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β-lactolin, a Monoamine Oxidase B Inhibitory Lactopeptide, Suppresses Reactive Oxygen Species Production in Lipopolysaccharide-Stimulated Astrocytes

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Featured Application: β-lactolin has protective effects on reactive astrocytes and mediates dopamine metabolism; it might thus aid in improving memory.

Abstract: Astrocytes are known to regulate normal brain function. Monoamine oxidase B (MAO-B), an enzyme highly expressed in astrocytes, metabolizes dopamine (DA) and induces reactive oxygen species (ROS) production. We have previously reported that β-lactolin, a whey-derived glycine–threonine–tryptophan–tyrosine tetrapeptide, improves memory impairment in mice by regulating the dopaminergic system; however, the effects of β-lactolin on astrocytes remain unclear. Herein, we investigated the effects of β-lactolin on cultured murine astrocytes. First, we measured intracellular ROS production in lipopolysaccharide-stimulated reactive astrocytes treated with or without β-lactolin, and then determined the role of β-lactolin in DA metabolism in astrocytes by measuring MAO-B enzyme activity and the levels of DA, and its metabolites, in DA-pretreated astrocytes. We found that β-lactolin significantly suppressed ROS production in lipopolysaccharide-stimulated reactive astrocytes ($p = 2.76 \times 10^{-6}$), inhibited MAO-B activity ($p = 2.65 \times 10^{-2}$) and increased intracellular DA levels ($p = 1.08 \times 10^{-3}$), suggesting that β-lactolin could inhibit DA metabolism in astrocytes. These results illustrate the novel protective effects of β-lactolin on reactive astrocytes and suggest their involvement in the memory-improving effects of β-lactolin via the dopaminergic system.

Keywords: β-lactolin; β-lactoglobulin; whey; peptide; astrocytes; reactive oxygen species; monoamine oxidase B; dopamine

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1. Introduction

Astrocytes are the most abundant glial cells with fundamental physiological functions in the brain, such as supporting neurons, modulating synaptic activities, and regulating the clearance of neurotransmitters, including dopamine (DA) [1–3]. Recent studies have indicated that astrocytes play important and complex roles in the central nervous system (CNS); hence, astrocyte dysfunction could contribute to the development of neurodegenerative diseases [4,5]. Reactive astrocytes are induced in response to pathological conditions, such as ischemia, brain injury, infection, and neurodegenerative diseases, such as Alzheimer's (AD), Parkinson's (PD) and Huntington's disease [6]. The induced astrocytes become harmful, as they lose their normal functions, upregulate the expression of inflammatory genes, and produce higher levels of reactive oxygen species (ROS) than astrocytes under resting conditions, causing further neurotoxicity and cell death [6]. Therefore, reactive astrocytes are considered as preventive and therapeutic targets in neurodegenerative diseases.

DA regulates several physiological processes, such as motor control and PD, a well-known neurodegenerative disease, is caused by DA depletion [7]. Moreover, accumulating

evidence has revealed that DA is crucial for cognitive functions. DA is directly associated with frontal cortex-dependent cognitive functions, including attention, executive function, learning and memory processes [8,9]. Several studies have shown that DA depletion and neuronal loss in the brain alter cognitive impairment in mice, indicating that restoring the DA system could contribute to the improvement of impaired cognitive performance [10,11].

The main enzymes responsible for DA metabolism are monoamine oxidase B (MAO-B) and catechol-*O*-methyltransferase (COMT), both of which are highly expressed in astrocytes [12,13]. MAO-B inhibitors are widely used for the treatment of PD, since MAO-B inhibition increases the synaptic DA concentration [7]. Recent studies have demonstrated that some MAO-B inhibitors have preventive and therapeutic effects on the development of AD [14,15]. In contrast, MAO-B is known to be an important source of ROS in astrocytes, because it generates hydrogen peroxide (H_2O_2) during enzymatic reactions [16,17]. Several studies have shown that H_2O_2 production in reactive astrocytes is increased in an MAO-B-dependent manner and an MAO-B inhibitor suppressed ROS production and neuronal loss in transgenic mouse models of AD and PD [14,18,19].

Recently, food-derived MAO-B inhibitory peptides have been identified [20]. β-lactolin, a β-lactopeptide of glycine–threonine–tryptophan–tyrosine (GTWY), is derived from β-lactoglobulin containing tryptophan–tyrosine (WY) sequences, which is abundantly present in fermented dairy products, such as camembert cheese, and in whey proteins digested by certain enzymes. We found that β-lactolin and its core sequence dipeptide, WY, inhibit the enzymatic activity of human recombinant MAO-B [20]. We demonstrated that β-lactolin administration in mice increased DA levels in the hippocampus and improved memory function [20], which was attenuated by knocking down D1-like receptors in the hippocampus [21]. These findings indicated that β-lactolin improves memory function by regulating the dopaminergic system in mice. In addition, β-lactolin prevented neural inflammation in the mouse model of AD [22] and β-lactolin supplementation improved cognitive function in randomized clinical trials [23,24]. However, the detailed mechanisms of β-lactolin, especially its direct effects on astrocytes, remain unclear.

In the present study, we investigated the effects of β-lactolin on cultured murine astrocytes. Lipopolysaccharide (LPS) and interferon gamma (IFN-ϒ) were used to induce reactive astrocytes as previously described [25]. We then evaluated the effects of β-lactolin on DA metabolism in astrocytes by measuring MAO-B enzyme activity and intracellular and extracellular monoamine levels in DA-pretreated astrocytes. This study demonstrated the novel effects of β-lactolin on astrocytes and indicates the involvement of astrocytes in the memory-improving effects of β-lactolin.

2. Materials and Methods

2.1. Cell Culture and Treatment

Murine astrocytes (CRL-2541; ATCC, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries, Beit-Haemek, Israel) and 100 U/mL penicillin–streptomycin (Gibco) at 37 °C under 5% CO_2. The cells were seeded in 6-well plates (IWAKI, Shizuoka, Japan) for reverse transcription quantitative polymerase chain reaction (RT-qPCR) and monoamine analysis or in 10-cm plates (Corning, Corning, NY, USA) for MAO and COMT activity assays at a density of 3.0×10^5 cells/mL and incubated overnight. β-lactolin (GTWY peptide; purity: 98%) was purchased from Bachem (Bubendorf, Switzerland). β-lactolin was dissolved in dimethyl sulfoxide (Wako, Osaka, Japan) and diluted in phosphate-buffered saline (PBS; TaKaRa Bio, Shiga, Japan), such that the final concentration in the culture medium was less than 1%.

2.2. ROS Analysis

Cells were seeded in 96-well plates (PerkinElmer, Waltham, MA, USA) coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) at a density of 1.5×10^4 cells/well and incubated for 8 h. Subsequently, the cells were treated with 0, 10, 50, and 100 ng/mL LPS (L3129; Sigma-Aldrich) dissolved in PBS and 50 U/L IFN-ϒ (R&D systems, Minneapolis, MN, USA) for 24 h. The cells were then treated with 0, 1, 5 and 10 µM β-lactolin for 6 h prior to staining, followed by incubation with 10 µM 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA; Invitrogen, Waltham, MA, USA) and 0.5 µg/mL Hoechst 33,342 (Dojindo, Kumamoto, Japan) in Hanks' balanced salt solution (Gibco) for 30 min at 37 °C. The culture medium was replaced with fresh Hanks' balanced salt solution. Fluorescence was measured using an Operetta CLS™ instrument (Perkin Elmer) at excitation and emission wavelengths of 460–490 nm and 500–550 nm (CM-H2DCFDA), and 355–385 nm and 430–500 nm (Hoechst), respectively. Fluorescence intensity per cell was analyzed using the Harmony 4.9 software (Perkin Elmer) and expressed in relative fluorescence units (RFU).

2.3. MAO Activity Assay

Cells were treated with 0, 1, 5, and 10 µM β-lactolin or 10 µM pargyline hydrochloride (Sigma-Aldrich), a selective MAO-B inhibitor, for 5 h. The cells were washed with PBS and collected by scraping the culture dishes. After centrifugation, mitochondria were isolated using the Mitochondria Isolation Kit for culture cells (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's protocol. Mitochondrial fractions were diluted in PBS and used to determine the MAO-B activity. Total protein concentration of each mitochondrial fraction was measured using the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific). Pierce™ Bovine Serum Albumin Standard Ampules (Thermo Fisher Scientific) were used as standards. MAO-B activity was measured using an MAO Assay Kit (Cell Biolabs, San Diego, CA, USA) as per the manufacturer's protocol. The substrate reacts with MAO-B isolated from the cells and generates H_2O_2, which reacts with the colorimetric probe and produces a red/pink product. The color change was quantified by measuring absorbance at 570 nm using a BioTek Eon microplate spectrophotometer (BioTek, Winooski, VT, USA) and expressed as MAO-B activity.

2.4. Monoamine Analysis

To measure the intracellular and extracellular levels of DA and metabolites in astrocytes, high-performance liquid chromatography (HPLC) analysis was performed as described previously [26]. Briefly, cells were pretreated with 200 µM DA hydrochloride (Sigma-Aldrich) dissolved in PBS for 2 h, followed by treatment with 0 or 10 µM β-lactolin for 3 h. The culture supernatant was diluted in an equal volume of 0.2 M ice-cold perchloric acid (Wako) containing 10 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich). The suspended cells were washed with PBS and homogenized with 0.2 M perchloric acid. After filtration through a 0.45-µm membrane (Merck Millipore, Burlington, MA, USA), the filtrate was used for HPLC analysis using an EICOMPAK SC-5ODS column (2.1 mm ø × 150 mm, 5 µm particle; Eicom, Kyoto, Japan) and a PREPAK column (Eicom) with electrochemical detection. A mobile phase containing 83% 0.1 M acetic acid (Wako) in citric acid (Nacalai Tesque, Kyoto, Japan) buffer (pH 3.5), 17% methanol (Wako), 190 mg/L sodium 1-octanesulfonate (Wako), and 5 mg/L EDTA-2Na (Dojindo) was infused at a flow rate of 0.5 mL/min. For electrochemical detection, the voltage applied was 700 mV against an Ag/AgCl reference electrode.

2.5. RT-qPCR Analysis

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA concentration and purity were assessed using a NanoDrop ND-1000 instrument (Thermo Fisher Scientific). RNA was reverse-transcribed using a SuperScript™ IV First-Strand Synthesis System (Invitrogen) as per the manufacturer's protocol. The cDNA was

then amplified using TB Green Premix Ex Taq II (TaKaRa Bio). The PCR was performed using a LightCycler 480 Instrument (Roche, Basel, Switzerland) under the following conditions: 95 °C for 10 s, followed by 45 cycles of 95 °C for 10 s, 61 °C for 5 s, and 72 °C for 9 s. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase. The primer sequences used for PCR are listed in Table 1.

Table 1. Primer list.

Gene	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (3′-5′)
Gapdh	GTCATCCCAGAGCTGAACGG	ATACTTGGCAGGTTTCTCCAGG
Mao-b	GCCCATTTCCACCAGTATGGA	CTGGGAATCTCTTGGCCCATC
Comt	GGTTGGTTTGAGTTCGTGCAG	TTTGCGTGTTGCTGCACATG

Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Mao-b, monoamine oxidase B; Comt, catechol-*O*-methyltransferase.

2.6. COMT Activity Assay

COMT activity was measured as described previously [27,28]. Briefly, cells were treated with 0 or 10 μM β-lactolin or 100 μM 3,4-dihydroxybenzoic acid (Sigma-Aldrich), a selective COMT inhibitor, for 24 h. The cells were washed with PBS and lysed in NP40 cell lysis buffer (Invitrogen) as per the manufacturer's protocol. After centrifugation, the supernatants were collected. Total protein concentration of each supernatant was adjusted to 200 μg/mL. Esculetin (Sigma-Aldrich) was dissolved in dimethyl sulfoxide and diluted in an aqueous buffer solution [38.7 mM NaH_2PO_4 (Wako), 61.3 mM Na_2HPO_4 (Wako), 5 mM $MgCl_2$ (Wako), 20 mM L-cysteine (Sigma-Aldrich), pH 7.4] at a final concentration of 40 μM in 120 μL of reaction mixture. A 96-well plate was placed on ice, and the cell lysate was added to achieve a final protein concentration of 100 μg/mL. The plate was preincubated in a SpectraMax M3 microplate reader (Molecular Devices, San Jose, CA, USA) preheated at 37 °C for 5 min. The reaction was initiated by the addition of *S*-(5′-adenosyl)-L-methionine (Sigma-Aldrich) at a final concentration of 200 μM. Fluorescence was measured at 3-min intervals for 60 min at 37 °C. The excitation and emission wavelengths were 380 and 460 nm, respectively. The change in the fluorescence intensity caused by enzymatic O-methylation of esculetin to form scopoletin was used to measure the enzyme activity. Additionally, samples without cell lysates were used as controls.

2.7. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using the Student's *t*-test or one-way analysis of variance followed by Bonferroni's post hoc test or Dunnett's test, as described in the figure legends. All statistical analyses were performed using the Ekuseru–Toukei Ver. 7.0 software (Social Survey Research Information, Tokyo, Japan). Values of p less than 0.05 were considered statistically significant.

3. Results

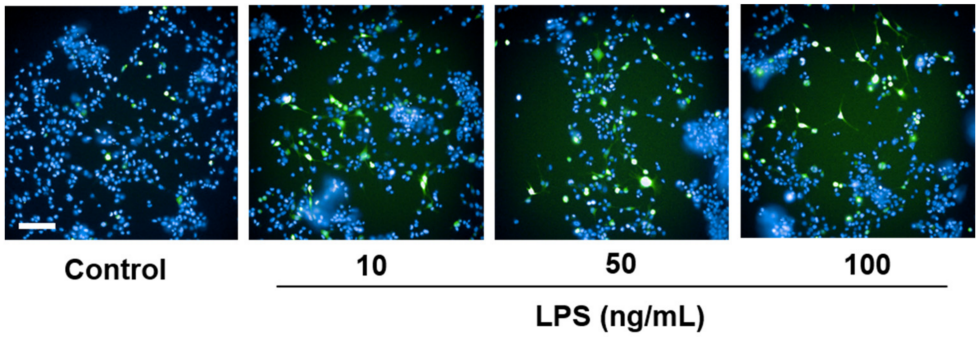
3.1. Effects of β-Lactolin on ROS Production in Astrocytes

To evaluate ROS production in astrocytes, murine astrocytes were treated with LPS and IFN-ϒ. Intracellular ROS levels were determined using the relative fluorescence intensity of CM-H2DCFDA. Referring to data from the literature [25,29], the used concentrations of LPS were as follows: 10, 50, and 100 ng/mL. Treatment with LPS caused a significant increase in the intracellular ROS levels compared to the control (control: 2077 ± 34.94 RFU; LPS 10 ng/mL: 2688 ± 60.58 RFU, $p = 4.30 \times 10^{-6}$; LPS 50 ng/mL: 2652 ± 77.46 RFU, $p = 8.72 \times 10^{-6}$; and LPS 100 ng/mL: 2710 ± 107.3 RFU, $p = 3.41 \times 10^{-6}$; Figure 1A,B). To investigate the effects of β-lactolin on ROS production in astrocytes, cells were treated with β-lactolin for 6 h after LPS stimulation. LPS treatment significantly increased the intracellular ROS levels (control: 3371 ± 38.43 RFU; LPS: 4687 ± 81.89 RFU, $p = 2.76 \times 10^{-6}$), whereas treatment with β-lactolin significantly reduced intracellular ROS levels (β-lactolin 1 μM: 4092 ± 89.52 RFU, $p = 3.35 \times 10^{-6}$; 5 μM: 4098 ± 60.45 RFU, $p = 2.91 \times 10^{-6}$; and 10 μM: 3771 ± 45.65 RFU, $p = 2.76 \times 10^{-6}$; Figure 1C,D). Of note, β-lactolin treatment alone

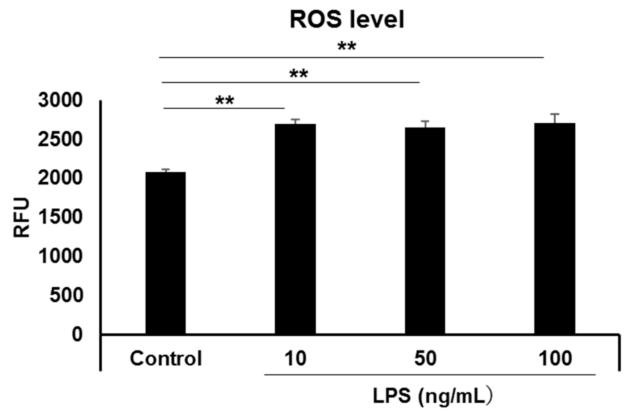
did not induce a significant change in the intracellular ROS levels compared to the control (Supplemental Figure S1A,B). These results indicate that β-lactolin treatment suppresses intracellular ROS production in LPS-treated astrocytes.

3.2. Effect of β-Lactolin on MAO-B in Astrocytes

Next, we evaluated the effect of β-lactolin on MAO-B enzyme activity in astrocytes. MAO-B activity in the astrocyte mitochondria treated with 10 μM β-lactolin was significantly lower than that in the control (100 ± 7.08% vs. 77.3 ± 5.88%, $p = 2.65 \times 10^{-2}$). Additionally, MAO-B activity in mitochondria treated with 10 μM pargyline was significantly lower than that in the control (68.96 ± 0.31%, $p = 3.90 \times 10^{-3}$; Figure 2).



(**A**)



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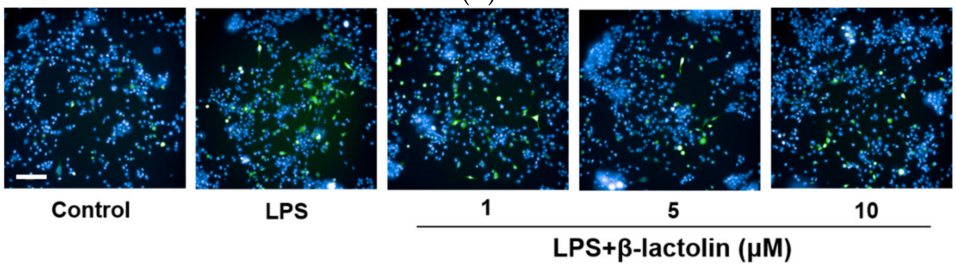
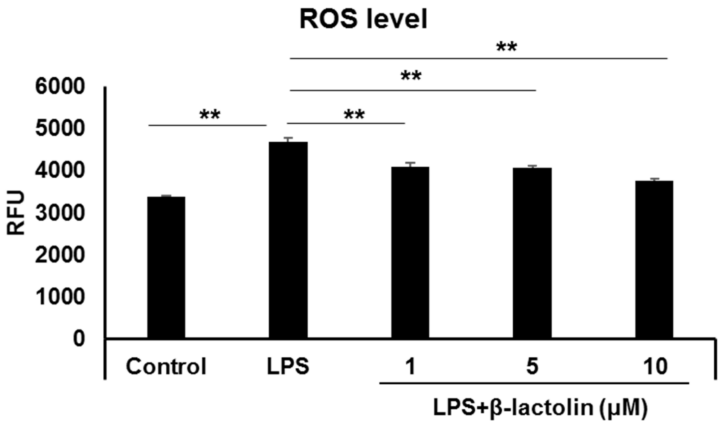


(**C**)

Figure 1. *Cont.*



(**D**)

Figure 1. Reactive oxygen species (ROS) production in astrocytes treated with LPS and β-lactolin. (**A**,**C**) Representative images show intracellular ROS detected using CM-H2DCFDA (green). The nuclei were stained with Hoechst 33,342 (blue). Scale bar is 100 μm at ×20 magnification. (**A**) Murine astrocytes were treated with 0, 10, 50, or 100 ng/mL lipopolysaccharide (LPS) and 50 U/L IFN-ϒ for 24 h. (**C**) Cells were treated with PBS, 10 ng/mL LPS, and 50 U/L IFN-ϒ for 24 h, then treated with 0, 1, 5, or 10 μM β-lactolin for 6 h prior to staining. (**B**,**D**) Quantification of ROS levels by measuring the relative CM-H2DCFDA fluorescence intensity per nucleus. Data represent the mean ± SEM (n = 12). The p values were calculated using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. ** $p < 0.01$. ROS, reactive oxygen species; CM-H2DCFDA, 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester; LPS, lipopolysaccharide; IFN-ϒ, interferon gamma; PBS, phosphate-buffered saline; ANOVA, analysis of variance; SEM, standard error of the mean.

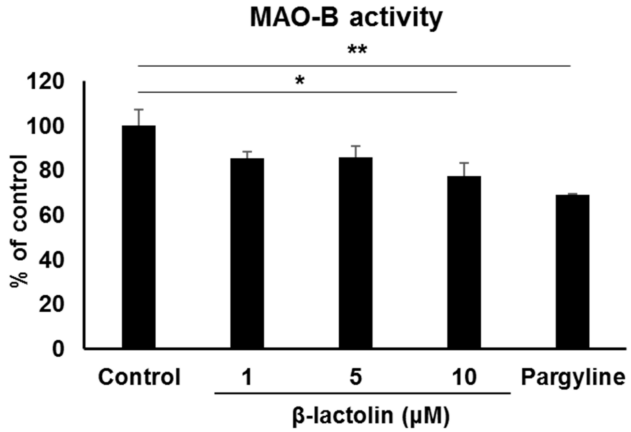


Figure 2. The effect of β-lactolin on MAO-B activity in astrocyte mitochondria. Cells were treated with 0, 1, 5, or 10 μM β-lactolin or 10 μM pargyline for 5 h. MAO activity assay was performed on mitochondria isolated from astrocytes. Pargyline, a selective MAO inhibitor, was used as a positive control. Data represent the mean ± SEM (n = 3). The p values were calculated using one-way ANOVA followed by Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.01$. MAO-B, monoamine oxidase B; ANOVA, analysis of variance; SEM, standard error of the mean.

3.3. Effects of β-Lactolin-Induced MAO-B Inhibition on DA Metabolism in Astrocytes

To evaluate the involvement of β-lactolin in DA metabolism in astrocytes, the levels of DA and its metabolites were measured using an HPLC-electrochemical detection system. Using RT-qPCR, we confirmed the gene expression of the two main enzymes that metabolize DA, *Mao-b* and *Comt*. The relative gene expression levels normalized to *Gapdh* were 1.23 ± 0.206 for *Mao-b* and 1.04 ± 8.4 × 10^{-3} for *Comt* (n = 3). Intracellular DA levels in astrocytes treated with β-lactolin were significantly higher than those in the control

group ($1.97 \times 10^{-2} \pm 8.59 \times 10^{-4}$ vs. $3.10 \times 10^{-2} \pm 4.01 \times 10^{-3}$ ppm, $p = 1.08 \times 10^{-3}$; Figure 3A). Intracellular levels of 3,4-dihydroxyphenylacetic acid (DOPAC) after treatment with β-lactolin were significantly higher than those in the control group ($9.54 \times 10^{-2} \pm 5.01 \times 10^{-3}$ vs. $0.104 \pm 4.14 \times 10^{-3}$ ppm, $p = 1.05 \times 10^{-2}$; Figure 3B). Homovanillic acid (HVA) levels were not changed by β-lactolin treatment (Figure 3C). The turnover of intracellular DA ([DOPAC + HVA]/DA) in β-lactolin-treated cells was significantly lower than that in control cells (5.01 ± 0.171 vs. 3.49 ± 0.443 ppm, $p = 2.27 \times 10^{-4}$; Figure 3D).

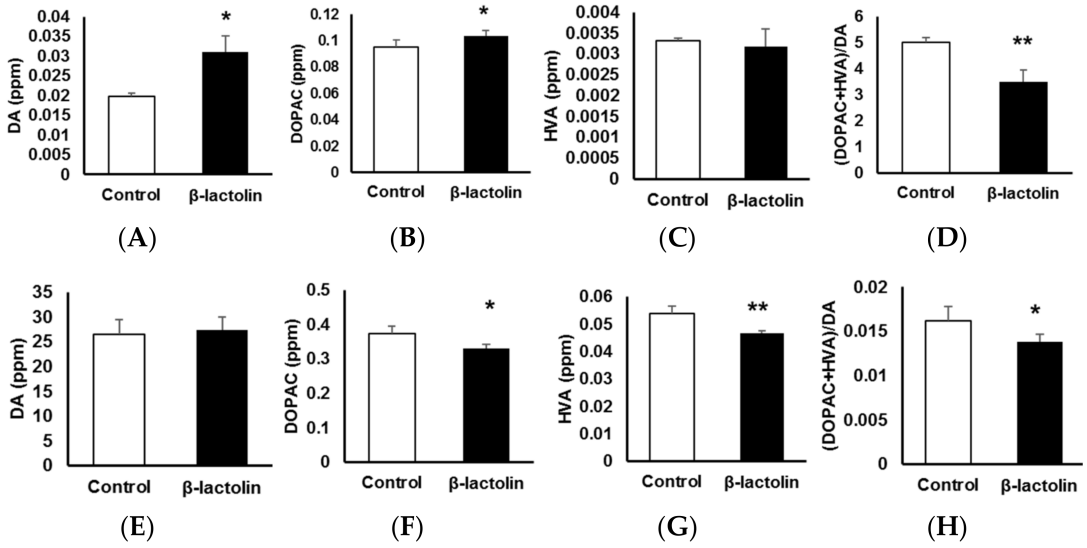


Figure 3. The effects of β-lactolin on dopamine (DA) metabolism in astrocytes. Cells were treated with 0 or 10 μM β-lactolin for 3 h, following treatment with DA for 2 h. The following monoamine levels in the cells (**A**–**D**) and in culture media (**E**–**H**) were measured using high-performance liquid chromatography (HPLC): DA (**A**,**E**), 3,4-dihydroxyphenylacetic acid (DOPAC) (**B**,**F**), and homovanillic acid (HVA) (**C**,**G**). The (DOPAC + HVA)/DA ratio (**D**,**H**) was also determined. Data represent the mean ± SEM ($n = 6$). The p values were calculated using the Student's t-test. * $p < 0.05$, ** $p < 0.01$. DA, dopamine; HPLC, high-performance liquid chromatography; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; SEM, standard error of the mean.

Extracellular DA levels in the culture supernatant of astrocytes were not changed by β-lactolin treatment (Figure 3E). However, extracellular DOPAC levels in the supernatant of cells treated with β-lactolin were significantly lower than those in the control ($0.374 \pm 2.13 \times 10^{-2}$ vs. $0.329 \pm 1.28 \times 10^{-2}$ ppm, $p = 2.20 \times 10^{-3}$; Figure 3F). Furthermore, extracellular HVA levels in the supernatant of cells treated with β-lactolin were significantly lower than those in the control ($5.40 \times 10^{-2} \pm 2.53 \times 10^{-3}$ vs. $4.67 \times 10^{-2} \pm 1.03 \times 10^{-3}$ ppm, $p = 3.12 \times 10^{-4}$; Figure 3G). The turnover of extracellular DA ([DOPAC + HVA]/DA) in the β-lactolin-treated group was significantly lower than that in the control group ($1.62 \times 10^{-2} \pm 1.58 \times 10^{-4}$ vs. $1.38 \times 10^{-2} \pm 8.15 \times 10^{-4}$ ppm, $p = 1.31 \times 10^{-2}$; Figure 3H). Notably, COMT activity was not affected by treatment with β-lactolin but was inhibited by treating the cells with 3,4-dihydroxybenzoic acid ($100 \pm 12.84\%$ vs. $68.70 \pm 13.04\%$, $p = 7.60 \times 10^{-3}$; Figure S2). These results indicate that β-lactolin suppresses DA metabolism in astrocytes.

4. Discussion

Astrocytes perform various crucial functions in the central nervous system (CNS) and reactive astrocytes are considered as therapeutic targets in neurodegenerative diseases. We have previously demonstrated that orally administered β-lactolin is delivered to the brain and improves memory function in in vivo models [20]. However, the effects of β-lactolin on astrocytes have not been investigated. This study showed that β-lactolin suppresses intracellular ROS production in LPS-treated astrocytes. Several studies have shown that excessive ROS production in astrocytes is a characteristic of reactive astrocytes, which

mediates the pathogenesis of neurodegenerative diseases, such as AD and PD [30,31]. The increase in ROS production in astrocytes triggers the release of pro-inflammatory cytokines, leading to neuronal dysfunction in the CNS [32]. H_2O_2, in particular, is an ROS that has a high membrane permeability; thus, its toxicity affects not only the astrocytes producing it but also their neighboring neurons [33]. Previous in vivo studies showed that LPS induces neuronal apoptosis, which is rescued by treatment with some bioactive products that attenuate astrocyte reactivity, including ROS production [34–36]. Similar neuroprotective effects of chemicals and/or bioactive compounds that suppress the reactivity of LPS-treated astrocytes were observed in ex vivo studies conducted on neuron-astrocyte co-culture models or neurons cultured in astrocyte-conditioned medium [37,38]. Hence, the present results indicate potential neuroprotective effects of β-lactolin on astrocytes. Therefore, further studies are warranted to determine the effects of β-lactolin on the crosstalk between LPS-treated astrocytes and neurons.

Next, we evaluated the inhibitory effect of β-lactolin on MAO-B in astrocytes. Consistent with the results of our previous study using human recombinant MAO-B [20], β-lactolin inhibited MAO-B enzyme activity in astrocytes. We assume that β-lactolin directly inhibits the enzyme activity because β-lactolin and WY peptide showed MAO-B inhibitory effects, whereas tryptophan did not in the previous studies [20,39]. Astrocytes highly express MAO-B, generating H_2O_2 as a result of its enzymatic reaction. Accumulating evidence has indicated that astrocytic MAO-B activity could regulate ROS production in astrocytes [18]. Indeed, recent studies have shown that some MAO-B inhibitors suppress intracellular ROS production in astrocytes [14,19]. Moreover, some food constituents that attenuate MAO-B activity showed protective effects by suppressing ROS production in these cells [40–42]. Therefore, the inhibitory effect of β-lactolin on MAO-B might also suppress ROS production. It is notable that some MAO-B inhibitory food constituents have antioxidant activity [40–42]. Given that whey-derived peptides containing WY showed strong antioxidant activity [43,44], the antioxidant activity of β-lactolin could be worth investigating. Furthermore, MAO-B mediates the synthesis of γ-aminobutyric acid (GABA) in astrocytes through the putrescine degradation pathway [45]. Reactive astrocytes produce excess GABA in an MAO-B-dependent manner, leading to impairment in synaptic plasticity and memory function, which is rescued by selective MAO-B inhibitors [45,46]. Therefore, the inhibitory effect of β-lactolin on MAO-B may affect GABA production in astrocytes. Further studies are warranted to investigate this possibility.

Lastly, we demonstrated that β-lactolin suppresses DA metabolism in astrocytes. The results are consistent with our previous report suggesting that β-lactolin improves memory function and depression-like behavior [47] by increasing DA levels in the hippocampus and frontal cortex of mice [20]. It has been reported that DA plays essential roles in cognitive control. Previous studies have demonstrated that DA levels in the prefrontal cortex are associated with cognitive performance, including working memory and attention [8,9]. Moreover, DA modulates synaptic plasticity in the hippocampus and enhances DA binding to DA receptors, thereby improving cognitive performance in mice [48,49]. In fact, the DA precursor levodopa improved the rate of task-based learning and task performances in elderly people [50]. Astrocytes are the main cells involved in DA metabolism in the CNS, as they express high levels of DA-metabolizing enzymes, such as MAO-B and COMT [13]. Moreover, it has been reported that DA metabolism dysfunction in astrocytes of the prefrontal cortex leads to cognitive impairment [51]. This evidence suggests that the suppressive effects of β-lactolin on DA metabolism in astrocytes might contribute to the memory-improving effects of β-lactolin. Notably, even though astrocytes express both MAO-B and COMT, the suppression of DA metabolism by β-lactolin was mainly considered to occur due to its inhibitory effect on MAO-B, since it did not significantly inhibit COMT activity, as demonstrated in this study.

However, there are some limitations to the present study. First, we did not clarify the mechanism underlying the inhibition of ROS production by β-lactolin. A mechanism that might lead to reduced ROS levels is the upregulation of antioxidant activity. Although we

demonstrated that MAO-B activity was suppressed by β-lactolin, we did not evaluate the effect of MAO-B inhibitors on ROS production in this study. Therefore, further studies are warranted to investigate the mechanisms involved in the decrease in ROS production in detail. Next, since we used only cultured astrocytes in this study, the direct effects of β-lactolin on astrocytes in in vivo models have not been evaluated. For instance, it is not clear to what extent the suppressive effects of β-lactolin on DA metabolism contribute to the memory-improving effects of β-lactolin via the dopaminergic system in mice. To evaluate this possibility, astrocyte-specific MAO-B knockdown using adenovirus needs to be performed in future in vivo studies.

In conclusion, this study revealed novel effects of β-lactolin on astrocytes, including its protective effects on astrocytes under pathologic conditions, and suggested the involvement of astrocytes in the memory-improving effects of β-lactolin.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11073034/s1, Figure S1: ROS production in astrocytes treated with β-lactolin, Figure S2: Effect of β-lactolin on COMT activity in astrocytes.

Author Contributions: Conceptualization, Y.A., and S.A.; methodology, S.A.; investigation, S.A., C.T., and R.O.; writing—original draft preparation, S.A.; writing—review and editing, Y.A., and T.A.; visualization, S.A.; supervision, Y.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors (S.A., T.A., C.T., R.O., Y.A.) are employed by Kirin Holdings Company, Limited. All authors declare no other competing interests.

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