Imidazolines increase the levels of the autophagosomal marker LC3-II in macrophage-like RAW264.7 cells

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Brief Report

**Imidazolines increase the levels of the autophagosomal marker LC3-II in macrophage-like RAW264.7 cells**

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Abstract

This study evaluated whether imidazolines can induce autophagy in the murine macrophage-like cell line RAW264.7. Idazoxan increased the content of LC3-II, an autophagosomal marker, in RAW264.7 cells. To determine whether this effect was due to the induction of its synthesis or inhibition of its degradation, idazoxan-treatment was performed in the presence of bafilomycin A\textsubscript{1}, which blocks autophagosome-lysosome fusion, as well as Pepstatin A and E-64d, both of which block protein degradation in autolysosomes. An increased content of LC3-II was observed in the presence of bafilomycin A\textsubscript{1} as well as the protease inhibitors. Furthermore, an increased number of autophagosomes was observed following idazoxan-treatment using an autophagosome-specific dye. This indicated that idazoxan induced autophagy. Other imidazolines, such as efaroxan, clonidine, and 2-(2-Benzofuranyl)-2-imidazoline, also increased the LC3-II content in RAW264.7 cells in the presence of bafilomycin A\textsubscript{1}. Taken together, these results indicate that some imidazolines, including idazoxan, can induce autophagy in RAW264.7 cells.

**Key words**: idazoxan; bafilomycin A\textsubscript{1}; autophagosome; LC3-II; cellular responses
**Introduction**

Imidazolines exhibit many pharmacological activities such as antihypertensive and sedative effects, via α₂-adrenergic receptors and imidazoline receptors that are classified as I₁-, I₂-, and I₃-receptor subtypes (Head and Mayorov 2006). Imidazolines display varied affinity and selectivity for their receptors, and their selectivity determines the cellular and pharmacological effects.

Some imidazolines demonstrate anti-inflammatory effects on immune cells (Regunathan et al. 1999). Idazoxan, which is an I₂-receptor agonist and I₁-receptor antagonist, exhibits anti-inflammatory effects via inhibition of nitric oxide production by macrophages; however, the molecular machinery underlying this inhibition remains undefined (Feinstein et al. 1999), partly because macrophages are unlikely to express I₁- or I₂-receptors (Feinstein et al. 1999; Regunathan et al. 1999).

Rapamycin, an inhibitor of mammalian target of rapamycin (mTOR) (Ravikumar et al. 2004), displays many cellular effects, including induction of autophagy and inhibition of nitric oxide production by macrophages (Jin et al. 2009; Tunon et al. 2003). Although the molecular basis of the induction of autophagy by rapamycin is well-studied, there is little information regarding its role in the inhibition of inflammatory responses. Given the similarities between the anti-inflammatory effects of idazoxan and rapamycin, we sought to determine whether idazoxan induced autophagy in macrophage-like cells.
Materials and Methods

Reagents

The anti-LC3 antibody (8E10) was purchased from Medical & Biological Laboratories (Nagoya, Japan). Anti-p70 S6 kinase and anti-phospho-p70 S6 kinase (Ser371) antibodies were purchased from Cell Signaling Technology Japan (Tokyo, Japan). Anti-c-Jun N-terminal kinase (JNK, C-17) and anti-phospho-JNK (G-7) antibodies, as well as idazoxan, vinblastine, rilmenidine, and clonidine were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anisomycin and rapamycin were purchased from Nacalai Tesque (Kyoto, Japan). Pepstatin A was purchased from Enzo Life Sciences (Farmingdale, NY, USA). E-64d was purchased from the Peptide Institute (Osaka, Japan). Bafilomycin A₁ and 2-(2-Benzofuranyl)-2-imidazoline (2-BFI) were purchased from Tocris Bioscience (Bristol, UK). Dexmedetomidine was purchased from Wako Pure Chemical Industries (Osaka, Japan). Efaroxan was purchased from LKT Laboratories (Saint Paul, MN, USA).

Cell culture

RAW264.7 cells (DS Pharma Biomedical, Suita, Osaka, Japan) were maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin (Thermo Fisher Scientific, Waltham, MA, USA), and 100 µg/mL streptomycin (Thermo Fisher Scientific) at 37 °C with 5% CO₂.

Preparation of cell lysates

Whole cell lysates were prepared by sonically disrupting cells suspended in ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.47 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4). Alternatively, cells were incubated on ice
for 15 minutes in 50 mM Tris-HCl (pH 7.5) containing 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, phosphatase inhibitor cocktail (Nacalai Tesque), and a protease inhibitor cocktail (Complete™ Mini, F.Hoffman-La Roche Ltd., Basel, Switzerland). Then, the cell suspensions were centrifuged at 10,000 g for 10 minutes and the supernatants used as cell lysates.

**Immuno blotting analysis**

Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were electroblotted onto nitrocellulose or polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with non-fat dry milk or bovine serum albumin, the membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The signals were analyzed using the LAS-3000mini image analysis system (Fujifilm, Tokyo, Japan).

**Confocal microscopy and flow cytometry**

RAW264.7 cells were treated with idazoxan for 16 hours. The cells were then stained using the Cyto-ID® autophagy detection kit (Enzo Life Sciences). Cells were observed and analyzed using an A1R confocal microscope (Nikon, Tokyo, Japan) or by flow cytometry using a FACSCalibur™ instrument (BD Biosciences, Franklin Lakes, NJ, USA).

**Results and Discussion**

*Idazoxan induced autophagy in murine macrophage-like cells*

We examined the induction of the autophagosomal protein LC3-II by idazoxan in the murine macrophage-like cell line RAW264.7 (Mizushima and Yoshimori 2007). As shown in Figure 1a and Supplementary Figure 1, idazoxan
treatment increased LC3-II levels in RAW264.7 cells. An increase in LC3-II levels was observed from 4 hours to 24 hours after idazoxan treatment (Supplementary Figure 1b). To determine whether this increase was due to the induction of autophagy or inhibition of LC3-II degradation in autolysosomes, generated by autophagosome-lysosome fusion, the effects of idazoxan were examined in the presence of bafilomycin A₁, a drug that blocks autophagosome-lysosome fusion (Yamamoto et al. 1998). Idazoxan increased LC3-II content in the presence of bafilomycin A₁ (Figure 1b), suggesting that the effect observed was due to the induction of autophagy.

A similar increase of LC3-II content was also observed in response to rapamycin, a well-known autophagy inducer, in the presence or absence of bafilomycin A₁ (Figure 1c). Furthermore, idazoxan increased LC3-II content in the presence of Pepstatin A and E-64d, two protease inhibitors that block protein degradation in autolysosomes (Tanida et al. 2005) (Figure 1d).

To confirm the effects of bafilomycin A₁ in our system, we examined the effects of other inhibitors of protein degradation on the formation of autophagosomes. Vinblastine, an inhibitor of autophagosome-lysosome fusion (Punnonen and Reunanen 1990), increased LC3-II content in RAW264.7 cells (Supplementary Figure 2a). Similarly, Pepstatin A and E-64d increased the LC3-II content in RAW264.7 cells (Supplementary Figure 2b). However, these increases, which were due to the inhibition of LC3-II degradation in autolysosomes, were not observed in the presence of bafilomycin A₁ (Supplementary Figure 2a and b). These observations indicate that inhibitors of autophagosome-lysosome fusion or LC3-II degradation in autolysosomes did not increase LC3-II content in RAW264.7 cells in the presence of bafilomycin A₁. Therefore, the increase in LC3-II content following
idazoxan treatment of RAW264.7 cells in the presence of bafilomycin A₁ (Figure 1b), most likely, was not due to the inhibition of protein degradation.

To determine whether idazoxan increased the formation of autophagosomes, we determined their number in RAW264.7 cells treated with idazoxan using a fluorescent dye, Cyto-ID®. Cyto-ID® is a recently developed cationic amphiphilic tracer dye, which labels autophagic compartments with minimum staining of lysosomes and endosomes (Chan et al. 2012; Guo et al. 2015; Klionsky et al. 2016). As shown in Figure 2a and b, the number of autophagosomes increased in response to rapamycin or idazoxan. The increased number of autophagosomes by idazoxan was confirmed by quantitative analysis of the fluorescence (Figure 2c and 2d). Collectively, these results further support the hypothesis that idazoxan induces autophagy in RAW264.7 cells.

*Imidazolines increased LC3-II levels in RAW264.7 cells*

We examined whether other imidazolines (Supplementary Figure 3) induced autophagy in RAW264.7 cells. Efaroxan, clonidine, and 2-BFI increased LC3-II levels in RAW264.7 cells in the presence or absence of bafilomycin A₁ (Figure 3a–c), indicating induction of autophagy. Similar effects of efaroxan, clonidine, and 2-BFI were observed in the presence of Pepstatin A and E-64d (Supplementary Figure 4a-c). In contrast, LC3-II levels did not increase following treatment with rilmenidine or dexmedetomidine, in the presence of bafilomycin A₁ (Figure 3d and e). Although the relationship between their chemical structures and ability to induce LC3-II is unknown, our results suggest that some imidazolines have the ability to induce autophagy in RAW264.7 cells.

*Induction of autophagy by idazoxan was not mediated by mTOR or the I₁-receptor.*
We examined the involvement of mTOR in idazoxan-induced autophagy. Rapamycin, but not idazoxan, inhibited the phosphorylation of p70 S6 kinase, a substrate of mTOR kinase (Figure 4a). Thus, the inhibition of mTOR was not involved in idazoxan-induced autophagy in RAW264.7 cells. Previously, the imidazoline compound clonidine was shown to induce autophagy through the I₁-receptor (Williams et al. 2008). Involvement of the I₁-receptor in idazoxan-induced autophagy was examined by monitoring phosphorylation of its downstream signaling molecule, JNK (Edwards et al. 2001). Phosphorylation of JNK was not observed in RAW264.7 cells treated with idazoxan or clonidine (Figure 4b and 4c). These observations suggested that the I₁-receptor was not involved in imidazoline-induced autophagy in RAW264.7 cells. Several stimuli, including oleic acid, have been shown to induce autophagy independently of mTOR. Analysis of such mTOR-independent autophagy induction might lead to an understanding of imidazoline-induced autophagy (Mei et al. 2011).

**Conclusion**

In summary, this study shows that some imidazolins, including idazoxan, efaroxan, clonidine, and 2-BFI, increase the levels of the autophagosomal marker LC3-II in macrophage-like RAW264.7 cells. Furthermore, idazoxan increases the number of autophagosomes in RAW264.7 cells. Taken together, these observations indicate that some imidazolins induce autophagy in RAW264.7 cells; the cellular pathway inducing autophagy in these cells remains to be elucidated.

**Conflict of interest**

The authors declare no conflict of interest.
Acknowledgments

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References


Figure legends

Figure 1. Idazoxan increases LC3-II levels in RAW264.7 cells in the presence of bafilomycin A1.

LC3-I and LC3-II in whole cell lysates were analyzed by immunoblotting (upper panels). Proteins (10 µg/lane) in the lysates were analyzed by staining with Coomassie blue (lower panels). RAW264.7 cells were treated with idazoxan for 16 hours (a, b), or rapamycin for 4 hours (c). The indicated concentrations of bafilomycin A1 were added during the final 4 hours of the treatments (b and c). (d) RAW264.7 cells were treated with Pepstatin A and E-64d, and then with idazoxan or rapamycin for 24 hours. Triplicate samples were analyzed.

Figure 2. Idazoxan increases the number of autophagosomes in RAW264.7 cells.

(a) RAW264.7 cells were treated with or without 100 µM idazoxan for 16 hours. The cells were then stained with Cyto-ID® to visualize autophagosomes (excitation 488 nm, emission 500–550 nm), and Hoechst 33342 to visualize the nuclei (excitation 405 nm, emission 425–475 nm). Scale bars indicate 25 µm. (b) RAW264.7 cells were treated with or without 100 nM rapamycin for 4 hours. The cells were then stained with Cyto-ID® to visualize autophagosomes, and Hoechst 33342 to visualize the nuclei. Scale bars indicate 25 µm. (c) Fluorescence of Cyto-ID® in cells treated as in (a) was analyzed by flow cytometry. The results shown are representative of triplicate experiments. (d) Fluorescence in (c) is shown as the geometric means ± standard deviations for triplicate
analyses.

**Figure 3. Imidazolines increase LC3-II levels in RAW264.7 cells in the presence of bafilomycin A₁.**

LC3-I and LC3-II in whole cell lysates were analyzed by immunoblotting (upper panels). Proteins (10 µg/lane) in the lysates were analyzed by staining with Coomassie blue (lower panels). RAW264.7 cells were treated with efaroxan for 8 hours (a), clonidine for 4 hours (b), 2-(2-Benzofuranyl)-2-imidazoline (2-BFI) for 16 hours (c), rilmenidine for 4 hours (d), or dexmedetomidine for 4 hours (e). The indicated concentrations of bafilomycin A₁ were added during the final 4 hours of the treatments.

**Figure 4. Phosphorylation of p70 S6 kinase and c-Jun N-terminal kinase (JNK) is not altered by idazoxan.**

(a) RAW264.7 cells were treated without (control) or with 100 µM idazoxan or 100 nM rapamycin for 24 hours. Whole cell lysates were analyzed by immunoblotting for phosphorylated p70 S6 kinase (p-p70S6K) and p70 S6 kinase (p70S6K). RAW264.7 cells were treated with 25 ng/mL anisomycin for 30 minutes plus 100 µM idazoxan (b) or 1 mM clonidine (c) for the indicated times. A translational inhibitor anisomycin activates stress-activated protein kinases including JNK. JNK and phosphorylated JNK (p-JNK) in cell lysates were analyzed by immunoblotting.
Idazoxan

LC3-Ⅱ

Cyto-ID® fluorescent dye

Autophagy in RAW 264.7

Cyto-ID® fluorescent image

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a

Idazoxan (µM)
0 10 30 100

LC3- I
LC3- II
(kDa)
100 50 25 15 10

b

Bafilomycin A₁ (nM)
0 12.5 25 50

Idazoxan (µM)
0 100 0 100 0 100 0 100

LC3- I
LC3- II
(kDa)
100 50 25 15 10

c

Bafilomycin A₁ (nM)
0 25

Rapamycin (nM)
0 100

LC3- I
LC3- II
(kDa)
100 50 25 15 10

d

Pepstatin A, E-64d

Rapamycin (nM)
0 100 0 100 0 100 0 100 0

Idazoxan (µM)
0 100 0 100 0 100 0 100

LC3- I
LC3- II
(kDa)
100 50 25 15 10
Figure 1. Fluorescence images showing the effects of idazoxan and rapamycin on differential interference contrast (DIC), Cyto-ID®, Hoechst 33342, and overlay images.

(a) Fluorescence images of cells treated with idazoxan.+(a) Fluorescence images of cells treated with rapamycin.

Counts (c) and fluorescence (d) measurements showing the effect of idazoxan on fluorescence intensity.
Efaroxan (mM) 0 1 0 1
Bafilomycin A₁ (nM) 0 25

Clonidine (mM) 0 1 0 1
Bafilomycin A₁ (nM) 0 25

2-BFI (mM) 0 0.1 0.5 1
Bafilomycin A₁ (nM) 0 25

Dexmedetomidine (mM) 0 0.1 0.3 0.5
Bafilomycin A₁ (nM) 0 25

LC3- I LC3- II
(kDa) 100 25 15 10

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