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FABP7 Protects Astrocytes Against ROS Toxicity via Lipid Droplet Formation

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Abstract

Fatty acid-binding proteins (FABPs) bind and internalize long-chain fatty acids, controlling lipid dynamics. Recent studies have proposed the involvement of FABPs, particularly FABP7, in lipid droplet (LD) formation in glioma, but the physiological significance of LDs is poorly understood. In this study, we sought to examine the role of FABP7 in primary mouse astrocytes, focusing on its protective effect against reactive oxygen species (ROS) stress. In FABP7 knockout (KO) astrocytes, ROS induction significantly decreased LD accumulation, elevated ROS toxicity, and impaired thioredoxin (TRX) but not peroxiredoxin 1 (PRX1) signalling compared to ROS induction in wild-type astrocytes. Consequently, activation of apoptosis signalling molecules, including p38 mitogen-activated protein kinase (MAPK) and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and increased expression of cleaved caspase 3 were observed in FABP7 KO astrocytes under ROS stress. N-acetyl L-cysteine (NAC) application successfully rescued the ROS toxicity in FABP7 KO astrocytes. Furthermore, FABP7 overexpression in U87 human glioma cell line revealed higher LD accumulation and higher antioxidant defence enzyme (TRX, TRX reductase 1 [TRXRD1]) expression than mock transfection and protected against apoptosis signalling (p38 MAPK, SAPK/JNK and cleaved caspase 3) activation. Taken together, these data suggest that FABP7 protects astrocytes from ROS toxicity through LD formation, providing new insights linking FABP7, lipid homeostasis, and neuropsychiatric/neurodegenerative disorders, including Alzheimer's disease and schizophrenia.

Keywords Fatty acid-binding protein 7 · Astrocytes · Lipid droplet · Thioredoxin · U87

Introduction

Astrocytes are star-shaped cells present in most of the brain. These cells are the most abundant glial cells in the central nervous system (CNS) and play pivotal roles in normal brain organization through regulation of neuronal development, homeostatic synaptic scaling and modulation of neurophysiological mechanisms implicated in the governing of complex behavioural processes [1, 2]. Astrocytes also pivotally influence the structural and synaptic plasticity of neurons either directly through physical contact or indirectly by releasing humoral factors, such as growth factors, thrombospondins and cholesterol [3–6]. Recent studies have revealed that a substantial fraction of the lipids incorporated into CNS myelin are contributed by astrocytes [7]. The continuous increase of myelin vulnerability resulting from the human brain's delayed myelination underlies underappreciated communalities between different disease phenotypes ranging from developmental ones such as schizophrenia (SZ) and bipolar disorder (BD) to degenerative ones such as Alzheimer's disease (AD).

Fatty acid-binding proteins (FABPs) are low-molecular-weight proteins (approximately 15 kDa) that serve as cellular chaperons for hydrophobic molecules, such as fatty acids, in a large variety of cells and organs [8–13]. By controlling the

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uptake and intracellular distribution of fatty acids, FABPs are thought to be involved in metabolism, signal transduction and gene regulation [14]. Studies in cultured cells have suggested a potential role of FABPs in fatty acid import, storage and export as well as cholesterol and phospholipid metabolism [15]. Recent studies have revealed that nuclear phospholipase C isoenzyme imbalance leads to pathologies in brain, hematologic, neuromuscular and fertility disorders [16]. FABP7, which preferentially binds n-3 polyunsaturated fatty acids (PUFAs), is expressed in neural stem cells and radial glia of the developing brain [17] and is essential for neurogenesis as a positive regulator of proliferation in neural stem progenitor cells [18]. In the embryonic brain, FABP7 is essential for the maintenance and proliferation of neural stem progenitor cells [19]. In the adult brain, FABP7 is expressed by astrocytes and oligodendrocyte progenitor cells (OPCs), whereas it is not detected in neurons, microglia or mature oligodendrocytes [12, 13]. FABP7 KO mice display a consistently lower prepulse inhibition than wild-type (WT) mice, which is one of the endophenotypes of schizophrenia, as well as altered emotional behaviours, including increased anxiety and hyperlocomotion [20]. Previous studies have shown that FABP7 expression is significantly altered in the brains of patients suffering from multiple sclerosis [21] and schizophrenia [22]. We recently found that FABP7 governs lipid raft function in astrocytes through regulation of caveolin-1 expression, and FABP7 deficiency results in an altered response of astrocytes to external stimuli [23]. Our subsequent study highlighted glial FABP7 as a critical regulator of astrocyte proliferation involved in the control of neuronal synaptic plasticity and leptin sensitivity [13, 24–26]. These outcomes have presented FABP7 as an important biological watchdog for glial cells, although several aspects of this involvement remain unknown.

Lipid droplets (LDs) are cytoplasmic lipid storage organelles made of a neutral lipid core, mainly triglycerides and esterified cholesterol, surrounded by a monolayer of polar lipids [27]. These stored lipids can be used in times of need to generate energy, membrane components and signalling lipids. Studies in cultured cells have suggested a potential role of FABPs in fatty acid storage (as LDs) as well as cholesterol and phospholipid metabolism [28, 29]. Astrocytes are the most abundant and diverse glial cells in the brain, synthesize and metabolize lipids [30], positioning them as major players in the regulation of lipid sensing and metabolism in the CNS. Recent study suggests that glial LD formation may take place through lactate-pyruvate-acetyl CoA-fatty acid pathway via apolipoproteins [31]. In addition to that FABP7 has been observed to be involved in higher LD accumulation in glioblastoma multiforme cells [28]. Genetic variants of FABP7 show significant associations with neuropsychiatric diseases [32]. Therefore, understanding the mechanisms controlling LD homeostasis in FABP7-deficient astrocytes is essential to determine the role of LDs in the onset and progression of a variety of neurological conditions.

Oxidative stress has been suggested as one of the potential common etiologies of various neurodegenerative diseases including AD, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD), glaucoma and multiple sclerosis (MS) which are characterized by the progressive loss of neurons, causing irreversible damage to patients [33]. In addition, brain cancers such as astrocytoma and glioma multiforme (GBM) are hypoxic cancers where oxidative stress increases cell vulnerability. Furthermore, astrocyte defects could be triggered by the oxidative stress that occurs during physiological ageing. Recent evidence has highlighted that intracellular or mitochondrial reactive oxygen species (ROS) at physiological levels in astrocytes can cause hippocampal (neuronal) dysfunction [34]. The vulnerability of cultured astrocytes is markedly increased by hypoxic conditions relevant to ischaemic injury [35]. Damage to astrocytes could therefore be deleterious to neuronal function. However, the pathophysiological function of FABP7 in conserving the integrity of astrocytes under ROS stress remains largely unknown.

In this study, we explored the role of FABP7 in the protection of primary mouse astrocytes under ROS stress using FABP7 KO mice and U87 human glioma cell line. We revealed that FABP7 in astrocytes and U87 cells is involved in the protection of astrocytes under both normoxic and hypoxic ROS stress through LD accumulation, thereby modulating antioxidant defence mechanisms and the apoptotic signalling cascade.

Materials and Methods

Reagents and Antibodies

The following reagents were purchased from the indicated sources: Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, USA), foetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA), Hanks' balanced salt solution (HBSS, Wako, Japan), penicillin/streptomycin (Gibco, Life Technologies, USA), L-glutamine (Gibco, Life Technologies), trypsin (Gibco, Life Technologies), phosphate-buffered saline (PBS, Wako, Japan), cell count reagent SF (Nacalai Tesque, Japan), lactate dehydrogenase (LDH) cytotoxicity detection kit (Takara, Japan), Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA), 4,6-diamidino-2-phenylindole (DAPI, Invitrogen), dihydroethidium (DHE, Thermo Fisher Scientific), MitoSOX Red mitochondrial superoxide indicator (Molecular Probe, Invitrogen) and BODIPY 493/503 (Thermo Fisher Scientific, MA, USA). Rabbit polyclonal anti-mouse FABP7 and 5 antibodies were established in our laboratory [36, 37]. Guinea pig polyclonal anti-mouse adipophilin/adipose differentiation-related peptide (ADRP)/perilipin 2 (PLIN2; Progen Biotechnik GmbH, Germany),

rat anti-mouse glial fibrillary acidic protein (GFAP; Invitrogen) and rabbit anti-mouse peroxiredoxin (PRX) pathway (thioredoxin [TRX], thioredoxin reductase 1 (TRXRD1), PRX1) antibodies, as well as antibodies targeting p38 mitogen-activated protein kinase (MAPK), phosphorylated (phospho-)p38 MAPK (Thr180/Tyr182), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), phospho-SAPK/JNK (Thr183/Tyr185) and cleaved caspase 3 (Asp175), were purchased from Cell Signaling Technology (MA, USA); rabbit anti-mouse hypoxia-inducible factor (HIF)-1α antibodies were from Novas Biological, USA, and anti-β-actin antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA. The western blot cocktail was from Abcam.

Primary Cells and Cell Line

The generation of FABP7 gene KO mice was described previously (Owada et al., 2006). C57BL/6 WT and FABP7 KO primary mouse astrocytes of the same genetic background (FABP7 KO) were used in this study. All experimental protocols were reviewed by the Ethics Committee for Animal Experimentation of Tohoku University Graduate School of Medicine and carried out according to the Guidelines for Animal Experimentation of the Tohoku University Graduate School of Medicine and under the law and notification requirements of the Japanese government. U87 human glioma cell line (HTB-14; American Type Culture Collection) was cultured at 37 °C in a 5% CO_2 atmosphere in DMEM high-glucose medium supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine. The medium was changed every other day.

Vector and Transfection

The coding region of human FABP7 was amplified by PCR, and amplified cDNA was subcloned into the mammalian expression vector. The constructed vectors were transfected into U87 cells using Lipofectamine® 2000 following the manufacturer's instructions. FABP7 overexpression was confirmed by western blotting after 48 h of transfection. ROS measurement was performed after 24 h of ROS induction.

Purified Astrocyte Cultures

Primary astrocytes were prepared from the cerebral cortex of 0- to 1-day-old WT and FABP7 KO mice as described previously with slight modifications [23]. In brief, following the isolation of cortices and removal of the meninges, olfactory bulb and hippocampus, dissociated cells were treated with 2.5% *w/v* of trypsin for 10 min. The cells were resuspended in DMEM containing 10% *v/v* of heat-inactivated FBS, and 1% *v/v* of penicillin/streptomycin and filtered through a 100-mm cell strainer (BD Falcon, NJ). Finally, the cells were seeded in T75 flasks (BD Falcon) at a density of 2×10^7 cells. The medium was replaced every third day. After 7–9 days in vitro, flasks were shaken for 24 h at 200 rpm to remove microglia and OPCs. The remaining astrocytes on the adherent monolayer were detached with 0.05% *w/v* of trypsin and 0.02% *w/v* of EDTA (Sigma-Aldrich, Japan), seeded onto appropriate plates and dishes and grown for 6–7 days until confluent under normal O_2 (20%) and 5% CO_2 conditions. The purity of astrocytes was confirmed to be > 95% by GFAP immunostaining. Primary cultured astrocytes for both genotypes were isolated, passaged and analysed at the same time to minimize bias.

ROS Induction

After passage, astrocyte cells were seeded in a multi-well plate/chamber slide following the protocol described earlier [38] with modification. For ROS measurement, the dihydroethidium (DHE) and mitosox red (MitoSOX) chemical probes were used; cells were seeded at 5×10^3 cells/well in 96-well plates, while an 8-chamber glass slide (LabTek) was used to microphotograph ROS staining using DHE/MitoSOX red. After reaching 60–70% confluence, normal high-glucose medium was changed to glucose-deprived medium [39]. The plates were transferred to either normoxic (20% O_2) or hypoxic (1% O_2) chambers [28] after the medium was replaced with conditioned medium. Here, glucose-deprived normoxic conditions were considered a single ROS stress, while glucose-deprived hypoxic conditions were considered a double ROS stress. ROS production was monitored by measuring the excitation/emission fluorescence at 518 nm/605 nm for DHE and 510 nm/595 nm for MitoSox red at 24, 48 and 72 h. The cells were stained after 72 h of incubation, followed by washing with Dulbecco's PBS (D-PBS) and counterstaining with Hoechst for nuclear staining. A rescue experiment was also performed under the same experimental conditions using N-acetyl cysteine (NAC, Sigma-Aldrich) as the ROS scavenging molecule at a 20-mM concentration.

Cell Viability and Apoptosis Assay

To evaluate the number of viable cells, the cell count reagent SF (Nacalai Tesque) was used. Briefly, cells were plated into 96-well plates in 100 mL DMEM at a concentration of 5×10^4 cells/ml. After reaching 60–70% confluence, normal glucose-enriched medium was changed to glucose-deprived medium [39]. The plates were transferred to either normoxic (20% O_2) or hypoxic (1% O_2) incubation [28]. Viability of primary astrocyte cells was checked after the 3rd, 5th and 7th day of incubation under experimental conditions by the addition of cell count reagent SF following the manufacturer's guidelines, and the absorbance was recorded at 450 nm. On the other hand, 24 h ROS stress was used for U87 glioma cell line.

Apoptotic activity was examined by western blotting for phospho-p38 MAPK, phospho-SAPK/JNK and cleaved caspase 3 activity.

Cell Cytotoxicity

To evaluate the cell cytotoxicity level after ROS induction, an LDH cytotoxicity detection kit (Takara, Japan) was used following the manufacturer's guidelines under the same experimental conditions of ROS induction and viability measurement. Cell cytotoxicity was measured on the 3rd, 5th and 7th days of ROS induction at 490 nm.

Western Blotting

Cell lysates were prepared in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The lysates were resolved on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Merck Millipore). The membrane was blocked using either 5% skim milk or 5% BSA in TBST (Tris buffered saline, Tween 20) according to the manufacturer's protocol and incubated with a primary antibody overnight at 4 °C followed by incubation with the secondary antibody. The detection was performed with the ECL Western Blot Detection Kit (ChemiDoc imaging system, BioRad, USA). β-actin was used as an internal control in all cases. All the band intensities were measured using ImageJ plugin software (NIH, USA), and the values were normalized to the respective control.

Immunofluorescence

Culture dishes (astrocyte/U87 cells) containing cells were washed with D-PBS (−) twice and fixed with 4% *w*/*v* of paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Fixed cells were permeabilized with 0.1% *v*/*v* of Triton X-100 in PBS and blocked with 5% *v*/*v* of goat serum in PBS. The reaction with primary antibodies (GFAP, FABP7) was performed overnight at 4 °C, and the reaction with secondary antibodies and DAPI was performed for 1 h at room temperature. Finally, the cells were incubated with 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) 493/503 in PBS for 15 min. The cells were microphotographed by confocal scanning laser microscopy (Zeiss LSM780 META, Carl Zeiss, Germany).

Statistical Analysis

Values are shown as the mean ± SEM of the number of independent experiments indicated ($n = 3$) or as examples of representative experiments performed on at least three separate occasions. Data were analysed using Student's 2-tailed unpaired *t* test (SPSS software version 16.0). p values < 0.05 were considered significant.

Results

Decreased LD Accumulation in FABP7 KO Astrocytes

To assess the role of FABP7 in LD accumulation in astrocytes, we performed BODIPY staining under normoxic (20% O_2) and hypoxic (1% O_2) conditions either in normal glucose- or glucose-deprived medium. Here, normoxic glucose-deprived conditions were considered nutritional ROS stress conditions [39], while hypoxic glucose-deprived conditions were considered double (nutritional and environmental) ROS stress conditions. We examined whether FABP7 depletion (Fig. S1) decreased LD accumulation in astrocytes under ROS stress after 72 h ROS induction (Fig. 1a). LD accumulation was prominently decreased in FABP7 KO astrocytes surrounding the nucleus under ROS stress in both normoxia and hypoxia under glucose-deprived condition. To determine whether the gene expression of other FABPs was altered in astrocytes under normoxic/hypoxic conditions, western blotting was performed for FABP7 and FABP5 [12]; however, FABP7 and FABP5 expression did not change under these experimental conditions (Fig. S3). We also carefully examined morphological alterations under experimental conditions, but no big difference was observed between WT and FABP7 KO astrocytes (Fig. S2). To further confirm LD accumulation in astrocytes, we performed western blotting using the LD-specific antibody targeting adipophilin/ADRP/PLIN2. Under ROS stress, LD accumulation was significantly lower in FABP7 KO astrocytes than in WT astrocytes (Fig. 1b). Therefore, FABP7 deficiency may decrease LD accumulation in astrocytes under ROS stress.

Increased ROS Generation Under Stress in FABP7 KO Astrocytes

To microphotograph ROS production in astrocytes under either normoxic or hypoxic ROS stress conditions, the cells were cultured in glucose-deprived conditions and incubated in normoxic (20% O_2) or hypoxic (1% O_2) conditions for 3 consecutive days while ROS generation was monitored at 24, 48 and 72 h using the DHE probe and measuring fluorescence at an excitation/emission of 518 nm/605 nm. After 72 h of incubation, the cells were incubated with DHE for 1 h, and the nucleus was counterstained with Hoechst 33342, followed by visualization under a fluorescence microscope LSM780 (Carl Zeiss, Germany). Minimal ROS generation was observed in normoxic conditions in astrocytes. ROS generation was elevated in glucose-deprived conditions (both normoxic/hypoxic) in FABP7 KO astrocytes compared to that in WT astrocytes (Fig. 2a). To quantify the percent change in ROS

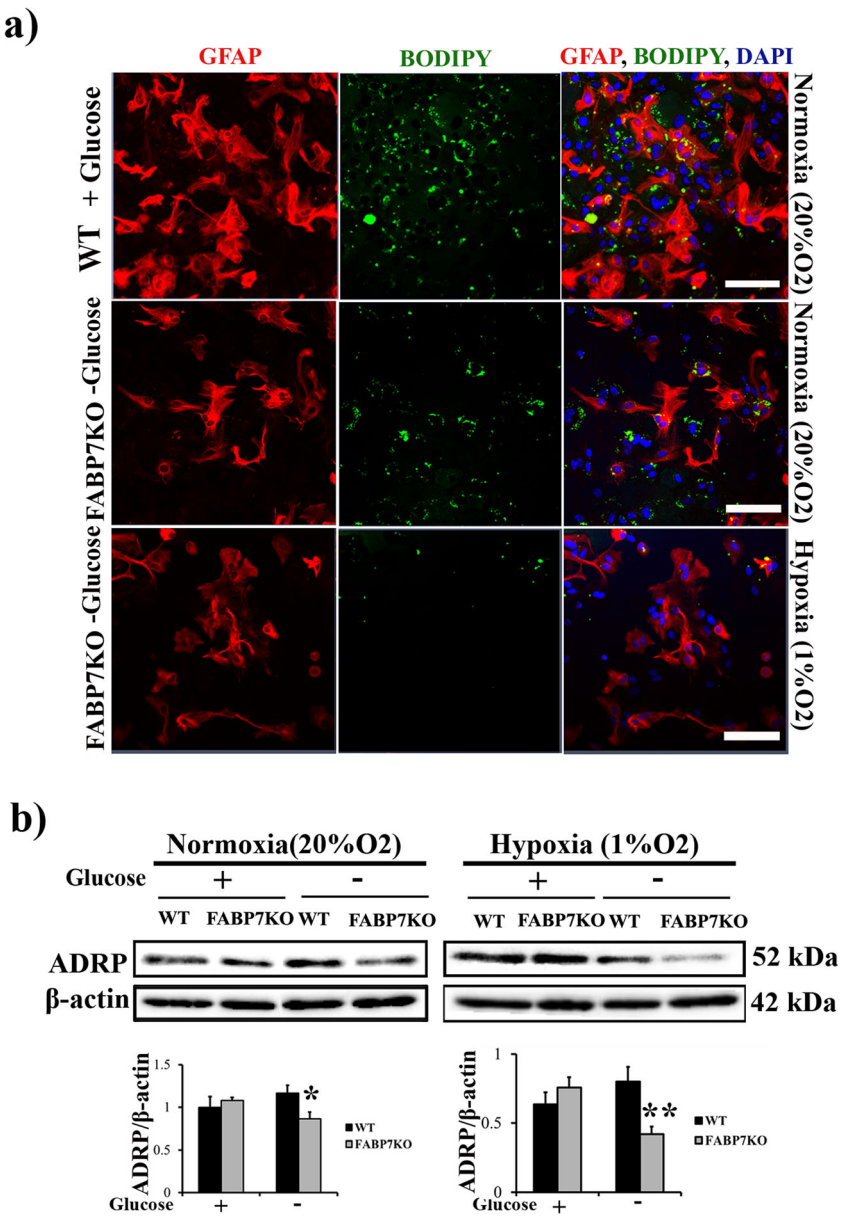


Fig. 1 Effect of ROS stress on LD accumulation in primary astrocytes. **a** Localization of LDs (BODIPY, stained green) was identified in astrocytes, stained red with GFAP either in normoxic (20% O_2) or hypoxic (1% O_2) at 72 h ROS stress conditions. Scale bar 25 μm. **b** Expression of adipophilin/ADRP/PLIN2 (a ubiquitous component of LDs) protein under normoxic and hypoxic ROS stress conditions. The band intensity of ADRP protein was quantified by NIH ImageJ software. The value was normalized to the β-actin expression level. The data shown are the means ± SEM and representative of three independent experiments. *$p < 0.05$; Student's t test compared to WT astrocytes

generation, the fluorescence of FABP7 KO astrocytes was normalized to that of WT astrocytes, revealing that ROS generation was significantly increased in FABP7 KO astrocytes at 24–72 h (Fig. 2b). These results suggest that FABP7 KO astrocytes generate higher ROS than WT astrocytes under ROS stress.

Increased Mitochondrial ROS Generation in FABP7 KO Astrocytes

Mitochondria are the primary sources of energy and ATP for any cell via the electron transport chain, which generates ROS as a natural by-product. We used the MitoSOX™ Red mitochondrial superoxide indicator to stain and image untreated (control) or ROS stress-induced astrocyte cultures with the same experimental settings as the DHE measurements. ROS induction dramatically increased superoxide production (substantial red stain) (Fig. S4a), while the uninduced control showed minimal superoxide production (no positive red stain). Similar to the DHE data, mitochondrial superoxide ROS generation was significantly higher in FABP7 KO astrocytes than in WT astrocytes at 24–72 h (Fig. S4b). These results suggest that mitochondria also contribute to ROS release under ROS stress.

Decreased Cell Viability and Increased LDH Toxicity in FABP7 KO Astrocytes Under ROS Stress

To investigate cell viability and toxicity, the cells were incubated under ROS stress for 3–7 days in experimental settings.

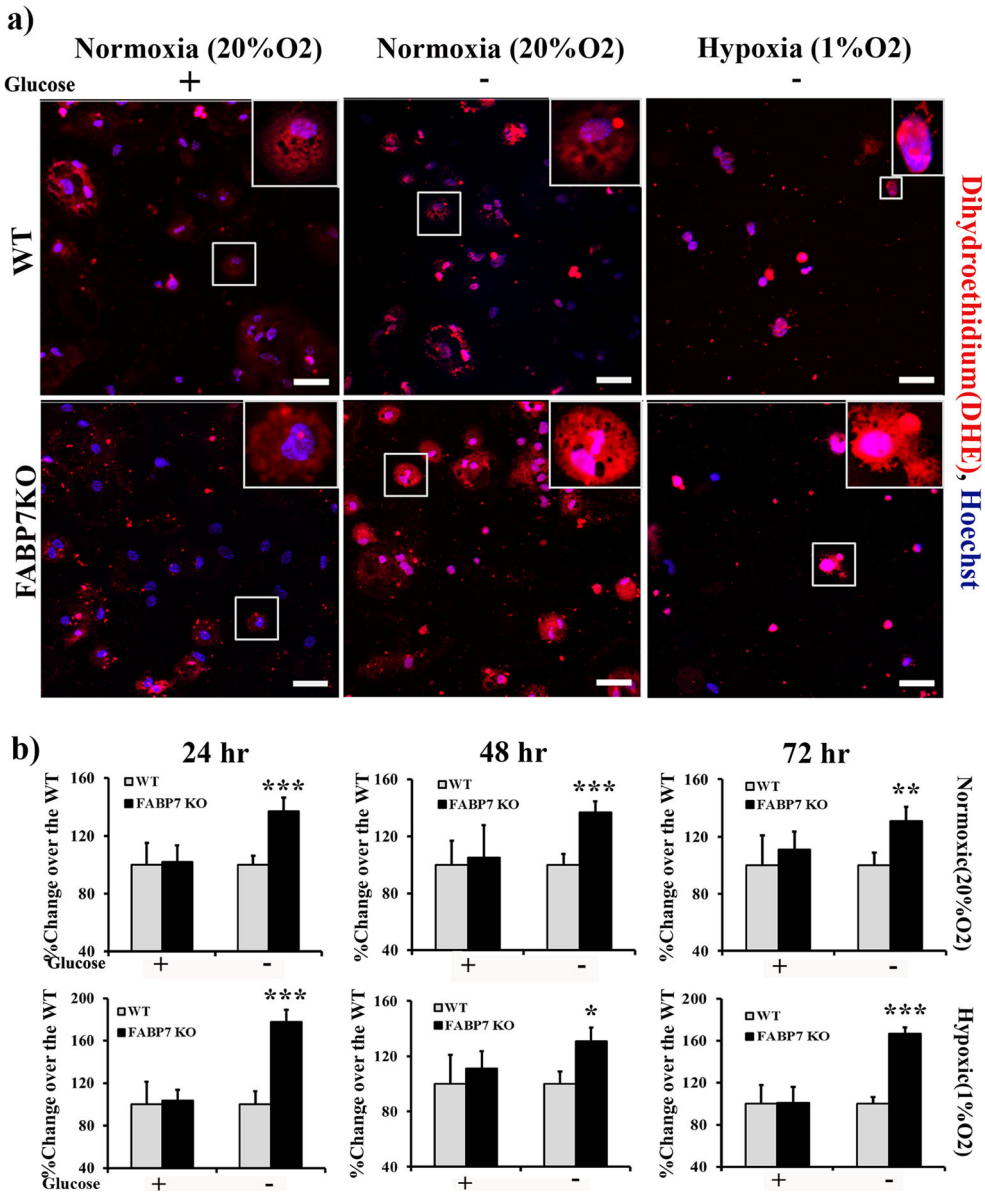


Fig. 2 ROS generation under ROS stress in FABP7 KO astrocytes. **a** ROS production was detected using DHE staining (red) of live astrocytes under ROS stress. The nucleus was counterstained with Hoechst 33342, washed and mounted to be microphotographed under a fluorescence microscope. **b** ROS production from astrocytes measured with DHE (a common superoxide indicator) after 24, 48 and 72 h incubation with conditioned medium. Fluorescence was measured at $Ex^{485\ nm}$ and $Em^{612\ nm}$. The data presented are the mean ± SEM of triplicate experiments using astrocytes derived from different mouse brain specimens. $*p < 0.05$ versus the respective WT control. The data are presented here as % change over the WT control

This experimental paradigm was chosen to obtain clear data without any large loss of astrocytes. After 4 h incubation of astrocyte cells on days 3, 5 and 7 with cell count reagent SF (Nacalai Tesque, Japan), the viable cell count was checked at 450 nm, and the FABP7 KO value was normalized to the respective WT control value. The cell viability data clearly showed decreased cell viability of FABP7 KO astrocytes compared to that of WT astrocytes under ROS stress (Fig. 3a), suggesting that FABP7 deficiency may decrease cell viability under ROS stress.

Disintegration of plasma membrane integrity and enhanced permeability leads to liberation of LDH; therefore, LDH leakage is a marker of cell viability, determined by the LDH toxicity assay. Incubation of primary astrocytes with LDH detection kit for 30 min under the same experimental cell viability paradigm (days 3, 5 and 7) revealed that LDH toxicity was consistently higher in FABP7 KO astrocytes under ROS stress than in WT astrocytes under the same conditions (Fig. 3b), suggesting a role of FABP7 in the protection of astrocytes under ROS stress.

Downregulated Antioxidant Defence Enzyme Expression in FABP7 KO Astrocytes Under ROS Stress

The TRX system plays a major role in cellular redox balance, which is essential for the maintenance of the intracellular redox system. TRX systems achieve their antioxidant function by transferring electrons to the PRXs that then use them to detoxify hydroperoxides in cells [40]. To investigate the possible impact of ROS elevation

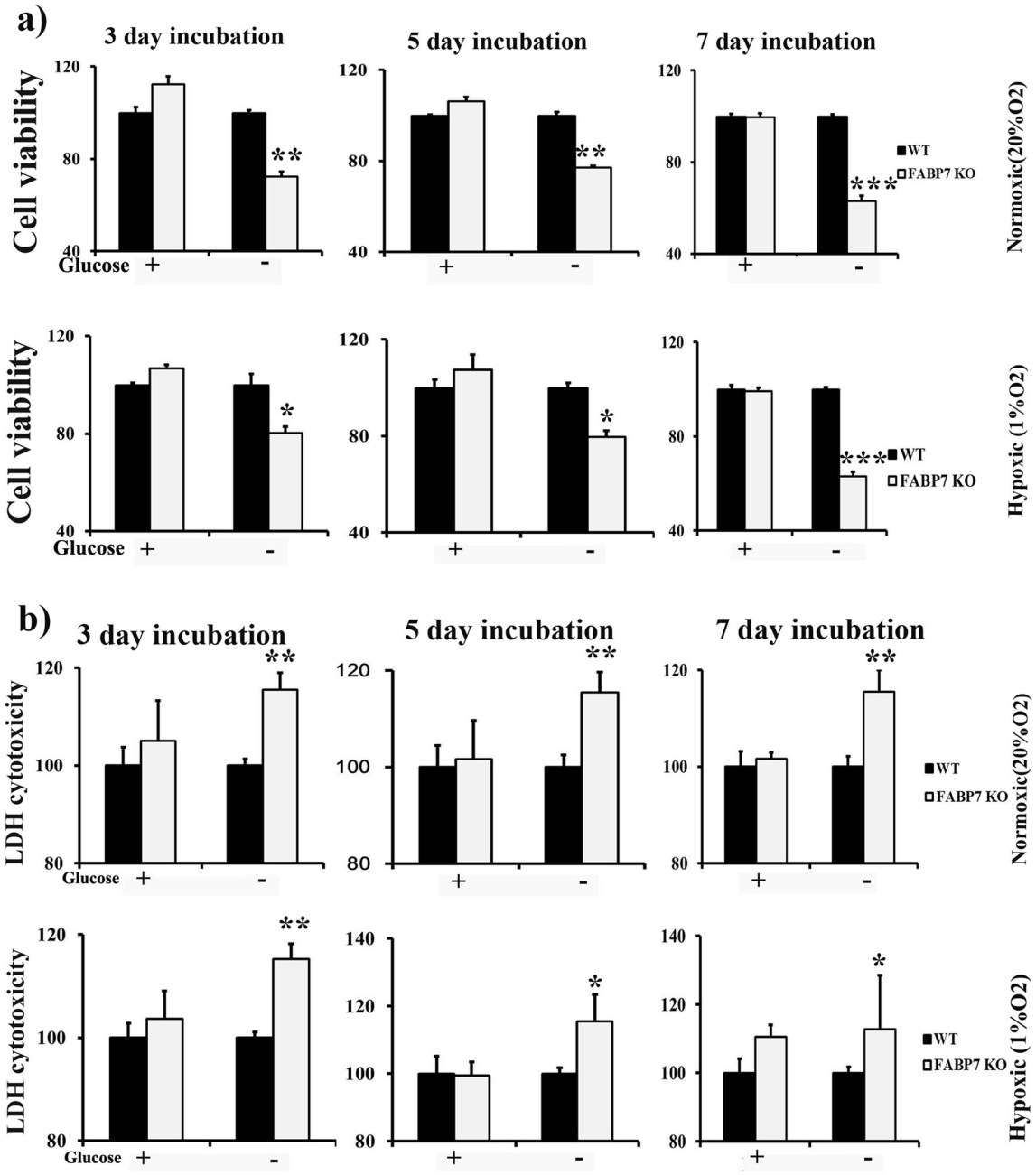


Fig. 3 Cytotoxicity and cell viability of FABP7 KO astrocytes under ROS stress. **a** Cell viability of astrocytes was measured after 4 h of ROS induction at days 3, 5 and 7 by absorbance at 450 nm. **b** LDH cytotoxicity of astrocytes was measured after 30 min of ROS induction by absorbance at 490 nm at days 3, 5 and 7. The data presented are the mean ± SEM of triplicate experiments using astrocytes derived from different mouse brain specimens. $*p < 0.05$ versus the respective WT control. The data presented here as % change over the WT control

in FABP7 KO astrocytes, we examined redox signalling status under experimental conditions. TRX and TRXRD1 signalling was significantly lower in FABP7 KO astrocytes under ROS stress than in WT astrocytes under the same conditions (Fig. 4a and b), suggesting that the TRX redox balance was compromised in FABP7 KO astrocytes under ROS stress, although PRX1 signalling was not affected.

Activation of the Apoptosis Signalling Pathway in FABP7 KO Astrocytes Under ROS Stress

As FABP7 deficiency in astrocytes resulted in decreased expression of antioxidant defence enzymes under ROS stress, we examined the expression of apoptosis signalling molecules (MAPK, c-JNK and cleaved caspase 3), all of which are downstream ROS-induced antioxidant signalling molecules [41].

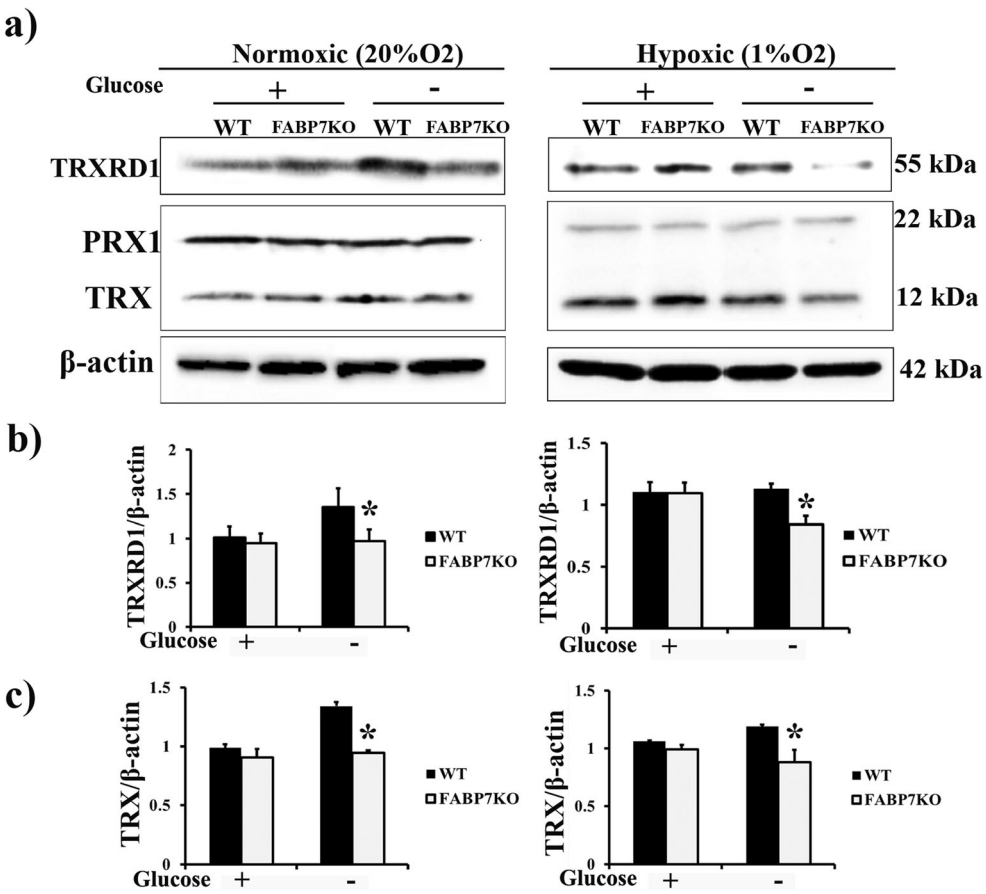


Fig. 4 Antioxidant signalling of FABP7 KO astrocytes under ROS stress. **a** Protein expression level of TRX, PRX and TRXRD1 antioxidant enzymes under conditioned medium (ROS stress) evaluated by western blotting. **b**, **c** The band intensity of TRX and TRXRD1 expression was analysed by NIH ImageJ software, and the value was normalized to the β-actin expression level. The data shown are the means ± SEM and representative of three independent experiments. *$p < 0.05$; Student's t test compared to WT astrocytes

Western blotting with antibodies targeting phospho-MAPK, p38 MAPK and SAPK/JNK (JNK) showed that activation (phosphorylation) of p38 MAPK and JNK was increased after 72 h of ROS induction in FABP7 KO astrocytes compared to that in WT astrocytes (Fig. 5a and b). Consistently, under ROS stress, cleaved caspase 3 activity was significantly increased in FABP7 KO astrocytes (Fig. 5c) compared to that in WT astrocytes, indicating that apoptosis signalling was activated in FABP7 KO astrocytes under ROS stress.

Rescue of ROS Generation in FABP7 KO Astrocytes After NAC Application

NAC is frequently employed as a source of sulfhydryl groups to cells as an acetylated precursor of reduced glutathione. NAC can also interact directly with ROS and nitrogen species because it is a scavenger of oxygen free radicals [42]. To determine whether antioxidant treatment can successfully rescue ROS toxicity, we cotreated astrocytes with NAC under ROS stress ROS generation (Fig. 6a) and LDH toxicity (Fig. 6b) were significantly decreased in the NAC-treated cells; consequently, cell viability (Fig. 6c) was significantly increased, suggesting that ROS generation due to FABP7 deficiency in astrocytes could be successfully restored by ROS scavenger application.

FABP7 Increases LD Accumulation in U87 Cells

Our FABP7 KO astrocyte data presented decreased LD accumulation upon glucose-deprived hypoxia induction, suggesting that FABP7 is involved in LD accumulation. FABP7 has been reported to be involved in fatty acid uptake and LD accumulation in an in vitro model [28]. To further confirm the involvement of FABP7 in LD accumulation in our experimental setting, we transfected the human FABP7 vector into the U87 human glioma cell line. Successful transfection of the human FABP7 vector (Fig. 7a) significantly increased LD accumulation in U87 cells identified by BOIDPY localization (Fig. 7b), as shown by confocal microscopy and confirmed by adipophilin expression (Fig. 7c) under the given experimental settings. Consistent with our FABP7 KO astrocyte data, ROS-induced control (mock) U87 cells displayed much less LD accumulation surrounding the nucleus than did ROS-induced FABP7-overexpressing cells.

FABP7 Elevates Antioxidant Defence Function in U87 Cells

Because a loss of antioxidant defence was observed in FABP7 KO astrocytes after ROS induction, we examined whether cellular redox enzymes might be differentially affected in

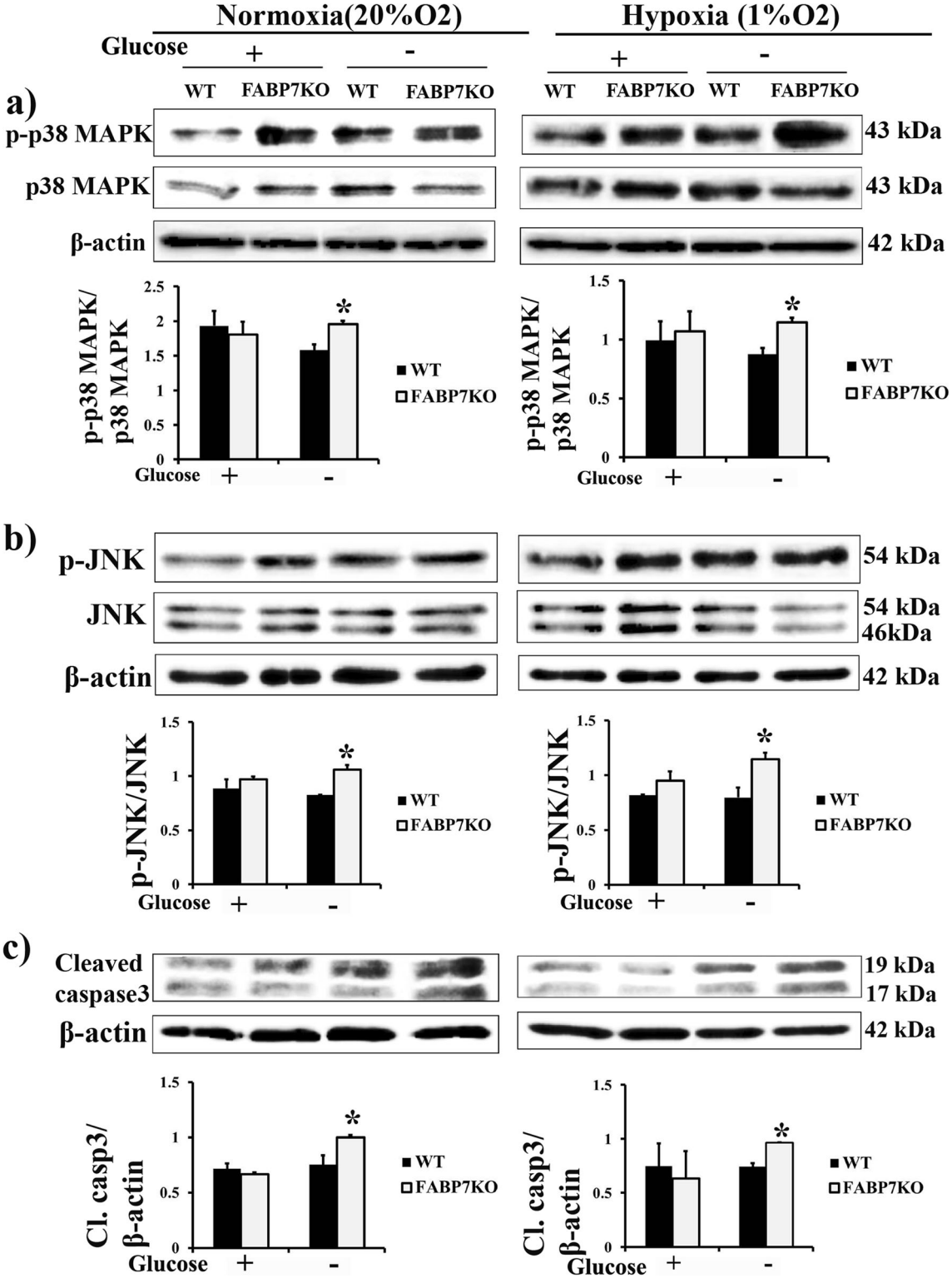
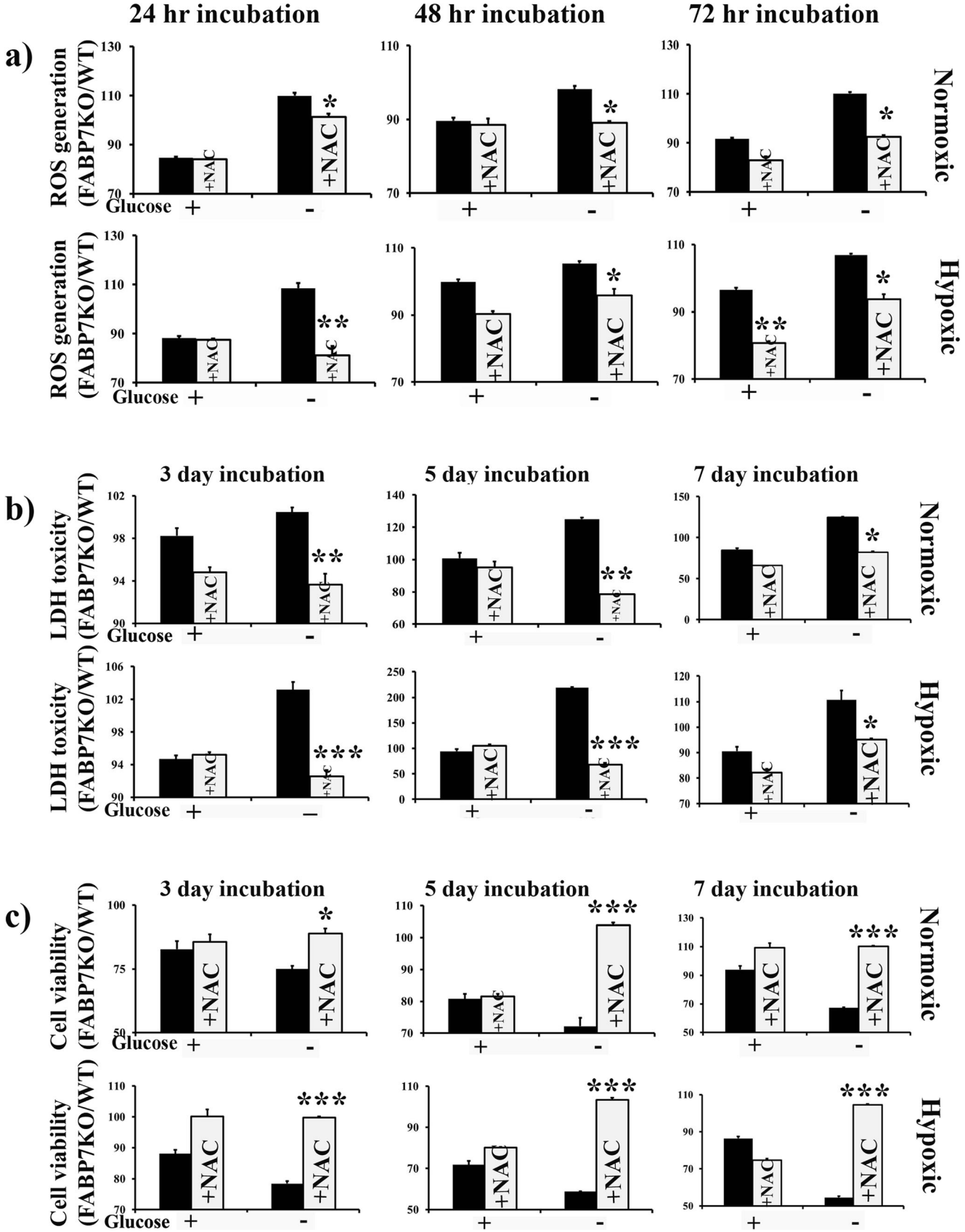


Fig. 5 Apoptosis signalling of FABP7 KO astrocytes under ROS stress. Protein expression level of phospho-p38 MAP kinase (**a**), phospho-SAPK/JNK (**b**) and cleaved caspase 3 (**c**) (apoptosis signalling indicator) under conditioned medium (ROS stress) evaluated by western blotting. Band intensity of phospho-p38 MAPK/p38 MAPK, phospho-JNK/JNK and cleaved caspase 3/β-actin expression was analysed by NIH ImageJ software. The data shown are the means ± SEM and representative of three independent experiments. *$p < 0.05$; Student's t test compared to WT astrocytes


24 hr incubation
48 hr incubation
72 hr incubation
a)
ROS generation (FABP7KO/WT)
Normoxic
Hypoxic
+NAC
Glucose + -
3 day incubation
5 day incubation
7 day incubation
b)
LDH toxicity (FABP7KO/WT)
c)
Cell viability (FABP7KO/WT)

◀ **Fig. 6** Effect of NAC application under ROS stress. **a** ROS generation of FABP7KO compared to WT astrocytes at 24, 48 and 72 h of incubation after NAC application under ROS stress, measured by DHE fluorescence reading. **b** LDH cytotoxicity of FABP7 KO was measured compared to WT astrocytes at 3, 5 and 7 days of incubation under ROS stress with an LDH kit at an absorbance of 490 nm. **c** Cell viability under ROS stress was checked using MTT cell count reagent measuring the absorbance at 450 nm. In all cases, the FABP7 KO/WT reading was compared between the NAC-treated groups and the nontreated group. The data shown are the means ± SEM and representative of three independent experiments. *$p < 0.05$; Student's t test comparing the NAC-treated group and the nontreated group

FABP7-overexpressing U87 cells under ROS stress. Lysates were collected from cells under both normoxic/hypoxic conditions with glucose- or glucose-deprived medium and evaluated for expression of TRXRD1, PRX1 and TRX (Fig. 8). In both normoxic/hypoxic conditions, TRXRD1 expression was elevated after FABP7 transfection in normal glucose conditions. In contrast, FABP7 transfection elevated the level of TRX in glucose-deprived medium in both normoxic and hypoxic conditions. Taken together, these data suggest that

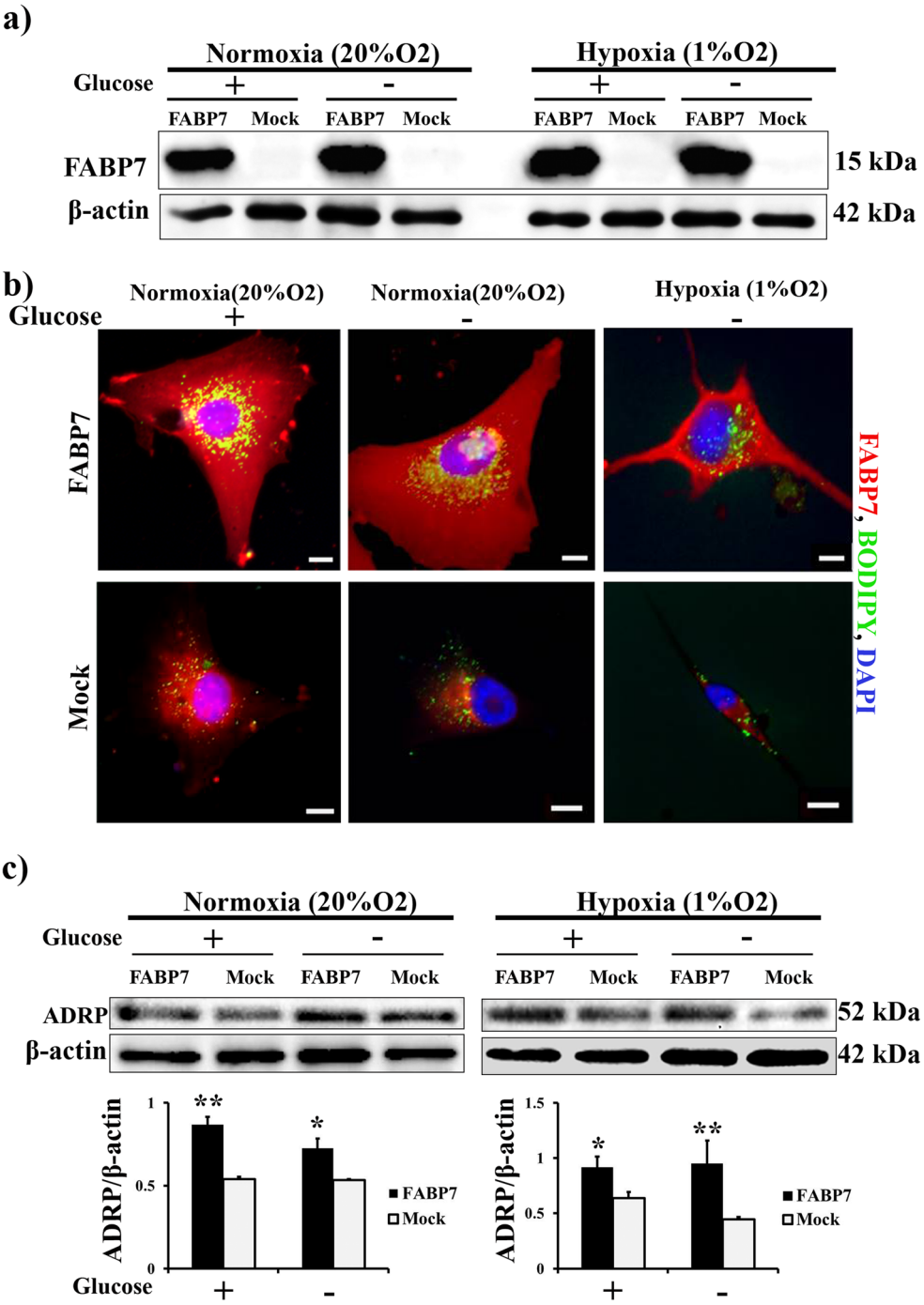


Fig. 7 FABP7 overexpression induces LD accumulation in U87 cells. **a** Overexpression of human FABP7 in U87 cells after 48 h of transfection. **b** Localization of LD (labelled green with BODIPY) and FABP7 (labelled red) in U87 cells under experimental conditions. **c** Expression of adipophilin/ADRP/PLIN2 (a ubiquitous component of LD) protein under normoxic and hypoxic ROS stress conditions for 24 h. The band intensity of ADRP protein was quantified by NIH ImageJ software. The value was normalized to the β-actin expression level. The data shown are the means ± SEM and representative of three independent experiments. *$p < 0.05$; Student's t test

antioxidant defence function was elevated by FABP7 transfection in U87 cells.

FABP7 Overexpression Prevents ROS-Induced Apoptosis in U87 Cells

The role of FABP7 in ROS-induced U87 cells was investigated after 16 h of transfection followed by 48 h of ROS induction. Western blotting was performed to examine the apoptosis-related protein expression of phospho-p38 MAPK (Fig. 9a), phospho-SAPK/JNK (Fig. 9b) and cleaved caspase 3 (Fig. 9c) in U87 cells incubated in normoxic or hypoxic glucose-deprived conditions for 48 h. Interestingly, apoptosis signalling molecules were significantly higher in normoxic/hypoxic, glucose/glucose-deprived conditions in nontransfected U87 cells than in FABP7-overexpressing U87 cells. In other words, FABP7 may protect cells from ROS stress. Consistent with previous observations regarding the protective role of FABP7 in breast cancer [43], we found that FABP7 overexpression suppressed apoptosis signalling in U87 cells, suggesting that overexpression of FABP7 in U87 cells may protect against apoptotic signalling. To determine the possible mechanism for the increase in apoptosis signalling in nontransfected U87 cells, we focused on ROS generation (Fig. S5). The ROS generation studies revealed that nontransfected U87 cells displayed higher ROS generation than FABP7-transfected U87 cells.

Discussion

This study revealed a new role for FABP7 in the protection of astrocytes against oxidative stress induced by glucose deprivation and hypoxia through LD accumulation. We first examined the effect of FABP7 ablation in astrocytes after oxidative stress and found that LD accumulation was significantly decreased, ROS toxicity was increased, antioxidant defence function was downregulated and apoptotic signalling was activated in FABP7 KO astrocytes compared to those in WT astrocytes. Next, we confirmed the involvement of FABP7

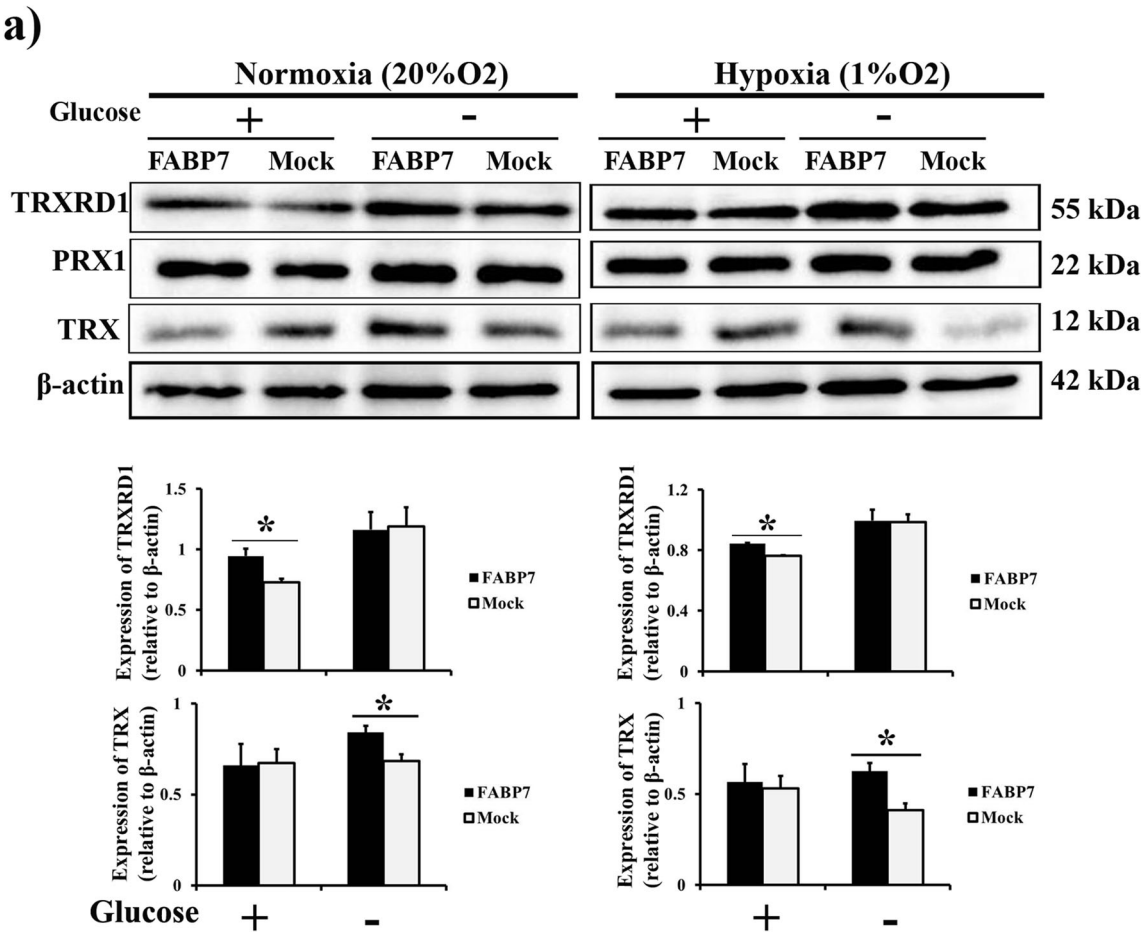


Fig. 8 FABP7 may preserve the antioxidant defence enzymes under ROS stress in U87 cells. **a** Expression of TRXRD1, PRX and TRX in FABP7-overexpressing U87 cells compared to the mock control. The band intensities of TRXRD1, PRX and TRX were quantified using NIH ImageJ software. The value was normalized to the β-actin expression level. The data shown are the means ± SEM and representative of three independent experiments. *$p < 0.05$; Student's t test

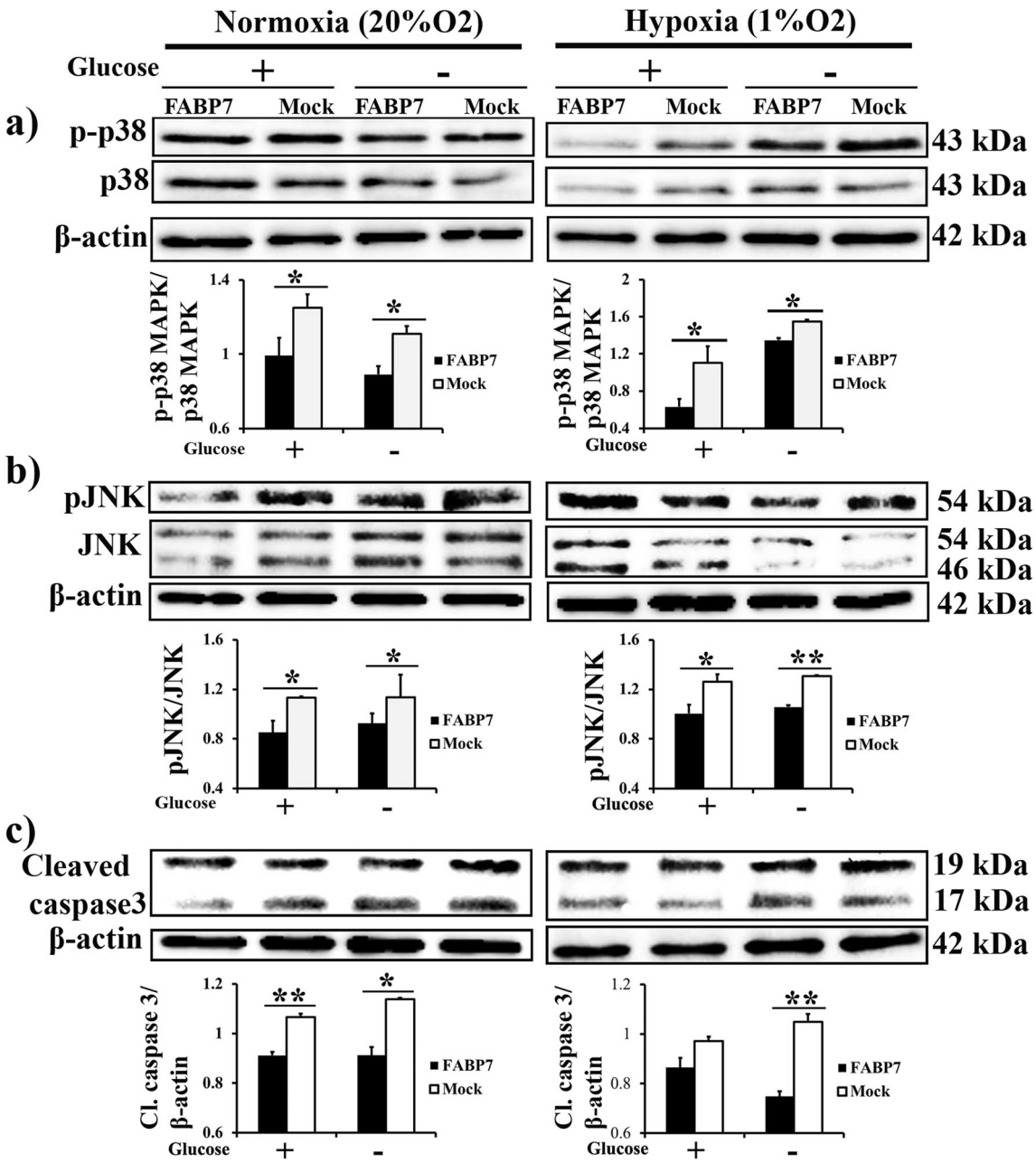


Fig. 9 FABP7 may protect FABP7-overexpressing U87 cells from apoptotic signalling under ROS stress. **a** p38 MAP kinase, **b** SAPK/JNK and **c** cleaved caspase 3 signalling were activated in mock-transfected U87 cells (using empty vector for transfection) under ROS stress, while overexpression of FABP7 protected U87 cells from apoptosis. Detection of β-actin was used to confirm successful subcellular fractionation. The results are representative of at least three independent experiments

in LD accumulation and its defensive role against oxidative stress using mammalian cell line studies. Based on these findings, we provided for the first time a novel role of FABP7 as a safeguard for astrocytes against ROS toxicity and cellular signal transduction (Fig. 10).

ROS produced under normal conditions can be managed, and homeostasis can be maintained without triggering injury to the host. However, ROS can damage cells under conditions such as ageing, inflammation or other pathological circumstances. In the CNS, activated microglial cells, the resident macrophages of the brain, are the main source of phagocytic ROS production [44]. Human astrocyte cell lines have also been shown to produce ROS through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity [45]. Here, we used oxygen glucose deprivation as a well-characterized in vitro model for the induction of astrocyte cell injury through ROS stress. FABP7 is localized in the cytoplasm of astrocytes and is involved in cellular LD accumulation [28]. Under physiological conditions, cellular lipid levels are maintained by a balance between uptake, de novo synthesis, consumption and

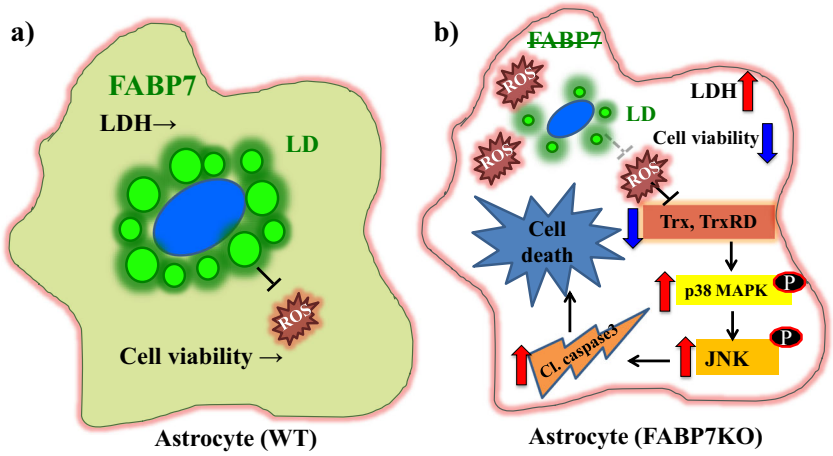


Fig. 10 ROS stress mediates apoptosis in FABP7 KO astrocytes. Schematic diagram of the signalling pathway that underpins ROS-mediated cell-specific apoptosis in a WT and b FABP7 KO astrocytes. In contrast to WT astrocytes **a**, lower lipid droplet accumulation in FABP7KO astrocytes **b** favors the higher ROS generation mediates the oxidation and inactivation of TRX signals, permitting the activation of p38 MAPK and the subsequent phosphorylation of JNK, which successively triggers the cleavage of caspase 3 activity that drives apoptosis

storage to obtain appropriate cellular functioning. LDs are key organelles that function to store the cellular surplus of lipid molecules in an esterified form [27]. Thus, we examined whether ROS stress may affect LD accumulation in FABP7 gene-ablated astrocytes. Interestingly, a significant decrease in LD accumulation was detected in FABP7 KO astrocytes under ROS stress compared to that in WT astrocytes under the same conditions, which was also confirmed by the ubiquitous component of LD PLIN2 (ADRP) protein expression. Several recent studies [46, 47] using a perilipin-deficient model suggested that fasting induces a dramatic decrease in lipid content through lipolysis. Taken together, the previous studies and the present findings of decreased LD accumulation in FABP7 KO astrocytes can be explained by increased lipolysis under glucose-deprived ROS stress. Indeed, human FABP7 overexpression significantly increased LD formation in the U87 GBM cell line.

Previous studies have suggested a possible link between lipolysis and ROS generation [48]. The addition of exogenous free fatty acids has also been shown to increase ROS generation in 3T3-L1 adipocytes [49]. In our study, to examine the role of FABP7 in astrocytes, we used isolated primary astrocytes (relatively immature astrocytes), which differ from mature (differentiated) astrocytes in PUFA composition [50] and response to external stimuli [38]. We observed that ROS induction significantly increased ROS generation in FABP7 KO astrocytes compared to that in WT astrocytes. Indeed, we confirmed hypoxia induction (Fig. S3) and unaltered FABP expression in FABP7 KO astrocytes. Furthermore, we revealed that in addition to common ROS generation, mitochondrial superoxide generation (Fig. S4) was also significantly elevated under ROS stress in FABP7 KO astrocytes. FABP7 transfection was also confirmed to decrease ROS generation in U87 cells (Fig. S5). Interestingly, PLIN5-deficient mice, which exhibit a significant decrease in LD accumulation, display increased ROS generation [51, 52]. Therefore, FABP7 has been suggested to play a protective role against ROS toxicity. Given that the expression of FABP7 is higher in the developing brain than in the adult brain [17], the significance of the FABP7-mediated protective function against ROS is highly possible in the proliferation of astrocytes, which is prominent during brain development.

The deleterious effects of excess ROS or oxidative stress eventually lead to cell death. The oxidation of fatty acids consumes significant amounts of oxygen, which can exacerbate astrocyte injury. Previous studies have indicated that excessive fatty acid oxidation elevates oxidative damage [53], which has been implicated in the development of ageing as well as neurodegenerative diseases [54, 55]. We therefore checked the cell viability of astrocytes after ROS induction. In this study, FABP7-deficient astrocytes displayed lower cell viability under ROS stress than did WT astrocytes. Under oxygen- and glucose-deprived conditions, energy metabolism shifts from mitochondrial aerobic oxidation to glycolysis to maintain cell survival [53]. Enhanced glycolysis increases the generation of lactate and decreases the intracellular pH [56]; meanwhile, the enzymes involved in the generation of ROS are unregulated and activated, and the electron transfer chain is switched on. Consistent with the cell viability results, cell LDH toxicity was significantly enhanced in FABP7-deficient astrocytes under ROS stress compared to that in WT astrocytes under the same conditions. Indeed, we confirmed a successful rescue from ROS toxicity after NAC application. Further studies on the phenotypes of FABP7 KO mice in an animal model of neuroinflammatory and neurodegenerative diseases are required to gain a better understanding of the role of astrocytic FABP7 in such diseases.

Moreover, we found that ROS stress elevates ROS toxicity while concurrently downregulating the antioxidant response (TRX and TRXRD1), causing activation of MAPK-JNK-cleaved caspase apoptosis signalling in FABP7-deficient

astrocytes. The generated ROS mediate the oxidation and inactivation of TRX (TRX-1), permitting activation of apoptosis signal-regulating kinase (ASK)-1 and subsequent phosphorylation of MAPK and SAPK/JNK [41] and triggering several pathways, including the cleaved caspase 3-dependent mitochondrial pathway, that drive apoptosis. Consequently, we were able to compensate for FABP7 deficiency using a FABP7 overexpression model in U87 cells and thus confirmed the protective role of FABP7 against ROS stress and apoptosis signalling. Recent studies revealed that glial LDs make an important contribution towards minimizing ROS damage in the CNS by storing linoleic acid and protecting it from peroxidation [57]. In addition, expression of an antioxidant enzyme or knockdown of JNK solely in neurons was able to reduce glial LD accumulation. Saturated and monounsaturated fatty acids are much less vulnerable than PUFAs to peroxidation, but they also accumulate in glial LDs during oxidative stress.

LDs allow cells to safely sequester otherwise toxic lipids. For example, as amphipathic molecules, overabundant fatty acids can severely compromise membrane integrity. Once turned into triglycerides and incorporated into LD, they are relatively inert, stable and harmless [58]. Recent evidence raises the important issue that PUFAs are more efficiently protected from peroxidation in the core of LDs than in membranes [57]. A growing amount of evidence supports the fact that cells protect themselves from fatty acid lipotoxicity by upregulating β-oxidation and neutral lipid storage in LDs [59, 60]. Directing fatty acids to LDs protects against lipid-induced cell damage in different cells and tissues. Interestingly, LDs in glia are smaller on average than those found in the major lipid storage depot of the fat body. Given that their biogenesis in glia requires diacylglycerol acyltransferase 1 (DGAT1), it is interesting that a previous study found that DGAT1 and DGAT2 are involved in the formation of small and large LDs, respectively [61]. Future biophysical investigations will be needed to determine the possible alteration of enzymes involved in glial lipogenesis and lipolysis in FABP7-deficient glial cells.

In summary, our investigations revealed a new molecular role of FABP7 in the protection of astrocytes under ROS through LD formation, explaining how the cellular redox state may affect glial cell cytotoxicity. LDs have been observed in many different cell types challenged with hypoxia, ischaemia or various metabolic imbalances. For example, hypoxia induces LDs in glioblastoma and breast cancer cells via a pathway involving HIF-1α [28]. This hypoxic induction mechanism in cancer cells appears different from the HIF-1α-independent pathway that has been shown in the *Drosophila* neural stem cell niche [57]. Nevertheless, in all contexts, inhibiting LD formation increases ROS toxicity and impairs cell proliferation. Therefore, future studies must determine whether FABP7-mediated LDs play antioxidant roles against neurodegenerative disorders.

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Compliance with Ethical Standards

All experimental procedures involving mice were approved by the Institute of Laboratory Animals of Tohoku University Graduate School of Medicine and carried out according to the Guidelines for Animal Experimentation of the Tohoku University Graduate School of Medicine and according to the laws and notification requirements of Japanese governments.

Conflict of Interest The authors declare that they have no conflict of interest.

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