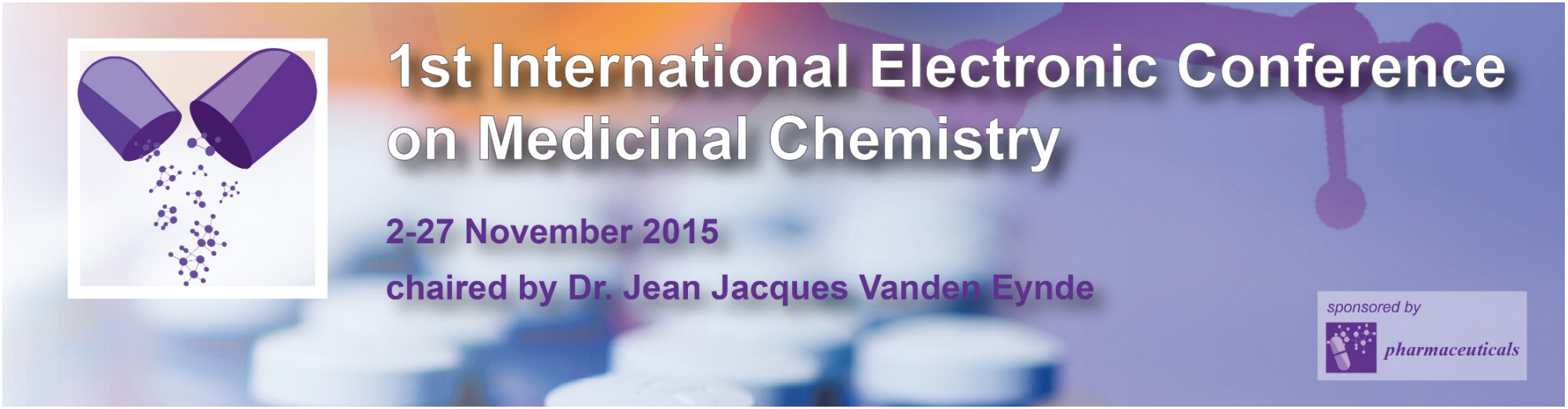

Induction of the expression of GABARAPL1 by hydrogen peroxide in C6 glioma cells

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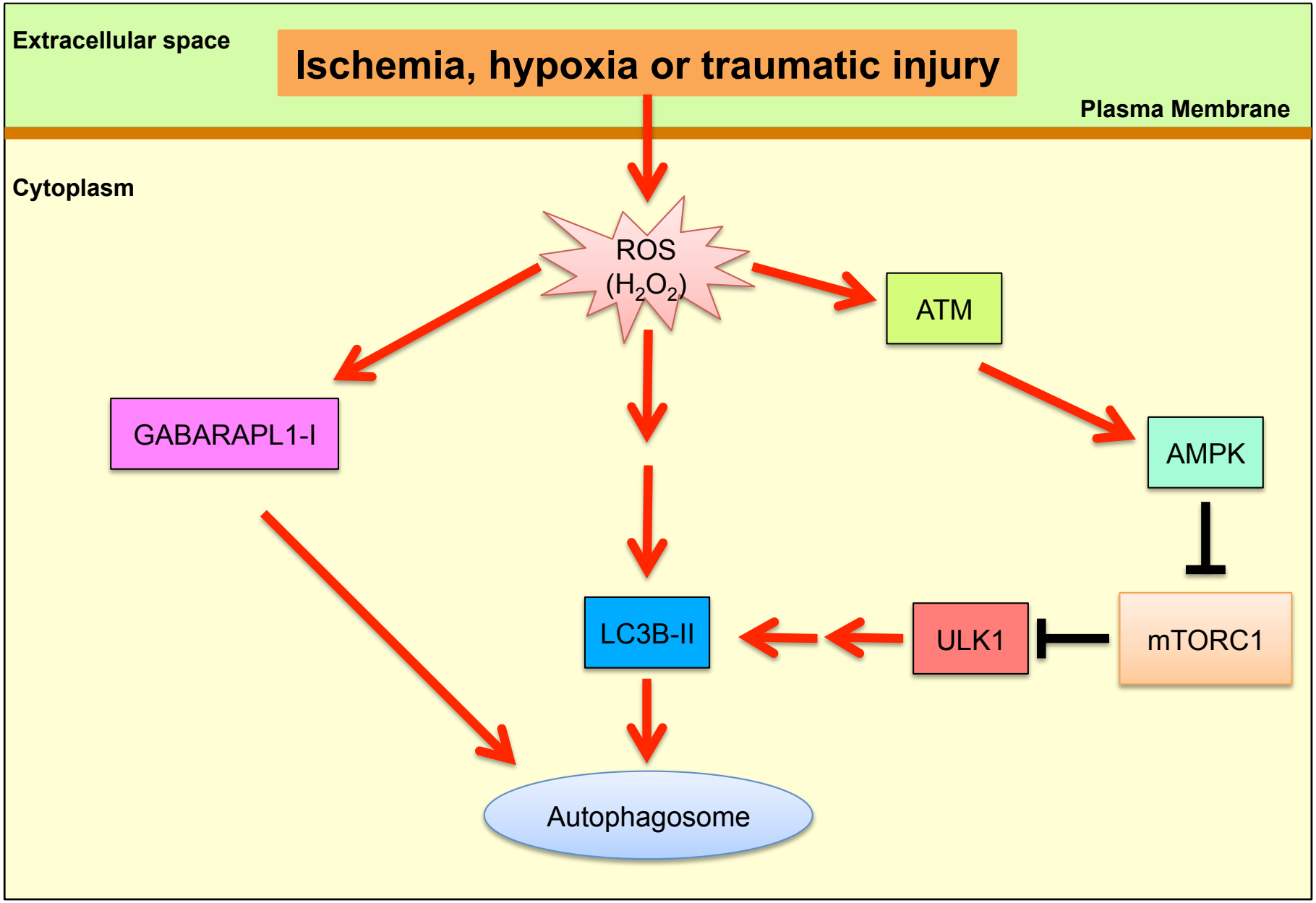
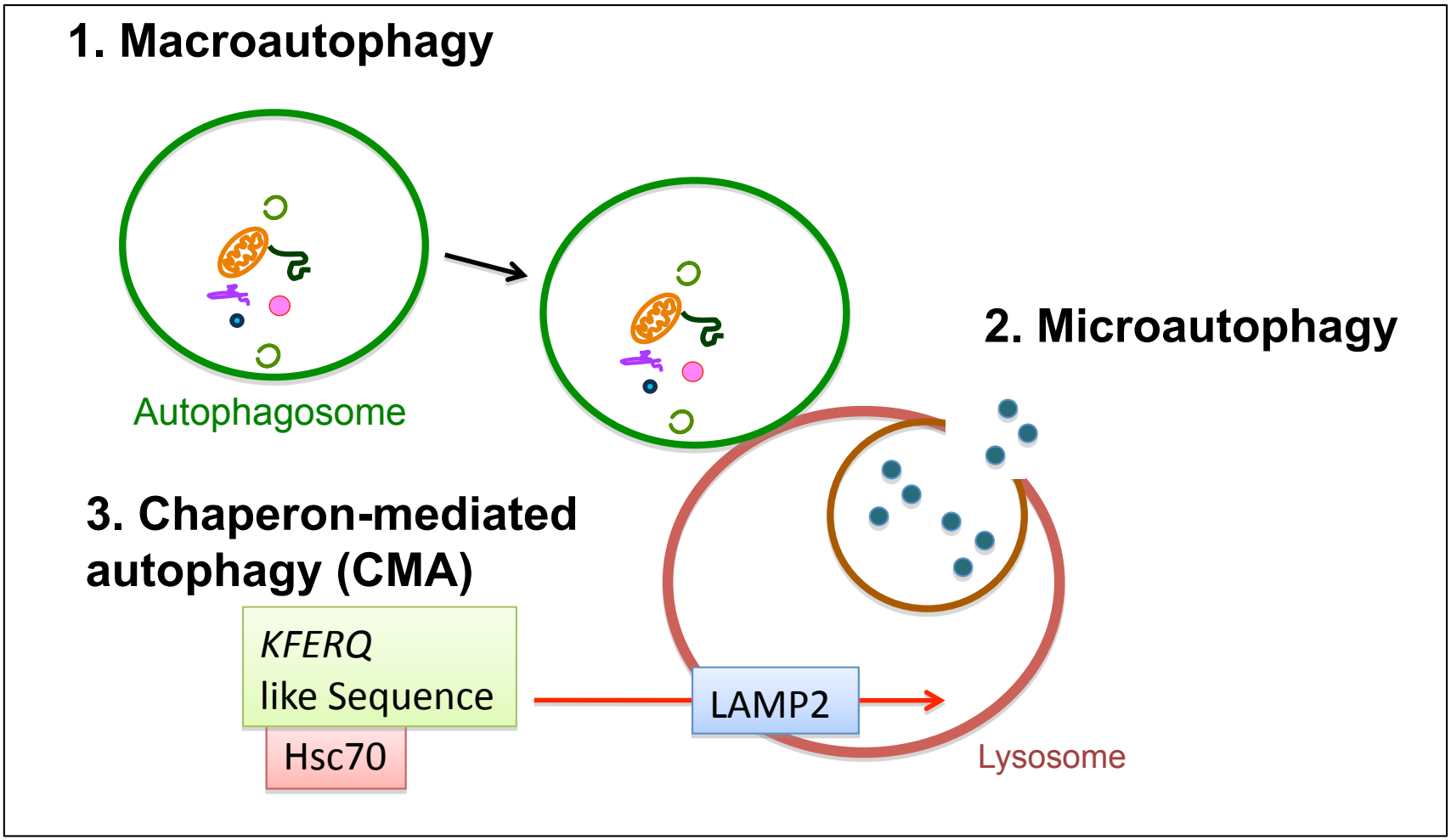


Abstract: Ischemia-reperfusion or traumatic brain injury induces the accumulation of reactive oxygen species (ROS) in brain. ROS causes oxidative stress to astrocytes as well as neurons and oxidative stress induces the damage to organelles, proteins or lipids. The removal of damaged cellular cytosolic components is indispensable for the cell to keep the homeostasis. Macroautophagy (hereafter referred to as autophagy) is the process to degrade defective proteins and damaged organelles. In the process of autophagy, damaged cellular cytosolic components are isolated by autophagosomes. Microtubule-associated protein 1 light chain 3B (LC3B) plays a significant role in the autophagosome formation and the conversion from unconjugated-LC3B (LC3B-I) to phosphatidylethanolamine (PE) conjugated-LC3B (LC3B-II) is the index of the activitation of autophagy. GABARAPL1 is the paralogue of LC3B and the function of GABARAPL1 is not fully understood.

In this study, we demonstrated GABARAPL1 mRNA and protein expression are up-regulated by H_2O_2 in rat C6 glioma cells and the induction of GABARAPL1 by H_2O_2 was accompanied with the conversion from LC3B-I to LC3B-II, indicating the formation of autophagosomes. Thus, GABARAPL1 may play a role in autophagic process, which is induced by H_2O_2. However, elucidation of the function of GABARAPL1 in autophagy will require further studies.

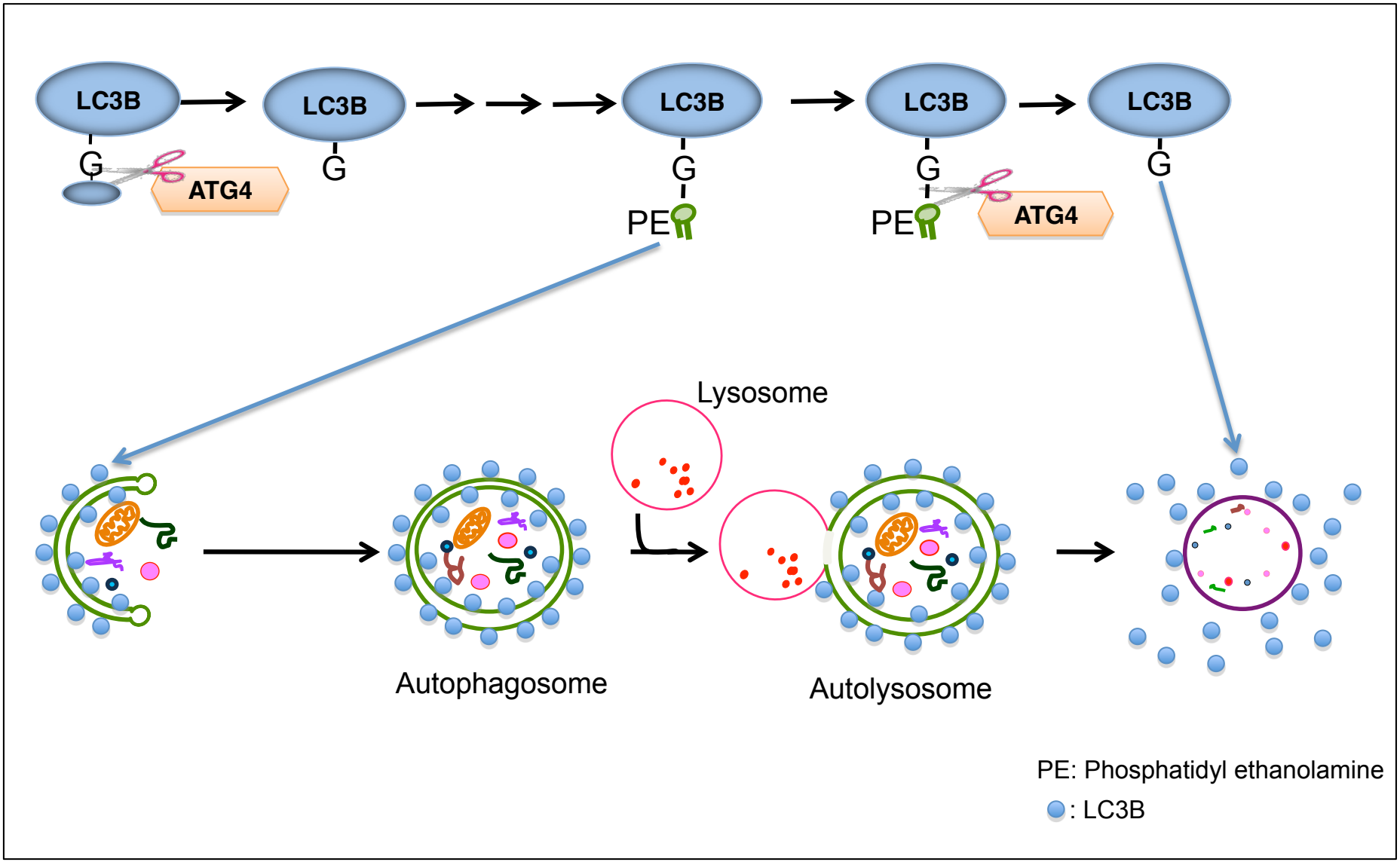
Keywords; autophagy, reactive oxygen species, LC3, GABARAP

Figure 1: Three different types of Autophagy (Macroautophagy, Microautophagy, and Chaperon-mediated autophagy)



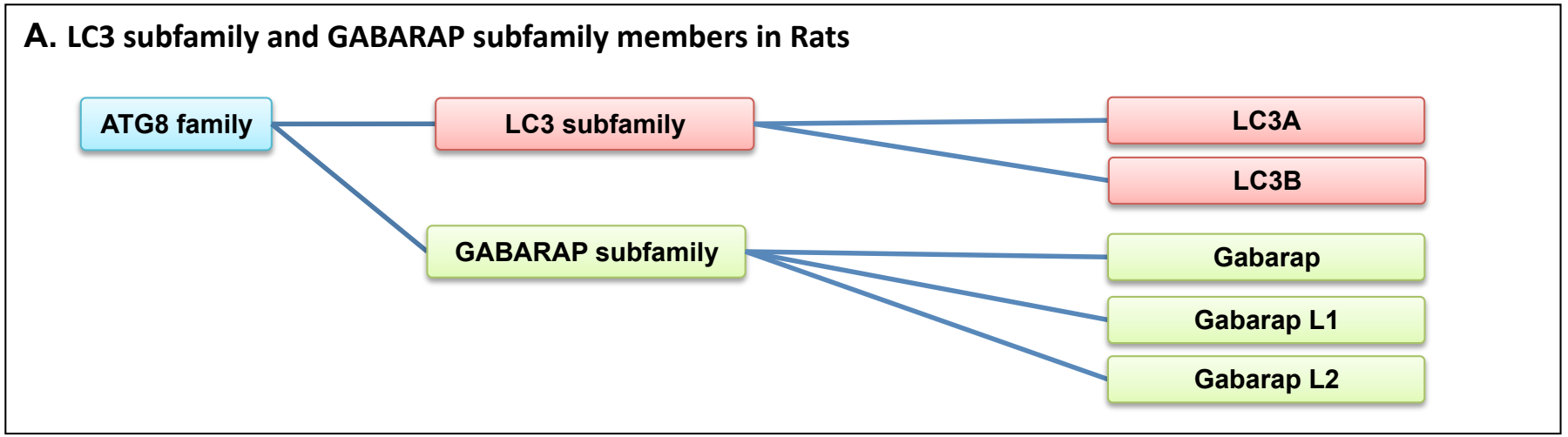
There are three different types of autophagy: 1. macroautophagy, 2. microautophagy, and 3. chaperone-mediated autophagy.
1. Macroautophagy: Cytosolic cellular components are isolated by an autophagosome, and they are carried to a lysosome.
2. Microautophagy: A Lysosome directly include cytosolic cellular components.
3. Chaperone-mediated autophagy: Hsc70 binds a protein that includes KFERQ like sequence and carries to a lysosome.

Figure 2: LC3B is the marker for the activity of autophagy



LC3B is an ubiquitin-like protein and is essential for the autophagosome formation. LC3B is catalyzed by Atg4 and then conjugated to phosphatidylethanolamine (PE). This LC3B-PE (LC3B-II) conjugate plays a crucial role in autophagosome formation. After Cytosolic cellular components are degraded, LC3B is released from an autolysosome by ATG4.

Figure 3: LC3 subfamily and GABARAP subfamily members in rats



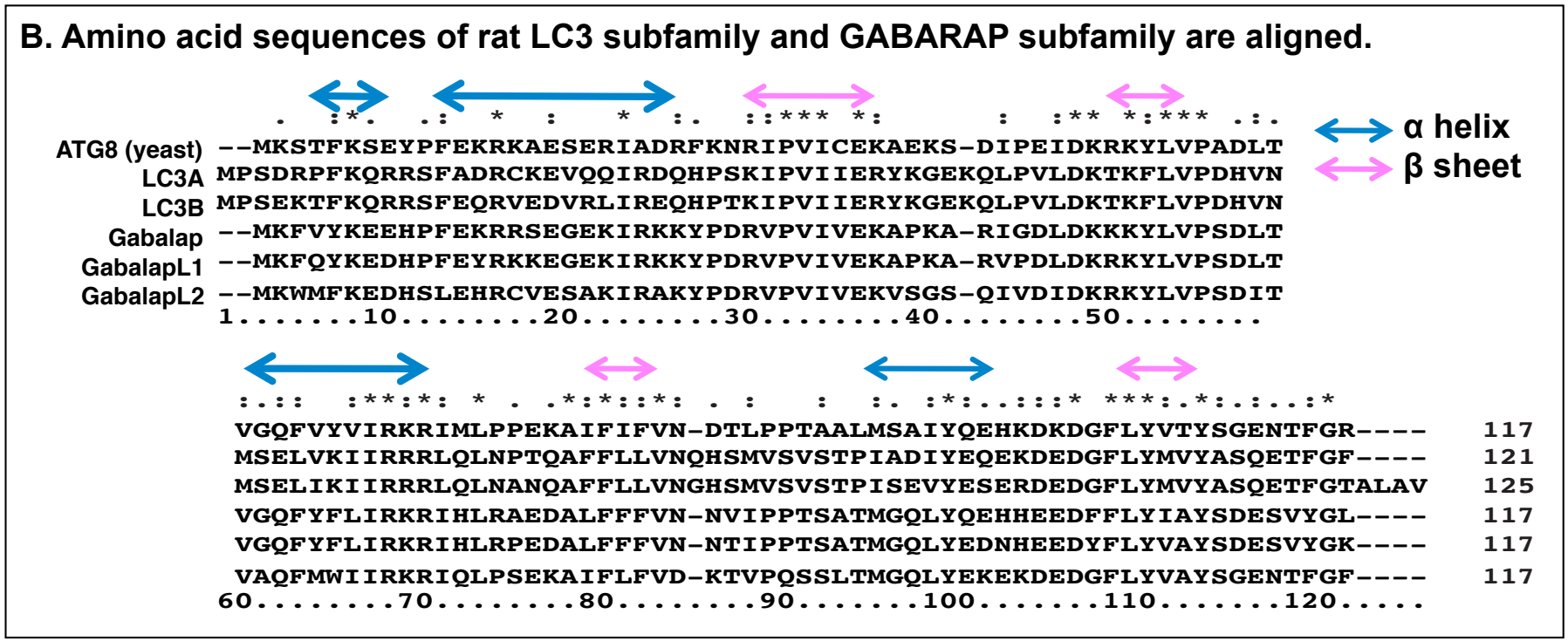
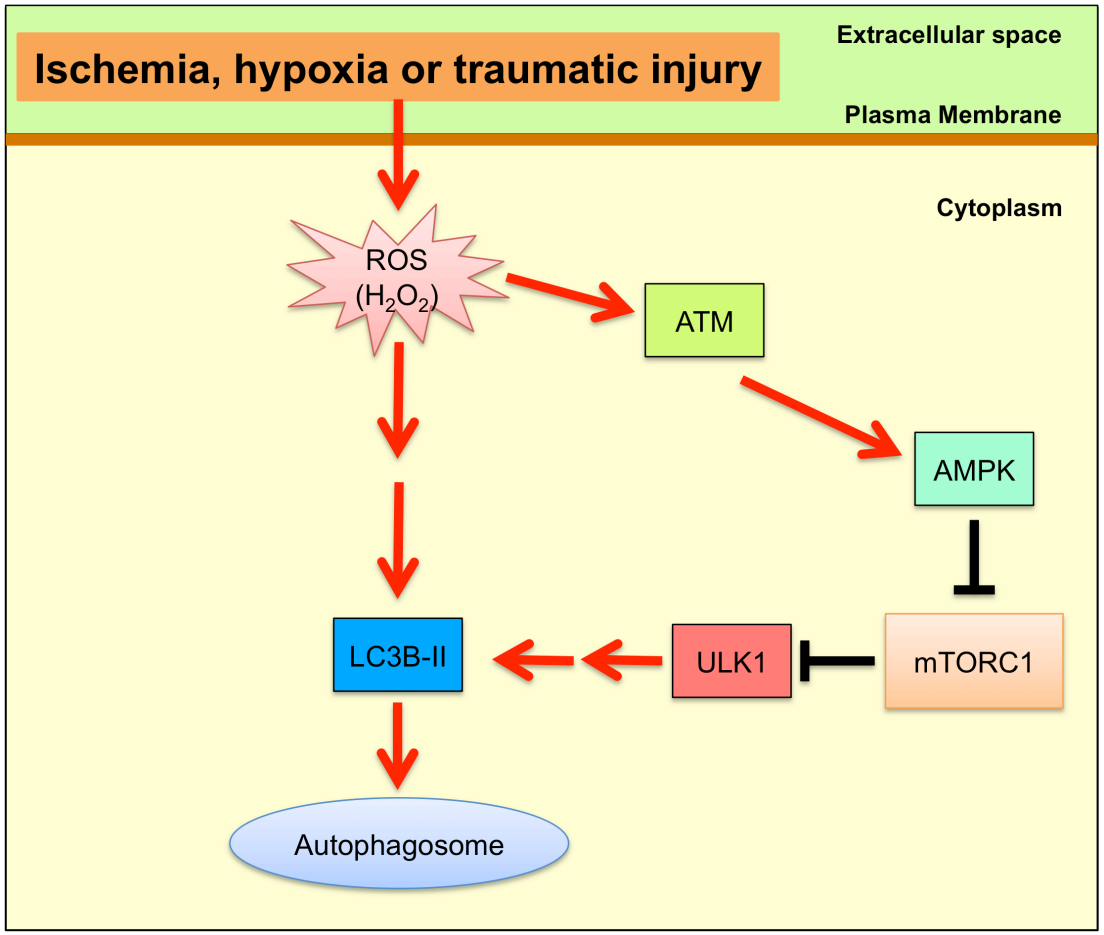


Figure 4: ROS (H_2O_2) induces LC3B-II expression and autophagosomes



Ischemia-reperfusion or traumatic brain injury induces the accumulation of reactive oxygen species (ROS) in astrocytes as well as neurons, and ROS induces the damage to organelles, proteins or lipids. In the process of autophagy, damaged cellular cytosolic components are isolated by autophagosomes. LC3B plays a significant role in the autophagosome formation, and the conversion from unconjugated-LC3B (LC3B-I) to phosphatidylethanolamine (PE) conjugated-LC3B (LC3B-II) is the index of the activation of autophagy. LC3B-II is induced via mTORC1-dependent and independent pathways. The roles of other LC3 subfamily and GABARAP subfamily members are not fully understood.

Materials and Methods

1.Cell culture and transfection

Rat C6 glioma cells were maintained in DMEM containing 10% FBS.

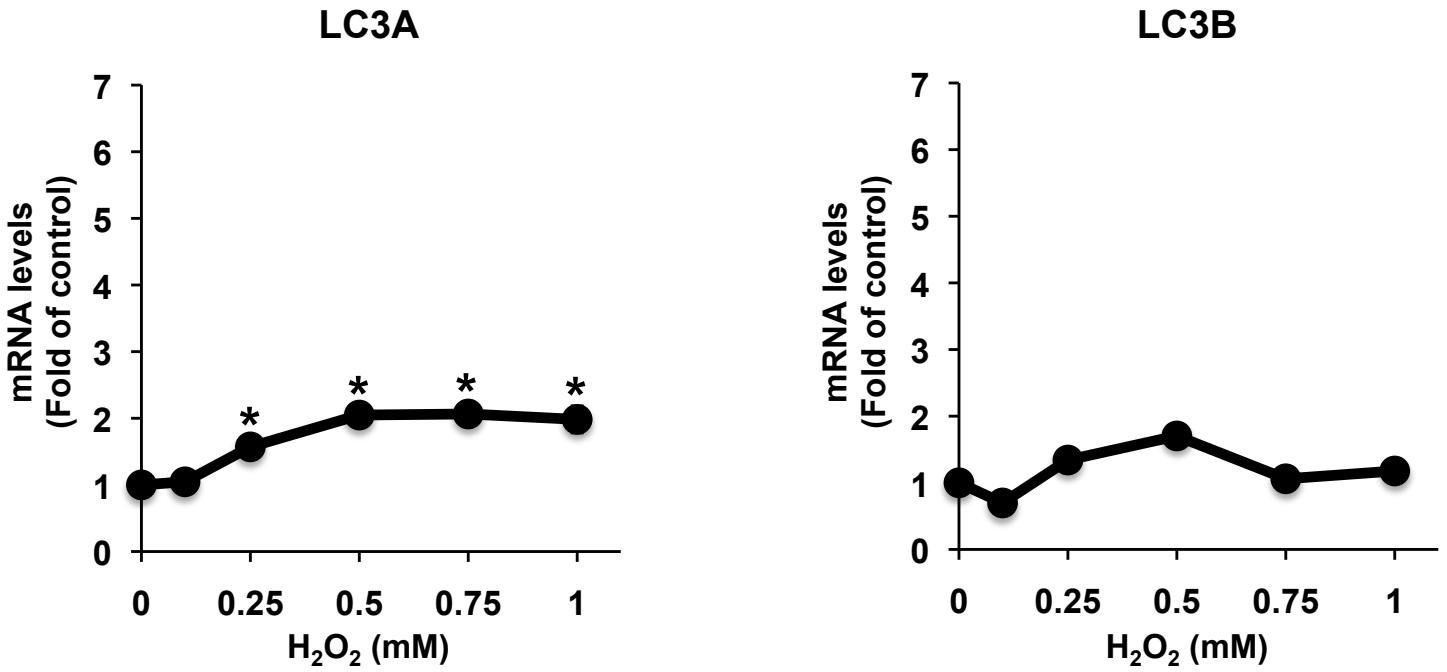
2. Real-time quantitative PCR

Real-time PCR was performed with cDNA isolated from C6 glioma cells using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) and LineGene (BioFlux, Tokyo, Japan). The expression level of a target gene was compared with the expression of *hydroxymethylbilane synthase (HMBS)* for each sample. The primers span more than one intron of target genes.

3.Western blot analysis

Protein extracts were separated by SDS-PAGE and transferred onto PVDF membranes, and were then probed with antibodies against GABARAPL1 (Proteitech, Chicago, IL, USA), LC3B (MBL, Nagoya, Japan) or actin (Sigma, St. Louis, MO, USA). Proteins of interest were detected with HRP-conjugated goat anti-rabbit or anti-mouse IgG antibody (MBL).

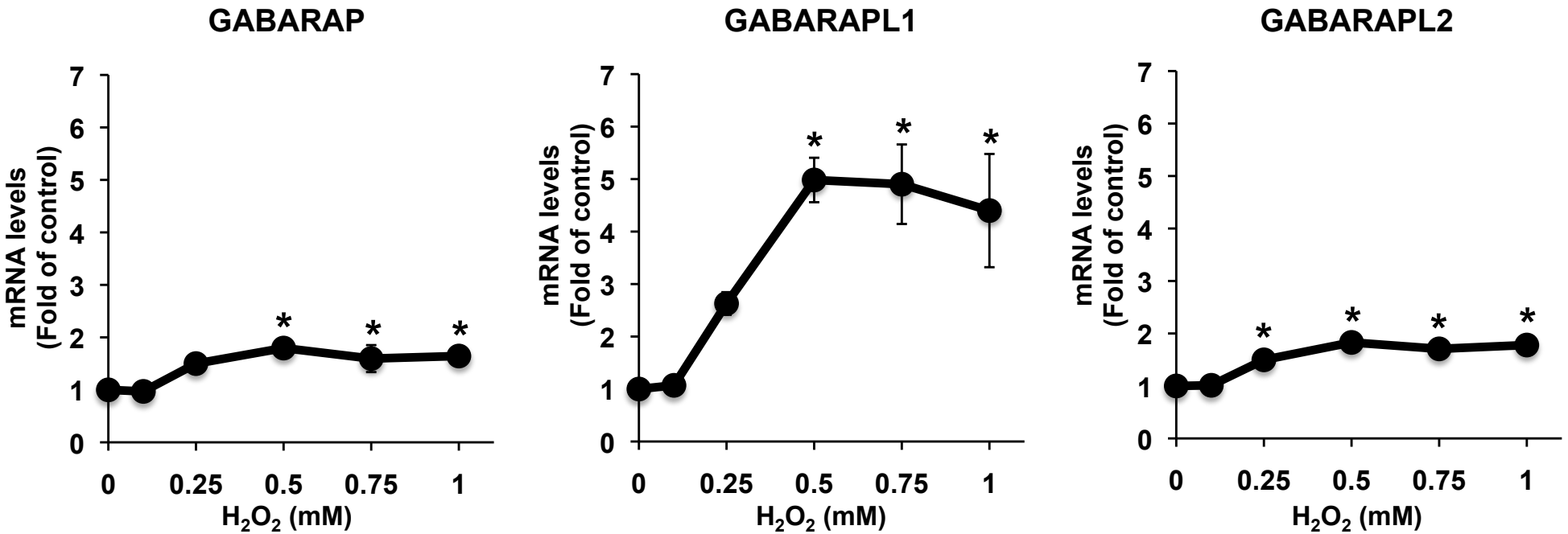
Figure 5:Quantification of mRNA of LC3 subfamily members by H_2O_2 in C6 glioma cells.



C6 glioma cells were treated with indicated concentrations of H_2O_2 for 6 h.
Total RNA was extracted and mRNA levels were analysed by real time PCR.
The expression levels were normalized to HMBS mRNA levels ($p<0.05$:*).

LC3A mRNA expression level increased to twice that of the control with 0.5 mM H_2O_2, and the increase in LC3A mRNA expression reached a maximum with 0.5 mM H_2O_2. However, the expression levels of LC3B mRNA were increased only slightly.

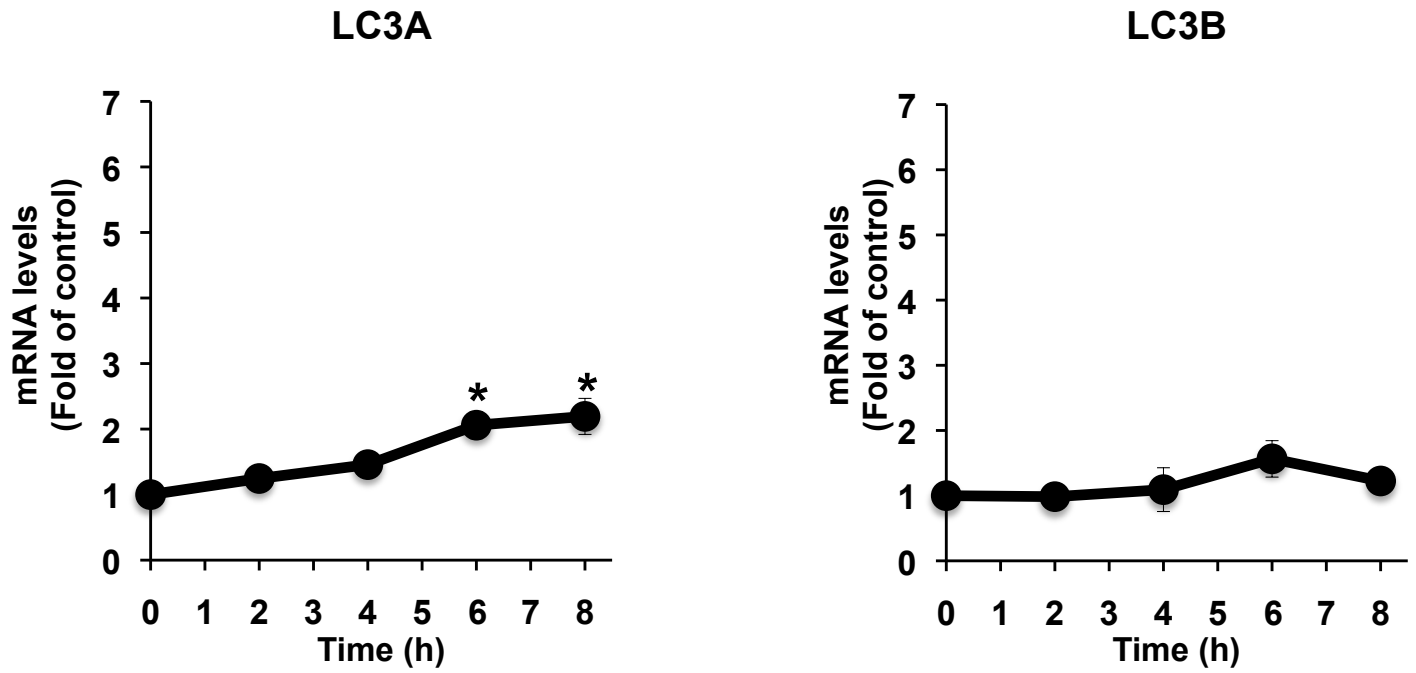
Figure 6:Quantification of mRNA of GABARAP subfamily members by H_2O_2 in C6 glioma cells



C6 glioma cells were treated with indicated concentrations of H_2O_2 for 6 h.
Total RNA was extracted and mRNA levels were analysed by real time PCR.
The expression levels were normalized to HMBS mRNA levels ($p<0.05$:*).

GABARAPL1 mRNA expression level increased to three times that of the control with 0.25 mM H_2O_2, and the increase in GABARAPL1 mRNA expression reached a maximum (five times that of the control) with 0.5 mM H_2O_2. However, the expression levels of mRNA for the remaining GABARAP subfamily members were increased only twice that of the control with H_2O_2

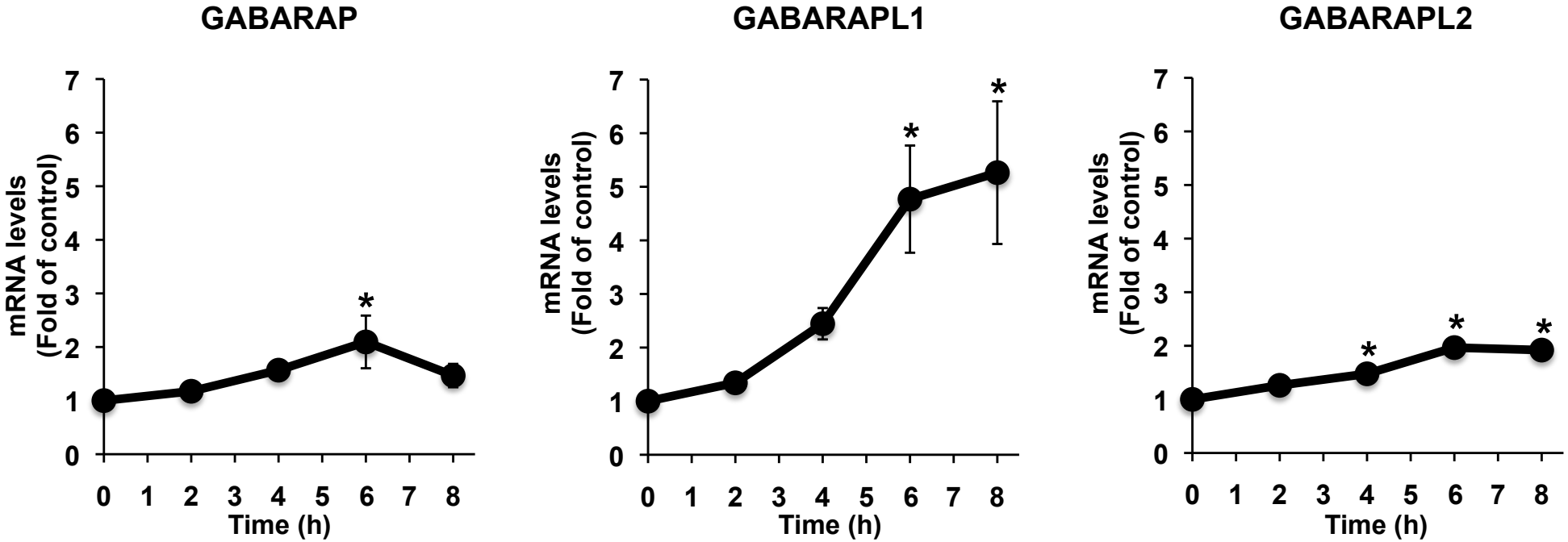
Figure 7:Time courses of the expression of mRNAs of LC3 subfamily members by H_2O_2 in C6 glioma cells



C6 glioma cells were treated with 1 mM H_2O_2 for the indicated times.
Total RNA was extracted and mRNA levels were analysed by real time PCR.
Expression levels were normalized to HMBS mRNA levels ($p<0.05$:*).

LC3A mRNA expression was twice that of the control with 1 mM H_2O_2 at 6 h, after which LC3A mRNA expression remained at this level. However, the expression levels of LC3B mRNA was only slightly increased with 1 mM H_2O_2.

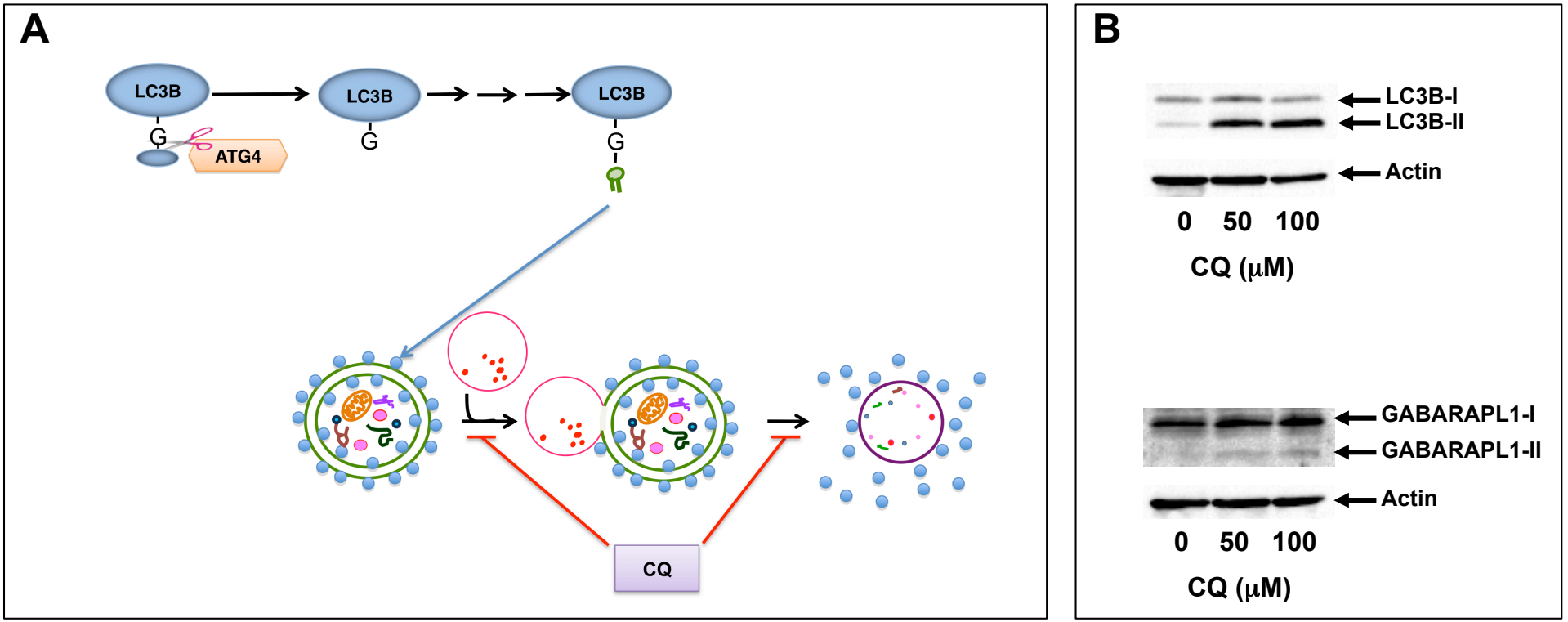
Figure 8:Time courses of the expression of mRNAs of GABARAP subfamily members by H_2O_2 in C6 glioma cells.



C6 glioma cells were treated with 1 mM H_2O_2 for the indicated times.
Total RNA was extracted and mRNA levels were analysed by real time PCR.
Expression levels were normalized to HMBS mRNA levels ($p<0.05$:*).

GABARAPL1 mRNA expression was five times that of the control with 1 mM H_2O_2 at 6 h, after which GABARAPL1 mRNA expression remained at this level. However, the expression levels of the mRNA for the remaining GABARAP subfamily members only slightly increased with 1 mM H_2O_2.

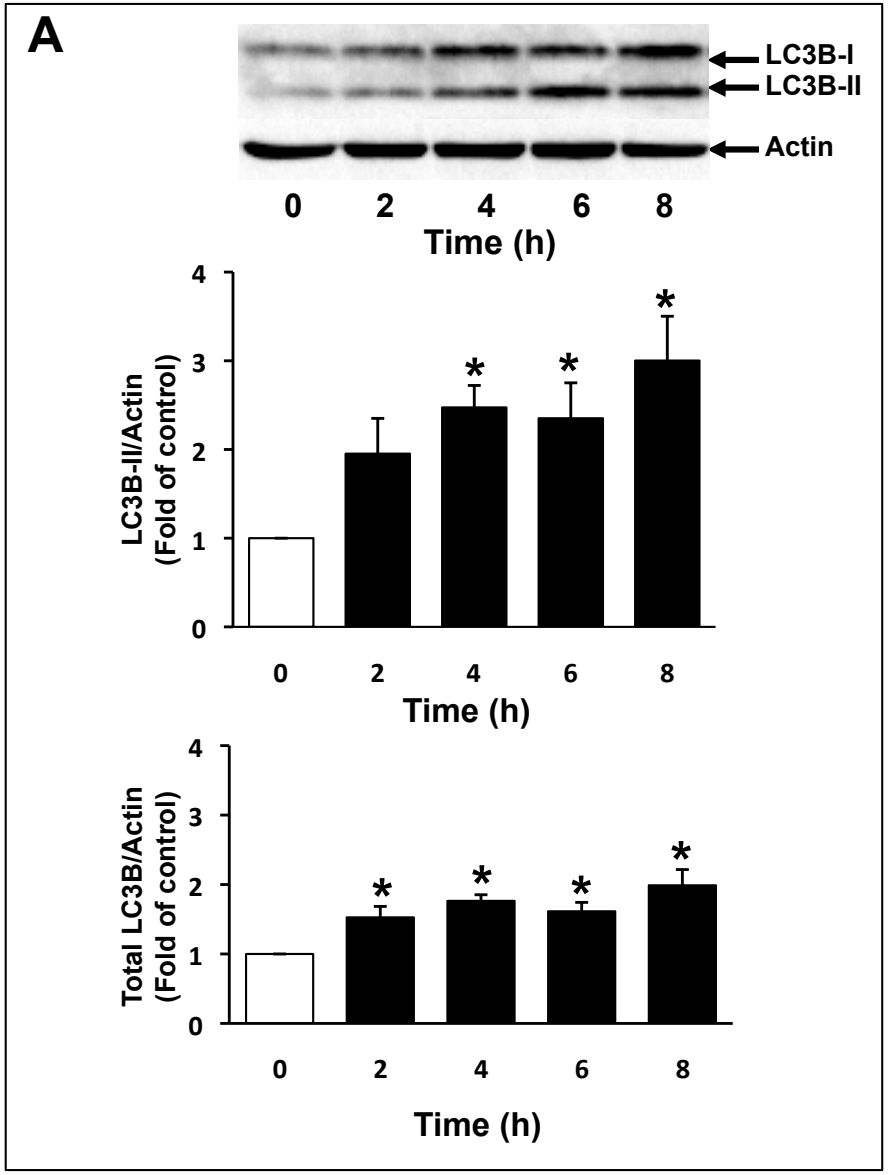
Figure 9: PE-conjugated form of LC3B (LC3B-II) and GABARAPL1 (GABARAPL1-II)

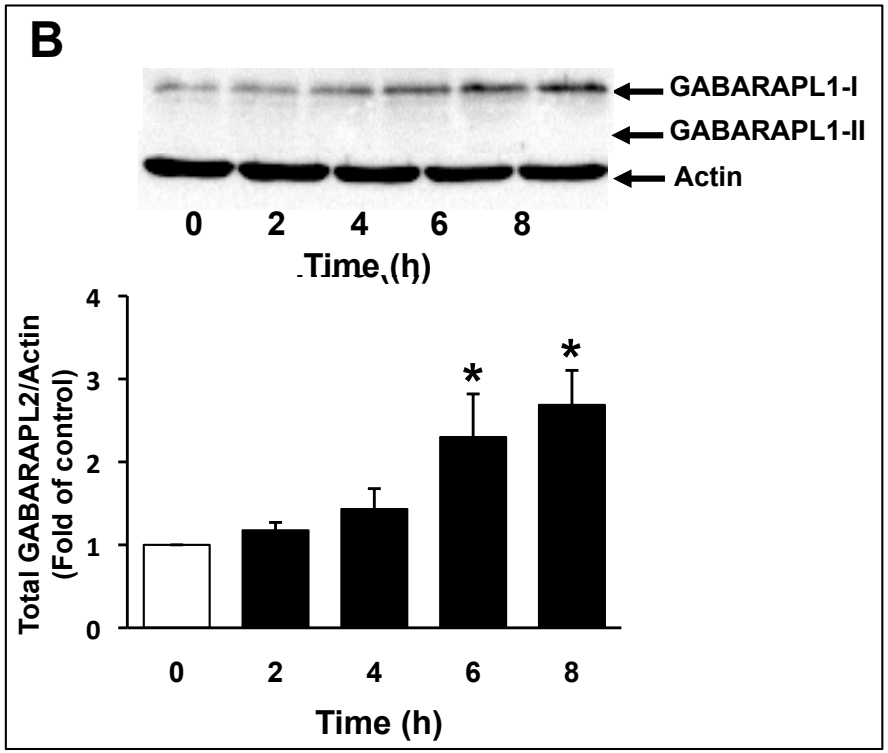


A. LC3B and GABARAPL1 is processed by ATG4 and then conjugated to phosphatidylethanolamine (PE) to form LC3B-II (LC3B-PE) and GABARAPL1-II (GABARAPL1-PE). Chloroquine (CQ) blocks lysosomal degradation and prevents the breakdown of the PE-conjugated form of LC3B (LC3B-II) and GABARAPL1 (GABARAPL1-II) in autolyssoemes.

B. C6 glioma cells were treated with the indicated concentration of CQ for 16 h. The PE-conjugated forms migrate faster than unconjugated forms. Although LC3B-II was detected without CQ, GABARAPL1-II was not detected without CQ.

Figure 10: Time courses of the expression of LC3B and GABARAPL1 by H_2O_2 in C6 glioma cells.





C6 glioma cells were treated with 1 mM H_2O_2 for the indicated times. Expression levels of LC3B or GABARAPL1 were normalized to actin.

A. The quantification of LC3B-II or total LC3B was performed ($p<0.05$:*). H_2O_2 significantly induced the levels of LC3B-II, and slightly induced the levels of total LC3B (LC3B+I and LC3B-II).

B. The quantification of GABARAPL1 was performed ($p<0.05$:*). H_2O_2 significantly induced the levels of GABARAPL1-I. However, GABARAPL1-II was not detected.

Conclusions

- LC3 subfamily and GABARAP subfamily members mRNAs are expressed in C6 glioma cells
- H_2O_2 significantly upregulated only GABARAPL1 mRNA among LC3 subfamily and GABARAP subfamily members.
- H_2O_2 did not have no effect on the levels of LC3B mRNA.
- H_2O_2 significantly induced LC3B-II and induced the levels of total LC3B (LC3B-I and LC3B-II) slightly. Thus, H_2O_2 induced the autophagy because the upregulation of LC3B-II indicates the induction of autophagy
- H_2O_2 significantly induced the levels of GABARAPL1-I, whereas H_2O_2 did not affect the induction of GABARAPL1-II.

GABARAPL1 may play a role in autophagic process, which is induced by H_2O_2. Because GABARAPL1-II, which is associated with membrane formation, was not upregulated, the function of GABARAPL1 in the autophagic process induced by H_2O_2 may be to carry cytosolic components to autophagosomes rather than to form of autophagosomes.