomes was also observed when we compared the U373MGsiGST6 and U373MG or U373MGpSR cells treated with 75 μM aminochrome ($P < 0.05$ and $P < 0.05$, respectively) (Figure 3E). These results suggest that aminochrome prevents colocalization between mitochondria and lysosomes when GSTM2 is silenced in U373MGsiGST6 cells.

Aminochrome has been reported to induce lysosomal dysfunction by increasing the pH of the lysosomes. Interestingly, bafilomycin A1, a reversible inhibitor of lysosomal H^+-ATPase, prevents the aminochrome-induced loss of lysosomal acidity.[14] It seems plausible that the effect of aminochrome on the lysosomes was dependent on the aminochrome-dependent inactivation of vacuolar-type H^+-ATPase, which pumps protons into the lysosomes. The possibility that the observed protective effect of bafilomycin A1, due to its ability to compete with aminochrome to bind with vacuolar H^+-ATPase, was determined by isolating the lysosomes. The isolated lysosomes were incubated during for 10 and 60 min with H3-aminochrome (75 μM), and no significant differences were observed. However, preincubating the lysosomes with 100 nM bafilomycin A1 for 1 h before adding the aminochrome led to the aminochrome being inhibited from binding to the lysosomes to the levels of 56% ($P < 0.001$) and 24% ($P < 0.001$) at 10 and 60 min, respectively (Figure 4). These results demonstrate that aminochrome also inhibits the vacuolar-type H+-ATPase, since the pretreatment of lysosomes with bafilomycin A1 inhibits the aminochrome from binding to the lysosomes.

Therefore, the presence of bafilomycin A1 should prevent aminochrome from inactivating the lysosomes by preventing it from binding to the vacuolar-type H^+-ATPase. We preincubated U373MG, U373MGpSR, and U373MGsiGST6 cells with 100 nM bafilomycin A1 for 2 h before the cells were treated with 75 μM aminochrome for 24 h, and the effect was the following: (i) A significant increase in ATP levels was observed in U373MGsiGST6 cells treated with 75 μM aminochrome preincubated with bafilomycin A1 ($P < 0.001$; Figure 1B). However, no effects of bafilomycin A1 were observed on U373MG and U373MGpSR cells in the presence of amino-

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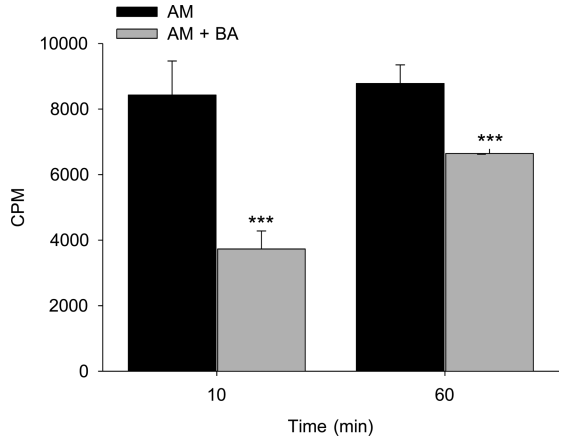

Figure 4. Inhibition of H^3-aminochrome binding to isolated lysosomes. Bafilomycin A1 (BA), a reversible inhibitor of vacuolar-type H^+-ATPase, inhibits H^3-aminochome (AM) binding to isolated lysosomes from U373MG cells. However, this inhibition decreases with time. Lysosomes were isolated as described under Methods. The significance was measured with ANOVA for multiple comparisons and Student's *t* test. (***$P < 0.01$).

chrome. (ii) The mitochondrial membrane potential of U373MG, U373MGpSR, and U373MGsiGST6 cells, pretreated with 100 nM bafilomycin A1 before treatment with 75 μM aminochrome, significantly increased in comparison with cells treated with 75 μM aminochrome (0.76 ± 0.04, $P < 0.001$; 0.84 ± 0.04, $P < 0.001$; 0.85 ± 0.07 JC-1 ratio of red/green fluorescence, respectively, Figure 2G–I). (iii) We determined that the inhibitory effect of 100 nM bafilomycin A1 had on the aminochrome-induced reduction in mitochondrial membrane potential should have a protective effect on cell death. A significant decrease in cell death was observed in U373MGsiGST6 cells pretreated with 100 nM bafilomycin A1 for 2 h before being treated with 75 μM aminochrome for 24 h (19 ± 2% cell death, $P < 0.001$) when compared with cells treated with 75 μM aminochrome alone (33 ± 2% cell death; Figure 2L). (iv) Pretreating the cells with 100 nM bafilomycin A1 also changed the colocalization behavior between the mitochondria and lysosomes; it decreased in U373MGsiGST6 cells treated with 75 μM aminochrome, since bafilomycin A1 induces a significant increase in colocalization between the mitochondria and lysosomes in U373MGsiGST6 cells treated with 75 μM aminochrome (Figure 3C,E). See Figure5 for a schematic describing lysosomal dysfunction in mitophagy.

Our results demonstrate that bafilomycin A1 exerts a certain level of protection against the neurotoxic effects of aminochrome by recovering ATP levels, mitochondrial membrane potential, mitophagy, and cell death. This seems to be paradoxical, since both bafilomycin A1 and aminochrome induce lysosomal dysfunction by inhibiting vacuolar-type H^+-ATPase.

This can be explained by the fact that bafilomycin A1 is a reversible inhibitor of lysosomal H+-ATPase. It has been reported that the inhibitory effect of bafilomycin A1 disappeared after 30 min after a 60 min pretreatment, and the vacuolar-type H^+-ATPase function was recovered.[29] Aminochrome is an irreversible inhibitor, but it is stable for only 40 min.[30] Aminochrome cannot wait until the binding sites on the vacuolar-type H^+-ATPase are free after pretreatment with bafilomycin A1 because aminochrome has other alternative metabolic routes.[15−23]

Therefore, the recovery of lysosomal pH allowed the damaged mitochondria to be degraded and promoted mitochondrial biogenesis since (i) bafilomycin A1 restores both ATP levels and the mitochondrial membrane potential, which finally resulted in a significant decrease in aminochrome-induced cell death,and (ii) pretreatment with bafilomycin A1 increased colocalization between the mitochondria and lysosomes as well as between Pink1 and LC-3, demonstrating that bafilomycin A1 promotes mitophagy under aminochrome treatment. Our results support the importance of mitophagy in maintaining normal mitochondrial function. The neurotoxic action of aminochrome is doubled by inducing both mitochondrial and mitophagy dysfunction, preventing the mitochondria from being recycled and from functioning normally.

The results presented in this study also support the proposed protective role of GSTM2 against aminochrome toxicity.[27−29,14,31,32] GSTM2 has been found to protect cells against aminochrome-induced autophagy and lysosomal dysfunction.[14] Recently, it has been reported that astrocytes secrete GSTM2 into a conditioned medium where dopaminergic neurons internalize GSTM2 in order to protect the cell against aminochrome neurotoxicity.[33−36] This study demonstrates that GSTM2 protects against mitochondrial dysfunction and lysosomal/autophagy dysfunction, since these effects are only observed in the cell line that permanently expresses a siRNA that decreases GSTM2 expression by 74% in the U373MGsiGST6 cells.

In conclusion, this study remarks upon the importance of the lysosomal/autophagy system in maintaining normal mitochondrial function, where aminochrome-induced mitochondrial dysfunction is dependent upon the dysfunction of the lysosomal/autophagy system.

METHODS

Chemicals. The dopamine was purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). The aminochrome was synthesized according to Paris et al. (2010). The ATP kit, CellTiter-Glo Luminescent Cell Viability Assay, was obtained from Promega (Madison, WI, U.S.A.). The LIVE/DEAD Viability/Cytotoxicity kit and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC1) were purchased from Molecular Probes (Eugene, OR, U.S.A.). The aminochrome was synthesized as previously described.[37]

Cell Lines. We used U373MGsiGST6 cells that expressed a siRNA for GSTM2, which silences the expression of GSTM2 by 74%, as well as U373MG and U373MGpSR control cells. The cell cultures were performed as described before.[14]

Cell Death. Cell death was determined by incubating the cell in culture medium for 24 h in the presence of aminochrome, purified according to Paris et al.[33] Cell death was measured by counting the live and dead cells after staining them with 0.5 μM calcein AM and 5 μM ethidium homodimer-1 for 45 min at room temperature in the dark (using the LIVE/DEAD Viability/Cytotoxicity Kit from Molecular Probes). Calcein AM is a marker for live cells, while ethidium homodimer-1 intercalates into the DNA of dead cells. The cells were counted with a phase contrast microscope equipped with fluorescence using the following filters: for calcein AM, 450−490 nm (excitation) and 515−565 nm (emission), and for the ethidium homodimer-1, 510−560 nm (excitation) and LP-590 nm (emission).

ATP Determination. The level of ATP was measured in the U373MG, U373MGpSR, and U373MGsiGST6 cells. ATP was determined by using the CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation, Madison, WI, U.S.A.).

Immunofluorescence. The U373MG, U373MGpSR, and U373MGsiGST6 cells were incubated for 16 h with the CellLight Reagent BacMam 2.0 Molecular Probes (Life Technologies) to mark

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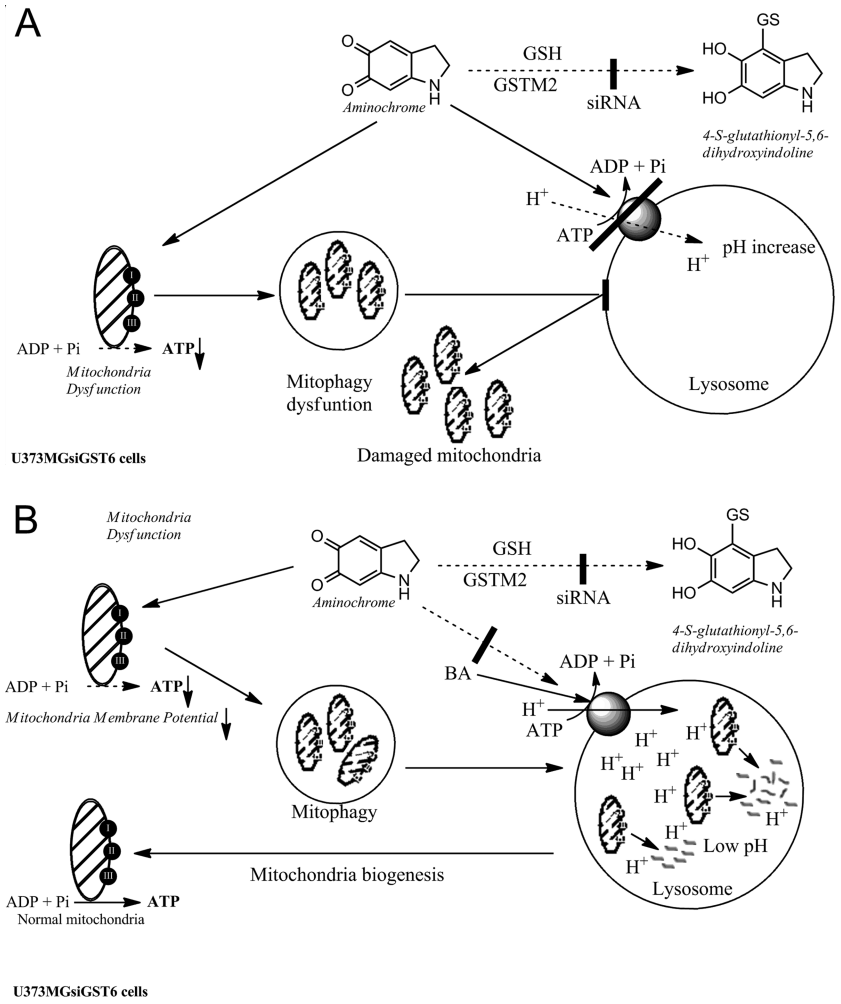

Figure 5. Possible role of lysosomal dysfunction in mitophagy. (A) Aminochrome induces both mitochondria and autophagy/lysosome dysfunction when GSTM2 is silencing with a constitutive siRNA expressed in U373MGsiGST6 cells. Aminochrome-induced mitochondrial dysfunction (mitochondria membrane-potential disruption and ATP level decrease) activates mitophagy, but mitochondria cannot be degraded due to the aminochrome-induced autophagy dysfunction caused by inactivating the vacuolar-type H^+-ATPase of lysosomes (Huenchuguala et al.). (B) Previous incubation of U373MGsiGST6 cells with 100 nM bafilomycin A1 prevents inactivation of vacuolar-type H^+-ATPase by aminochrome. Bafilomycin A1 inhibits vacuolar-type H^+-ATPase reversibly, and later, bafilomycin A1 is released from the vacuolar-type H^+-ATPase; lysosomes will be functional again, and mitophagy will end with mitochondria degradation. Under these conditions, mitochondria restore the membrane potential and ATP level, which significantly decreases cell death and probably promotes mitochondria biogenesis.

the mitochondria with green fluorescence. The lysosomes were marked with the primary antibody (anti-LAMP1 sc-17768 mouse IgG monoclonal, Santa Cruz Biotechnology). The PINK-1 was marked with anti-PINK1 (PARK6) Rabbit AP6406b (ABGENT). The LC-3 was marked with anti-MAPLC3β Mouse sc-376404 (Santa Cruz Biotechnology). The secondary antibody was marked with (goat antimouse Alexa fluor 546 (red) A11030) (Life Technologies) and grown to 70% confluence on glass coverslips. The cells were fixed with a solution of 4% *p*-formaldehyde for 20 min before being incubated with 0.2% Triton X-100 for 5 min to permeate the cell membrane. The cells were incubated at room temperature with a blocking solution (1% BSA in PBS) for 1 h after being washed with PBS and incubated with a solution containing the primary antibody (anti-LAMP1 sc-17768 mouse IgG monoclonal, Santa Cruz Biotechnology) at a dilution of 1/100 dissolved in PBS plus 1% BSA overnight at 4 °C. After the cells were washed, they were incubated with the secondary antibody (goat antimouse Alexa fluor 546 (red) A11030) (Life Technologies) at a dilution of 1/200 in PBS plus 1% BSA for 90 min, protected from light and at room temperature. The results were observed using an AxioVision microscope.

Isolation of Lysosomes and H^3-Aminochrome Binding. The isolation and enrichment of the intact lysosomes was performed using the Thermo Scientific Lysosome Enrichment Kit for Tissue and Cultured Cells (#89839). The lysosomes were isolated from the U373MG cells following the kit instructions. The H^3-aminochrome was prepared as previously described[37] but in the presence of both dopamine and 2 μL of H^3-dopamine. The concentration of H^3-aminochrome was determined spectrophotometrically by using the aminochrome molar extinction coefficient 3058 M^{-1} cm^{-1} at 475 nm.[38] The determination of whether H^3-aminochrome was binding to the lysosomes was performed after 10 and 60 min of incubation. The effect of bafilomycin on the binding of H^3-aminochrome to the lysosomes was determined by preincubating the lysosomes with 100 nM bafilomycin for 1 h before adding the H^3-aminochrome.

Determination of Mitochondrial Membrane Potential. The mitochondrial membrane potential of the cells was assessed using the JC-1 probe.[16]

Statistical Analysis. All data were expressed as mean ± SD values. Statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and a Student's *t* test.

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Author Contributions

S.H. did the majority of the experiments and the manuscript discussion. P.M. did a part of the experiments and manuscript discussion. J.S.-A. designed the experiment, wrote the manuscript, worked on the manuscript discussion and performed text editing.

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Notes

The authors declare no competing financial interest.

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