Behavior of labile ferrous ions and reactive oxygen species during ferroptotic response of cells

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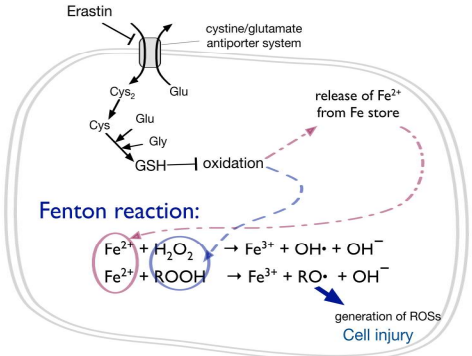
Abstract

Ferroptosis is a recently identified iron-dependent regulated cell death (Dixon and Stockwell, 2014). Understanding mechanisms and regulations of this oxidative cell injury may bring us new therapeutic methods for cancer and neurodegenerative diseases. The injury may be caused by reactive oxygen species (ROSs) which are generated by Fenton reaction between labile ferrous ions (Fe^{2+}) and peroxides. Therefore, Fenton reaction should be a key for ferroptotic responses of cells. However, other indirect pathways to generate ROSs should be also considered. To further understand the mechanisms how ROSs are generated during the ferroptotic responses, we tried to visualize labile Fe^{2+} and ROSs by using cell permeable, activatable fluorescent probes. As previously described, >20 μM of erastin caused ferroptosis of HT-1080 cells. To visualize the responses for an enough time-period (~9 hours), we chose the erastin concentration of 30 μM and observed the behavior of labile Fe^{2+} and ROSs by fluorescence microscopy. To detect labile Fe^{2+}, both commercially-available chemical probe FeRhoNox-1 (RhoNox-1, Hirayama, et al., 2013) and newly-synthesized RhoNox-4 which possesses a higher sensitivity with high specificity to labile Fe^{2+} were used. To detect ROSs, aminophenyl fluorescein (APF, Setsukinai et al., 2003) which fluoresces by either hydroxyradical, peroxinitrite, or hypochlorous acid, and OxiORANGE (Koide et al., 2007) which does by either hydroxyradical or hypochlorous acid were used. The fluorescence signals indicating labile Fe^{2+} reached the maximum at 3 hours after erastin stimulation. On the other hand, fluorescence signals of APF and OxiORANGE became maximal at 6 hours after the stimulation. The results were consistent with the simple model that hydroxyradical is generated by the reaction between cytoplasmic labile Fe^{2+} and peroxides and thus causes the ferroptotic cell death.

References
Dixon and Stockwell 2014, *Nat. Chem. Biol.* **10**:9-17
Hirayama, Okuda, Nagasawa, 2013 *Chem. Sci.* **4**, 1250-1256
Setsukinai, Urano, Kakinuma, Majima, Nagano (2003) *J. Biol. Chem.* **278**:3170-3175
Koide, Urano, Kenmoku, Kojima, Nagano (2007) *J. Am. Chem. Soc.* **129**:10324-10325

Background

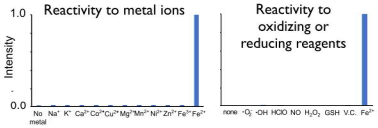
Erastin induces ferroptotic response of cells



Question: Do the increase in Fe^{2+} precedes the increase in ROSs?

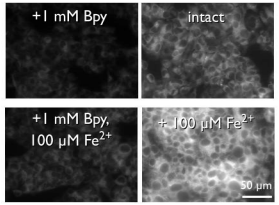
RhoNox-4, a new chemical probe to detect labile Fe^{2+}

Specifically reacts with Fe^{2+}.



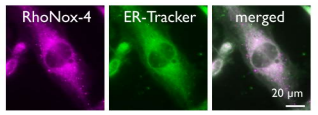
Left, 2 μM of RhoNox-4 was incubated with 1 mM of each ion in 50 mM HEPES buffer. pH=7.4 for 60 min. *Right*, 2 μM of RhoNox-4 was reacted with 100 μM KO_2 for $\cdot O_2^-$, $\cdot OH$ generated from 200 μM H_2O_2 and 20 μM $FeSO_4$, 100 μM NaOCl for HClO, NO generated by 100 μM NOC-12, 100 μM H_2O_2, 1 mM reduced glutathione, 1 mM ascorbic acid., and 20 μM $FeSO_4$. Fluorescence intensities at 575 nm were measured by fluorescence spectrophotometer with 530 nm excitation.

Intracellular Fe^{2+} can be detected



HepG2 cells pretreated with/without 100 μM $Fe(SO_4)_2(NH_4)_2$ for 30 min were washed to remove extracellular Fe^{2+}. Then the cells were reacted with 1 μM of RhoNox-4 for 30 min. 2,2'-Bipyridine (Bpy, metal-ion chelator) were added with RhoNox-4. The cells were observed by fluorescence microscopy (Ex. 530-560 nm, Em.573-647 nm).

Localizes to ER.



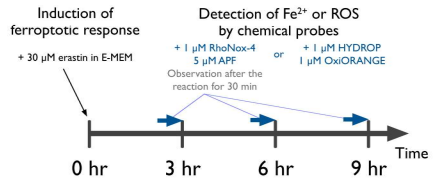
HT-1080 cells were co-stained with 125 nM ER-Tracker Green (Molecular Probes, Thermo) and 250 nM RhoNox-4. The cells were observed by fluorescence microscopy (Ex. 460-500 nm, Em. 512-542 nm for ER-Tracker; Ex. 530-560 nm, Em.573-647 nm for RhoNox-4).

Another labile Fe^{2+} probe has been also reported

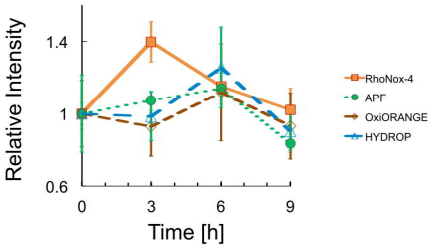
Allegra T. Aron et al., 2016
J. Am. Chem. Soc., 138: 14338-14346

Ferroptotic response of HT-1080 cells

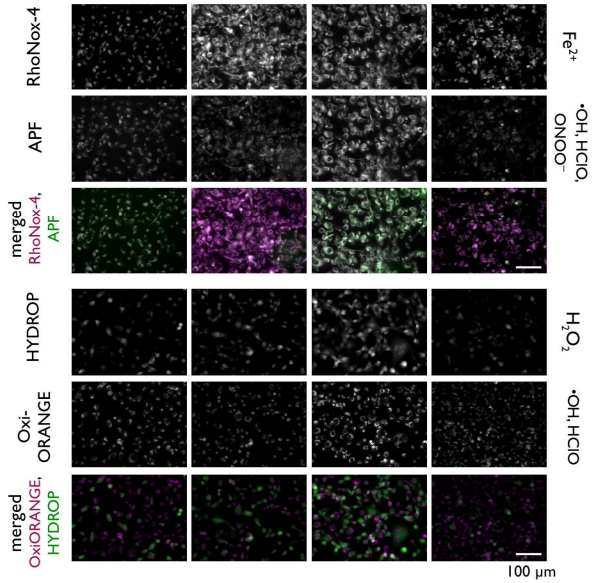
Experiment design



Fluorescence intensities obtained from the images

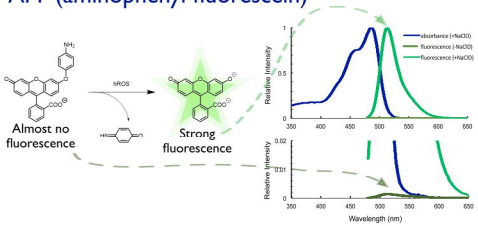


Microscopy results



Chemical probes used

APF (aminophenyl fluorescein)



Reactivity of ROS probes

Probes	Alt. name	Ex. (nm)	Em. (nm)	·OH	HClO	$ONOO^-$	H_2O_2
APF	aminophenyl fluorescein	490	515	○	○	○	×
OxiORANGE	MitoHR	553	577	○	○	×	×
HYDROP	NBzF DA	492	518	×	×	×	○

Discussion

1) Oxidation of organic molecules such as lipid peroxidation may play more important role than that of H_2O_2 during the ferroptotic response of HT-1080 cells.

2) The decrease in GSH caused by erastin does not directly increase the concentrations of ROSs.

Conclusion

1) Chemical probes including RhoNox-4 and ROS probes provide powerful tools to monitor the ferroptotic responses of cells.

2) Fe^{2+} concentration increases before the increase in ROSs including H_2O_2.