Effects of CCCP on the expression of GABARAPL2 in C6 glioma cells

Yoshimitsu Kiriyama, Airi Ozaki, Katsuhito Kino and Hiromi Nochi*
Tokushima Bunri University, Kagawa School of Pharmaceutical Sciences, Japan

Abstract
Astrocytes play an important role in the central nervous system. Ischemia, hypoxia, and traumatic injury cause mitochondrial damage in neurons and astrocytes. Damaged mitochondria are isolated and removed by macroautophagy (hereafter referred to as autophagy), which degrades cellular components with autophagosomal membranes. Autophagy that removes mitochondria is called mitophagy. Phosphatidylethanolamine (PE)-conjugated microtubule-associated protein 1 light chain 3B (LC3B) (LC3B-II) plays a crucial role in the formation of autophagosomes, and the conversion of unconjugated-LC3B (LC3B-I) to LC3B-II is the index of the induction of autophagy. Gamma-aminobutyric acid receptor-associated protein-like 2 (GABARAPL2, which is also known as GATE-16) is an outer mitochondrial membrane protein, binds LC3A, GABARAP, and selective pathways. The degradation of damaged mitochondria is considered as the selective autophagy (mitophagy) [21]. Nix, which is an outer mitochondrial membrane protein, binds LC3A, GABARAP, and GABARAPL2 but only weakly binds LC3B. Nix and GABARAP1 are engaged in mitophagy by carbonyl cyanide 3-chlorophenylhydrazone (CCCP) [22]. However, the functions of GABARAPL2 in damaged mitochondria remain unclear.

In this study, rat C6 glioma cells, which are often used as a model for astrocytes, were used to observe the expression of the mRNA of all the members of the LC3 subfamily and GABARAP subfamily. In addition, we examined the effects of CCCP, which causes mitochondrial damage, on the expression of GABARAPL2 in C6 glioma cells.

Materials and methods

Materials
CCCP and an anti-actin antibody were from Sigma (St. Louis, MO, USA). Anti-LC3B antibody and anti-GABARAPL2 (GATE-16) antibody was from MBL (Nagoya, Japan). Horseradish peroxidase-linked secondary antibodies were from Zymed Laboratories (South San Francisco, CA, USA). Mitotracker Red CMXRos Mitochondria Probe was purchased from Lonza Walkersville, Inc (Walkersville, MD, USA).

Correspondence to: Hiromi Nochi, Ph.D, Tokushima Bunri University, Kagawa School of Pharmaceutical Sciences, Shido 1314-1, Sanuki, Kagawa 769-2193, Japan; Tel: +81-87-899-7100; Fax: +81-87-894-0181; E-mail: nochi@kph.bunri-u.ac.jp

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Cell culture

Rat C6 glioma cells were obtained from JCRB cell bank (Osaka, Japan) and were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Reverse transcription - PCR

Total RNA isolated from cells was purified using RNeasy Mini Kits (Qiagen, Valencia, CA, USA). Reverse transcriptase reactions were performed on 1 µg of RNA using a PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan) with the following primers: LC3A, 5’-ATGCCCTCGACCCGCTTTCC-3’ and 5’-TCAGAGGCGGTCGTTGCTC-3’; LC3B, 5’-ATGCCGTCCGAGACCTTCT-3’ and 5’-TTACACGCGACGTGCTCC-3’; Gabarap, 5’-ATGAAGTGTCCGATAGTACAAAGA-3’ and 5’-TCAGAGGCGGTCGTTGCTC-3’; GabarapL1, 5’-ATGAAGTTTCAGTATAAAGA-3’ and 5’-TCGACCGCGACCTTCT-3’; GabarapL2, 5’-ATGAAGTGTCCGATAGTACAAAGA-3’ and 5’-TCGACCGCGACCTTCT-3’.

All primer sets were selected to span intron(s) to obtain mRNA-specific PCR products and to amplify between 300 bp and 400 bp.

Western blot analysis

C6 glioma cells treated with vehicle or CCCP were harvested and lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM diisopropylfluorophosphate, 10 µg/mL of leupeptin, 10 µg/mL of aprotinin, and 10 µg/mL of pepstatin) on ice for 20 min. Cell lysates were sonicated and centrifuged at 12,000×g at 4°C for 10 min. Proteins in the supernatants were electrophoresed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon P membranes (Millipore, Billerica, MA). The membranes were blocked with 1% skim milk in 0.1% Tween 20/PBS (PBS-T) and incubated with primary antibodies (dilutions based on manufacturer’s recommendations). The membranes were washed three times in
PBS-T and incubated for 1h with a horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected with ImmunoStar LD (Wako Pure Chemical Industries, Osaka, Japan).

Mitochondria staining

Rat C6 glioma cells were cultured on 35-mm glass-bottomed dishes (Iwaki, Shizuoka, Japan). The cells were treated with CCCP for 4h. Mitotracker Red CMXRos Mitochondria Probe (final concentrations of 50 nM) was added at 30 minutes before the end of incubation. Images were acquired using a Leica DMI 6000 B microscope with AF6000 software (Leica, Wetzlar, Germany).

Statistical analysis

Results of multiple observations are presented as means ± SEM. One-way analysis of variance (ANOVA) followed by the Dunnett’s test was used for multiple comparisons. P<0.05 was considered significant.

Results

The levels of mRNA expression of the members of the LC3 subfamily and GABARAP subfamily in C6 glioma cells were assessed by RT-PCR (Figure 1). The mRNAs of all of the members of the LC3 subfamily and GABARAP subfamily were expressed in C6 glioma cells. The mRNA of LC3B was expressed most abundantly, and the mRNAs of LC3A, GABARAP, and GABARAPL2 were moderately expressed. The mRNA of GABARAPL1 was slightly expressed. We examined the effects of CCCP on the expression of GABARAPL2-II and total GABARAPL2. GABARAPL2-II, which is the PE-conjugated form of GABARAPL2, can bind autophagosomes. Thus, the levels of GABARAPL2-II are considered to reflect the activity of GABARAPL2. GABARAPL2-I, which is the unconjugated form, migrates slower than GABARAPL2-II on SDS-PAGE [16]. GABARAPL2-II expression levels were increased seven times compared to that of the control in response to 20 µM of CCCP, and the increase in GABARAPL2-II expression reached a maximum (12 times that of the control) in response to 100 µM of CCCP. Total GABARAPL2 expression levels increased to twice that of the control in response to 100 µM of CCCP, and total GABARAPL2 expression in response to 120 µM of CCCP remained at this level (Figure 2A). In addition, we investigated the effects of 120 µM of CCCP on the expression of GABARAPL2-II and total GABARAPL2 at various time points up to 6 h. GABARAPL2-II was increased to 13 times that of the control in response to 120 µM of CCCP at 1 h, after which the expression level of GABARAPL2-II remained at this level. Total GABARAPL2 was increased about twice that of control in response to 120 µM of CCCP at 1h, after which the GABARAPL2 expression remained at this level.

The effects of 120 µM of CCCP on the expression of LC3 were examined at various time points up to 6 h (Figure 3A) because the conversion of LC3 from LC3-I to LC3-II reflects the induction of autophagy and is a key step in the forming of autophagosomes. Similar to the results of GABARAPL2, LC3B-II expression was increased about ten times that of the control in response to 120 µM of CCCP at 1h, after which it remained at this level. However, total LC3B expression levels were not affected by 120 µM of CCCP (Figure 3A). The fragmentation of mitochondria precedes mitophagy [23]. We confirmed that the reticular mitochondrial network was fragmented by 120 µM of CCCP (Figure 3B). These results indicated that mitochondrial dysfunction correlated with the expression and activity of GABARAPL2.
Discussion

The segregation and exclusion of damaged mitochondria are crucial to maintain the function of astrocytes. Damaged mitochondria are sequestered and eliminated by mitophagy, which is accompanied by the formation of autophagosomes. LC3B, which is a member of the LC3 subfamily and GABARAP subfamily, is a well-characterized protein that is involved in the formation of autophagosomes. However, the functions of the other members of the LC3 subfamily and GABARAP subfamily have not been completely elucidated. Although the physiological functions of GABARAPL2 in mitophagy are not well understood, it has been shown that GABARAPL2 is linked to phospholipids in the same manner as LC3 phospholipidation and may play a role in autophagy [16]. Moreover, GABARAPL2 binds to an ubiquitin-binding protein, p62 [24], and to a mitochondrion-localized protein, Nix [22]. Both p62 and Nix are involved in mitophagy that is induced by CCCP. In CCCP-induced mitophagy, voltage-dependent anion channels (VDACs), which are on the surface of mitochondria, are ubiquitinated by PINK1 and Parkin, and p62 then binds ubiquitinated-VDACs. LC3B carries p62 with ubiquitinated-VDACs to the autophagosome [21,25,26]. On the other hand, Nix interacts with GABARAPL1, and GABARAPL1 then brings the mitochondria to the autophagosome during the CCCP-induced mitophagy [22]. CCCP is the uncoupler that induces the fragmentation of mitochondria, which precedes mitophagy [23]. Therefore, we examined the effects of CCCP on the activation and expression of GABARAPL2. CCCP induced the upregulation of both GABARAPL2-II and total GABARAPL2. In contrast, the treatment of CCCP resulted in LC3-II upregulation, which is the indicator of the formation of the autophagosome, while CCCP did not affect the levels of expression of total LC3B. Recent studies have shown that LC3B plays a role in the early stage of autophagy (elongation of the phagophore membrane) and that GABARAPL2 is necessary for the late stage of autophagy (autophagosome closure) [20]. Therefore, GABARAPL2 may function in autophagosome closure during mitophagy, and each member of the LC3 subfamily and GABARAP subfamily may have distinct roles in the process of autophagy.

In conclusion, we demonstrated that both GABARAPL2-II and total GABARAPL2 were upregulated by CCCP in C6 glioma cells. GABARAPL2 possibly functions in the segregation of damaged mitochondria that occurs to protect against the dysfunction and death of astrocytes. The mechanism involving GABARAPL2 in the clearance of damaged mitochondria in astrocytes and the functions of GABARAPL2 require further investigations.

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