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REVIEW ARTICLE

Modulation of neuronal glutathione synthesis by EAAC1 and its interacting protein GTRAP3-18

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Abstract Glutathione (GSH) plays essential roles in different processes such as antioxidant defenses, cell signaling, cell proliferation, and apoptosis in the central nervous system. GSH is a tripeptide composed of glutamate, cysteine, and glycine. The concentration of cysteine in neurons is much lower than that of glutamate or glycine, so that cysteine is the rate-limiting substrate for neuronal GSH synthesis. Most neuronal cysteine uptake is mediated through the neuronal sodium-dependent glutamate transporter, known as excitatory amino acid carrier 1 (EAAC1). Glutamate transporters are vulnerable to oxidative stress and EAAC1 dysfunction impairs neuronal GSH synthesis by reducing cysteine uptake. This may start a vicious circle leading to neurodegeneration. Intracellular signaling molecules functionally regulate EAAC1. Glutamate transporter-associated protein 3-18 (GTRAP3-18) activation down-regulates EAAC1 function. Here, we focused on the interaction between EAAC1 and GTRAP3-18 at the plasma membrane to investigate their effects on neuronal GSH synthesis. Increased level of GTRAP3-18 protein induced a decrease in GSH level and, thereby, increased the vulnerability to oxidative stress, while decreased level of GTRAP3-18 protein induced an increase in GSH level in vitro. We also confirmed these results in vivo. Our studies demonstrate that GTRAP3-18 regulates neuronal GSH level by controlling the EAAC1-mediated uptake of cysteine.

Keywords Glutathione · Cysteine · EAAC1 · GTRAP3-18 · Neurodegeneration

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Introduction

J. de Rey-Pailhade first reported in 1888 a substance from yeast cells, which he named "*philothione*", a Greek word meaning sulfur loving, because of its reactivity with sulfur (de Rey-Pailhade 1888; Meister 1988). Later, F.G. Hopkins identified a dipeptide containing glutamate and cysteine and named it "*glutathione*" (Hopkins 1921). de Rey-Pailhade believed that glutathione (GSH) was part of philothione; however, it became clear that philothione was identical to GSH, which was finally found to be a tripeptide consisting of glutamate, cysteine, and glycine (Kendall et al. 1930). However, the function of GSH in living cells did not receive much attention, as described in a paper entitled "*Lest I forget thee, glutathione*…" in the 1960s (Kosower and Kosower 1969). Since the 1970s, accumulating lines of evidence have clarified the functions, metabolism, and regulation of glutathione. Currently, the functional importance of GSH has been demonstrated in a variety of cellular processes, including antioxidant defenses, cell signaling, gene expression, and apoptosis. However, the system that regulates GSH synthesis is still elusive, especially in the central nervous system (CNS).

GSH plays especially important roles in the CNS. Some neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, are associated with depletion of GSH in the brain (Ramassamy et al. 2000; Sian et al. 1994), which might be considered as an early event in these diseases (Jenner 1994). The brain level of GSH is lower than that of other organs (Commandeur et al. 1995) and declines with aging (Maher 2005). Notably, the basal GSH level in neurons is lower than that in astrocytes, microglia, and oligodendrocytes (Hirrlinger et al. 2002), indicating different mechanism(s) for maintaining GSH homeostasis between neurons and glial cells. In this review, we will

discuss the role played by the neuronal glutamate transporter, excitatory amino acid carrier 1 (EAAC1), in cysteine supply for neuronal GSH synthesis and its regulation by the interacting protein, glutamate transporter-associated protein 3-18 (GTRAP3-18), while other reviews in this issue will describe the role of system x_c^- for neuronal GSH synthesis (Lewerenz et al. 2011) or the mechanisms used by glial cells to maintain their antioxidant defenses (Gras et al. 2011; Had-Aissouni 2011; McBean 2011; Persson and Rönnbäck 2011).

GSH functions

GSH is a major low-molecular-weight thiol present in the brain (Dringen 2000) at a concentration of ~2–3 mM and exerts its functions in a number of cellular processes via several mechanisms. GSH functions predominantly to protect the brain against oxidative stress, neurotoxins, or other forms of stress causing neurodegeneration.

GSH reacts non-enzymatically with superoxide (Winterbourn and Metodiewa 1994), nitric oxide (Clancy et al. 1994), hydroxyl radical (Bains and Shaw 1997), and peroxynitrite anion (Koppal et al. 1999) and acts as an effective scavenging compound. GSH also serves as an electron donor for the reduction of H_2O_2 or other peroxides catalyzed by GSH peroxidase (EC 1.11.1.9) (Chance et al. 1979; Lei 2002). GSH is enzymatically conjugated with various endogenous and xenobiotic compounds by glutathione-S-transferase (EC 2.5.1.18) (Commandeur et al. 1995) to form mixed disulfides, which are exported outside of the cell. This process plays an important role in the detoxification of the cell.

GSH is the major redox buffer and maintains the redox homeostasis in the cell. Under oxidative stress, GSH can lead to the reversible formation of mixed disulfides between protein thiol groups (S-glutathionylation) to prevent irreversible oxidation of proteins (Giustarini et al. 2004). The protein sulfhydryl residues, mainly cysteine, are the targets of S-glutathionylation and the interaction with GSH affects protein ability to function as enzymes or receptors. The thiol redox state also regulates DNA synthesis, gene transcription, and programmed cell death (Arrigo 1999; Voehringer 1999).

GSH also plays critical roles in cell proliferation. The inhibition of GSH synthesis arrests the cell cycle in the S and G2 phases (Poot et al. 1995). A recent study demonstrated that proliferating cells in the S and G2 phases of the cell cycle showed increased GSH level in the nucleus and that Bcl-2, a member of the nuclear pore complex, might be involved in this change (Markovic et al. 2007). Indeed, Bcl-2 overexpression elevated GSH level in the nucleus (Voehringer et al. 1998). These results suggest that cells require GSH at appropriate period for their proliferation.

GSH synthesis

GSH is a tripeptide consisting of glutamate, cysteine, and glycine. The synthesis of GSH requires two ATP-dependent enzymatic reactions. Glutamate cysteine ligase (GCL, EC 6.3.2.2), also known as γ-glutamylcysteine synthetase, catalyzes the first step, which is the rate-limiting enzymatic step in GSH synthesis (Dringen 2000). GCL mediates the first reaction between glutamate and cysteine to form a dipeptide, γ-glutamylcysteine, which then reacts with glycine in a reaction catalyzed by glutathione synthase (EC 6.3.2.3) to produce GSH (Dringen 2000). GSH regulates its own synthesis by feedback inhibition of GCL (Richman and Meister 1975). GCL is composed of catalytic and modulatory subunits, GCLc and GCLm, respectively. GCLc, but not GCLm, has all the enzymatic activity and is also responsible for the feedback inhibition by GSH (Seelig et al. 1984). GCLc knockout was embryonic lethal in mice, demonstrating that this gene is essential for embryonic development (Dalton et al. 2004). The majority of GSH in a cell remains in the cytoplasm where it is synthesized (Sims et al. 2004). Mitochondria also contain GSH (Griffith and Meister 1985; Sims et al. 2004), although they cannot synthesize GSH by themselves because they lack GCL activity (Griffith and Meister 1985). The nucleus contains more GSH than the mitochondria (Bellomo et al. 1992; Soboll et al. 1995). A recent paper demonstrated that the nuclear synthesis of GSH is accomplished by shuttling of the GCLc subunit from the cytoplasm to the nucleus (Radyuk et al. 2009). Previous studies have demonstrated the precise enzymatic mechanisms for GSH synthesis in cells, but less interest has been focused on the uptake of substrates for this synthesis. The intracellular level of cysteine is much lower than those of the other two substrates, glutamate and glycine, and cysteine has been shown to be the rate-limiting substrate for neuronal GSH synthesis in vitro (Dringen et al. 1999a, b). The process of cysteine uptake lies upstream of the subsequent enzymatic reactions during GSH synthesis. Moreover, at high extracellular concentrations, cysteine may be toxic for neurons (Gazit et al. 2004; Janaky et al. 2000). Therefore, it is important to understand how cysteine is supplied to neurons.

Supply of GSH precursors to neurons

The liver synthesizes and stores the highest levels of GSH in the body (Commandeur et al. 1995). More than 80% of the total GSH efflux flows into the blood (Lauterburg et al. 1984; Kaplowitz et al. 1983) to provide GSH to the other organs. Rat liver can release 20–50% of the stored GSH in 60 min (Aw et al. 1986; Griffith and Meister 1979); however, the released GSH does not appear to reach the brain. Intravenously administered GSH is metabolized rapidly

(Ammon et al. 1986; Lash and Jones 1985) and GSH penetrates the blood–brain barrier (BBB) poorly, so that only 0.5% of radiolabeled GSH can be detected in the brain extract after intracarotid injection (Cornford et al. 1978). L-cysteine also penetrates the BBB poorly (Gazit et al. 2004). In contrast, the level of cystine (the disulfide form of cysteine) in plasma is higher than those of cysteine, GSH, or other thiol derivatives, suggesting that cystine serves to supply thiol from the liver to the brain (Wang and Cynader 2000).

Plasma cystine is transported into the brain via a cystine transporter, called system x_c^-, at the BBB (Hosoya et al. 2002). System x_c^- is a sodium-independent cystine/glutamate antiporter composed of two subunits, xCT and 4F2hc (Sato et al. 1999), also present on glial cells (Pow 2001; Qin et al. 2006) and reported to be expressed in neurons by some authors (see Lewerenz et al. 2011). However, as mature cultured neurons preferentially utilize cysteine, not cystine, for their GSH synthesis (Dringen and Hirrlinger 2003; Kranich et al. 1996; Sagara et al. 1993), it is thought that the rate of cystine uptake into the brain is especially important for maintaining GSH level in glial cells (Cho and Bannai 1990; Kranich et al. 1996, 1998; Sagara et al. 1996).

In the CNS, astrocytes display high level of GSH (Dringen and Hamprecht 1998) and release GSH in the extracellular space (Wang and Cynader 2000). Astrocytes can export about 10% of their intracellular GSH within 1 h (Dringen et al. 1997a, b) and continuously resynthesize GSH from various precursors (Dringen et al. 1997a, b; Dringen and Hamprecht 1998; Had-Aissouni 2011; McBean 2011). The GSH released by astrocytes may be an important source of cysteine through its hydrolysis by the consecutive action of two extracellular enzymes, γ-glutamyltransferase (EC 2.3.2.2) present on the membrane of astrocytes and aminopeptidase N (EC 3.4.11.2) present on the membrane of neurons (Dringen 2000). Released GSH may also react with cystine, which is transported from plasma via system x_c^-, to form cysteine and cysteine–GSH. These reactions increased cysteine levels in the CNS compared with those of plasma (Wang and Cynader 2000) and form an extracellular pool of cysteine that may be used by neurons to sustain their GSH synthesis. Therefore, neurons are thought to rely on astrocytes for their antioxidant defenses (Bolanos et al. 1996; Dringen et al. 1999a, b; Kranich et al. 1996; Sagara et al. 1993).

Excitatory amino acid transporter for GSH synthesis

In neurons, approximately 90% of the total cysteine uptake is mediated by sodium-dependent systems, mainly the excitatory amino acid transporter (EAAT), also known as system X_{AG}^- (Shanker et al. 2001). Originally, EAATs have been reported to play an important role in removing extracellular glutamate in the CNS (Anderson and Swanson 2000; Maragakis and Rothstein 2004). There are five EAATs termed GLAST (Glutamate Aspartate Transporter, also named EAAT1) (Storck et al. 1992), GLT-1 (Glutamate Transporter-1, also named EAAT2) (Pines et al. 1992), EAAC1 (also named EAAT3) (Kanai and Hediger 1992), EAAT4 (Fairman et al. 1995), and EAAT5 (Arriza et al. 1997). GLAST and GLT-1 are localized to astrocytes, while EAAC1, EAAT4, and EAAT5 are localized to neurons. The expression of EAAT4 and EAAT5 is restricted to the cerebellar Purkinje cells and retina, respectively, whereas EAAC1 is widely expressed in the CNS (Arriza et al. 1997; Rothstein et al. 1994; Yamada et al. 1996). EAATs co-transport three Na^+ ions and one H^+ ion with each glutamate and counter-transport one K^+ (Kanai and Hediger 2003). EAATs can use as substrate not only the excitatory amino acids glutamate and aspartate but also cysteine (Zerangue and Kavanaugh 1996). In particular, EAAC1 can transport cysteine at a rate comparable to that of glutamate with an affinity that is 10- to 20-fold higher than that of GLAST or GLT-1 (Zerangue and Kavanaugh 1996). Partial knock-down of EAAC1 resulted in approximately 20% decreases in cysteine uptake and GSH content in cultured neurons (Himi et al. 2003), and EAAC1-deficient mice showed an approximately 40% decrease in brain GSH content and neurodegeneration at advanced age (Aoyama et al. 2006). Interestingly, dopaminergic neurons of the substantia nigra which degenerate in Parkinson disease express high levels of EAAC1 (Plaitakis and Shashidharan 2000) and are particularly vulnerable to glutamate transporter dysfunction through an oxidative mechanism that potentiates NMDA receptor-mediated excitotoxicity (Nafia et al. 2008). These results suggest that EAAC1 is important as a cysteine transporter for neuronal GSH synthesis and that its dysfunction may contribute to neurodegenerative insults.

Regulation of EAAC1 activity

Under normal conditions, approximately 20% of EAAC1 expression is found on the plasma membrane (Fournier et al. 2004). EAAC1 activity is mainly related to expression on the cell surface, rather than de novo synthesis (Davis et al. 1998; Fournier et al. 2004). Protein kinase C (PKC) activation induces cell-surface expression of EAAC1 (Fournier et al. 2004; Gonzalez et al. 2002, 2003). Phorbol 12-myristate 13-acetate (PMA), a PKC activator, induces a nearly twofold increase in the cell-surface expression of EAAC1 within 15 min (Fournier et al. 2004). Particularly, PKC subtype α induces EAAC1 translocation to membrane surface, whereas PKCε mediates the increase

in EAAC1 activity without translocation to the membrane (Gonzalez et al. 2002). EAAC1 is up-regulated by serum- and glucocorticoid-inducible kinase SGK1 (Schniepp et al. 2004) and phosphoinositide-dependent kinase PDK1 (Rexhepaj et al. 2006), and down-regulated by direct interaction with δ-opioid receptor (Xia et al. 2006) or GTRAP3-18 (Lin et al. 2001). Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), also decreases the expression of EAAC1 on the cell surface (Davis et al. 1998), whereas platelet-derived growth factor (PDGF) increases the delivery of EAAC1 to the membrane via Akt/PI3K activation (Fournier et al. 2004; Sheldon et al. 2006). RTN2B, a member of the reticulon protein family, interacts with EAAC1 to facilitate the trafficking of EAAC1 out of the endoplasmic reticulum (ER) to the cell surface (Liu et al. 2008). All-*trans*-retinoic acid (ATRA) plays critical roles in the development and regeneration of the nervous system (Maden 2007). Specifically, ATRA induces the expression of EAAC1, although the distribution is mainly in the cytoplasmic region (Bianchi et al. 2008).

EAATs form homomultimers, mainly trimers, and each subunit works independently (Haugeto et al. 1996; Gendreau et al. 2004; Koch and Larsson 2005). The subunit has eight transmembrane domains with two membrane-inserted loops (Yernool et al. 2004). The carboxyl-terminal domain of EAAC1 is an intracellular tail and plays an essential role in trafficking to the membrane surface. A mutant EAAC1 lacking 20 carboxyl-terminal amino acids did not show trafficking to the cell surface stimulated by PMA or PDGF (Sheldon et al. 2006). Interestingly, PDGF did not induce trafficking of an EAAC1 chimera containing the carboxyl-terminal domain of GLT-1, while it induced trafficking of a GLT-1 chimera containing the carboxyl-terminal domain of EAAC1 (Sheldon et al. 2006). Another study showed that a short EAAC1 carboxyl-terminal motif, ^{502}YVN^{504}, was necessary for PDGF-induced redistribution to the plasma membrane (Sheldon et al. 2006). The phosphorylation of serine 465 in EAAC1 by PKCα activation increased the translocation to the plasma membrane, and mutation of serine 465 to aspartic acid also increased the expression in the plasma membrane (Huang et al. 2006). Mutation of arginine 447 of EAAC1 to neutral or negative amino acid residues completely blocked the transport of glutamate and aspartate without impairing cysteine transport (Bendahan et al. 2000), suggesting independent mechanisms for the uptake of glutamate and cysteine by EAAC1.

GTRAP3-18/addicsin/JWA

GTRAP3-18 is a protein of 188 amino acids that was initially identified as a negative modulator of EAAC1 by a yeast two-hybrid screen system from rat brain (Lin et al. 2001). Analysis of the distribution of GTRAP3-18 in brain tissue showed widespread expression colocalized to neurons. However, the subcellular localization of GTRAP3-18 is still controversial (Lin et al. 2001; Maier et al. 2009). GTRAP3-18 also has an inhibitory effect on Rab1, which is involved in ER-to-Golgi trafficking, and GTRAP3-18 overexpression inhibited neurite growth in vitro (Maier et al. 2009). The expression of GTRAP3-18 decreased significantly from embryonic day 17 to post-natal day 0 and in the adult rat (Maier et al. 2009). However, the physiologic role of GTRAP3-18 is still poorly understood.

The mRNA of mouse GTRAP3-18, which is also called addicsin, is up-regulated in amygdala after morphine treatment (Ikemoto et al. 2002). Although the regulatory mechanism(s) of GTRAP3-18 remains unclear, ADP-ribosylation factor-like 6 interacting protein 1 (Arl6ip1) interacts with GTRAP3-18 and inhibits the binding of GTRAP3-18 to EAAC1, which promotes EAAC1-mediated glutamate transport activity (Akiduki and Ikemoto 2008).

Human GTRAP3-18, also called JWA, is generally up-regulated by cell differentiation, heat shock, and oxidative stress in vitro in non-neuronal cells (Chen et al. 2007; Mao et al. 2004; Wang et al. 2003). JWA was also reported to be up-regulated in the thalamus of patients with schizophrenia, suggesting altered thalamic glutamatergic neurotransmission (Huerta et al. 2006). One study demonstrated that JWA-knock-down cells showed greater DNA damage from oxidative stress than control cells, and the authors concluded that JWA might serve as a stress sensor to protect cells against DNA damage by oxidative stress (Chen et al. 2007). In contrast, our data demonstrated that the increased expression of JWA rendered the cells more vulnerable to oxidative stress induced by H_2O_2 (Watabe et al. 2007). Further studies in neuronal cells are needed to conclude whether JWA acts in a neuroprotective or neurodegenerative manner.

We recently reported that the inhibition of GTRAP3-18 expression using antisense oligonucleotides increased the intracellular GSH content in vitro. In contrast, the increase in the expression of GTRAP3-18 caused by treatment with methyl-β-cyclodextrin (MeβCD) led to decreased GSH content without blocking the trafficking of EAAC1 to the membrane (Watabe et al. 2007). Immunocytochemical studies supported the conclusion that GTRAP3-18 was present in both the plasma membrane and the intracellular compartment (Watabe et al. 2007, 2008). However, recent reports showed that GTRAP3-18 was an integral membrane ER protein that prevented EAAC1 maturation by retaining EAAC1 at the ER as a regulator of trafficking (Ruggiero et al. 2008; Maier et al. 2009). It is still debated whether GTRAP3-18 is a component of the ER exclusively. We also studied GSH regulation by GTRAP3-18 in

vivo (Watabe et al. 2008). Continuous intracerebroventricular (ICV) injection with MeβCD increased the hippocampal expression of GTRAP3-18 with decreased GSH content. Continuous ICV injection with siRNA for GTRAP3-18 decreased the hippocampal expression leading to increased GSH content. Our results suggest that GTRAP3-18 is a potential target for increasing neuronal GSH level endogenously.

Conclusions

GSH is a major intracellular thiol that plays essential roles in protecting cells against oxidative stress, maintaining redox homeostasis, cell proliferation, and supporting detoxifying enzyme activities in the CNS. GSH depletion in the brain causes neurodegeneration. It is crucial to better understand the modulatory mechanisms governing neuronal GSH level. Neuronal GSH synthesis is regulated by cysteine uptake via EAAC1, which is negatively controlled by GTRAP3-18. Clarifying the regulatory mechanisms that control EAAC1/GTRAP3-18 interactions might reveal a promising method to increase neuronal GSH level selectively against neurodegeneration.

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