

The protective effects of bioactive (*R*₅)-glucoraphanin on the permeability of the mice blood-brain barrier following experimental autoimmune encephalomyelitis

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Abstract. – OBJECTIVES: Alterations in blood-brain barrier (BBB) permeability are due to the disruption of the Tight Junctions (TJs), large multiprotein complexes important for the maintenance of structural integrity and for permeability of the barrier.

In this experimental study we evaluated the neuroprotective role of (*R*₅)-glucoraphanin, a glucosinolate present in *Brassicaceae*, notably in Tuscan black kale, and bioactivated with myrosinase enzyme (bioactive *R*₅-GRA) (10 mg/kg/d intraperitoneally), to prevent the dysfunction of BBB, in an experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS).

MATERIALS AND METHODS: EAE was induced by immunization with myelin oligodendroglial glycoprotein peptide (MOG)₃₅₋₅₅ in mice.

By western blot analysis of brain tissues, we evaluated expression and distribution of the TJ-associated proteins, claudin-1, -3, -5 and ZO-1. Additionally, in order to gain a better insight into the mechanisms of action of bioactive *R*₅-GRA, we investigated Foxp3, ERK1/2 and caspase 3 expression associated both to inflammatory response as well as to apoptotic pathway.

RESULTS: Our results demonstrated that treatment with bioactive *R*₅-GRA counteracts the alteration of all these parameters and preserves TJ integrity through an antiinflammatory and anti-apoptotic activity during MS.

CONCLUSIONS: Bioactive *R*₅-GRA, could be a therapeutic perspective helpful in preventing dysfunction of the BBB.

Key Words:

Blood-brain barrier, (*R*₅)-glucoraphanin, Tight junctions, Multiple sclerosis, Inflammation, Apoptosis.

Introduction

Multiple sclerosis (MS) is a chronic, progressive inflammatory disorder of the central nervous system (CNS). In occidental countries, MS affects about 0.1% of the population and is the first cause of disability of non-traumatic origin in young adults¹.

The exact causes of MS are not yet precisely known, however, it is well established that activation of the adaptive immune system leads to activation and proliferation of T cells that can infiltrate the CNS and release cytokines causing oligodendrocyte damage, demyelination, axonal damage and disruption of the blood-brain barrier (BBB)²⁻⁴.

Under physiological conditions, the BBB maintains the homeostasis of the CNS strictly limiting the passive diffusion of polar substances from the blood to the brain and by restricting immune cell trafficking into the CNS by selective transport of molecules and cells from the systemic compartment⁵. This control is due to the unique structural elements of the microvasculature – endothelial cells of brain capillaries and their accessory structures, including the basement membrane, pericytes, and astrocytic end feet processes.

The intercellular space within tight and impermeable biological barriers is sealed by large molecular complexes called tight junctions (TJs), which form a continuous barrier to fluids across the epithelium and endothelium. They function in regulation of paracellular permeability and in the maintenance of cell polarity, blocking the movement of transmembrane proteins between the apical and basolateral cell surfaces⁶. In the BBB, TJs consist of three principal types of transmem-

brane proteins: occludin, claudins and junctional adhesion molecules (JAMs), involved in intercellular contacts and interactions with cytoplasmic scaffolding proteins such as zonula occludens (ZO) proteins, actin cytoskeleton and associated proteins, such as protein kinases, small GTPases and heterotrimeric G-proteins⁷⁻⁹.

Several studies have shown that the vascular integrity of the BBB plays a significant role in the progression of neurological disorders, like MS. It is well established that cerebral endothelial cells, the junctional complex assembly in BBB, as well as leukocyte recruitment, are essential in the mediation of BBB breakdown.

A wide range of inducers such as inflammatory mediators (TNF- α , IL-1 β IFN- γ), endothelial growth factors (VEGF), enzyme and oxidative stress have been identified in the initiation of BBB breakdown. These act by promoting the disruption of the tight junction assembly, leukocyte recruitment and directly damaging the microvasculature¹⁰⁻¹².

However, currently, effective treatments to counteract these BBB deficits don't exist. It is possible that persistent BBB permeability contributes to progressive demyelination, allowing the entry of infiltrating inflammatory cells or factors such as circulating fibrinogen to the CNS¹³.

Therefore, a potential therapeutic strategy to treat MS could be represented by inhibition of inflammatory cells infiltration into the CNS, enhancing BBB permeability¹⁴.

Because of the complexity MS etiology, a single animal model that exactly mimic the condition and symptoms of MS patients it is hard to develop. Several animal models have been developed in different animal species to focus on different aspects of the disease.

Experimental autoimmune encephalomyelitis (EAE) is the most commonly used model for MS¹⁵. It is induced by immunization of mice with a peptide of twenty-one aminoacids obtained from myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ followed by *Bordetella pertussis* injection. EAE mimics the main features of the disease, including paralysis, weight loss, demyelination, inflammation in the CNS and the breakdown of the BBB. In the EAE model, activated myelin-specific T cells, mainly Th1 and Th17 cells, contribute to the compromise of the BBB and migrate into the CNS. In the CNS, infiltrating and local antigen presenting cells (APCs) present antigens to reactive T cells, leading to further inflammation, demyelination, and axon damage¹⁶.

Convincing evidences, together with the more recent literature, show the beneficial effects of *Brassicaceae* vegetables, such as broccoli, cabbage, cauliflower and Brussels sprouts, etc., due to the dominant presence of glucosinolates (GLs) in this family of vegetables^{17,18}. GLs have been shown to be widely associated with both the inflammatory response and oxidative as well as for apoptotic pathway.

GLs structures share a common core of a β -D-glucopyrano moiety linked via a sulfur atom to a (Z)-N-hydroximiniosulfate ester and a variable aglycon side chain derived from the α -amino acid biosynthetic precursor. Several methionine-derived GLs, which constitute the largest group of GLs, bear in their side chain an additional sulfur atom at different oxidation states (sulfide, sulfoxide, or sulfone functions). Among them, one of the most studied is *R*_S-(-)-glucoraphanin [*R*_S-GRA; 4(*R*_S)-methylsulfinylbutyl glucosinolate] a compound found in *Brassicaceae*, notably in Tuscan black kale. Under neutral pH condition, Myrosinase (Myr) catalyses the GLs hydrolysis (> 99%) producing the corresponding isothiocyanates (ITCs), such as *R*_S-sulphoraphane (*R*_S-SFN) from *R*_S-GRA¹⁹.

The present study aims to investigate the possible neuroprotective role of bioactive *R*_S-GRA in the preventing BBB dysfunction following experimental EAE, through a mechanism that involves a modulation of inflammatory as well as apoptotic pathway and the subsequent physiologic responses.

Materials and Methods

Animals

Male adult C57BL/6 mice (Harlan Nossan, Milan, Italy) 20-25 g weight were used. Mice were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116/92) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986). Experimental procedures did not cause any significant animal suffering.

Reagents

The Myelin Oligodendrocyte Glycoprotein peptide (MOG)₃₅₋₅₅ (MEVGWYRSPFSRVVH-LYRNGK; Auspep, Melbourne, Australia) was synthesized and purified by Cambridge Research Biochemicals (Billingham, UK).

Complete Freund's Adjuvant (CFA) containing *Mycobacterium tuberculosis* (*M. tuberculosis* H37Ra) was purchased from Difco Laboratories (Sparks, MD, USA), while *Bordetella pertussis* (*B. pertussis*) toxin was from Sigma-Aldrich Company Ltd. (Milan, Italy).

Induction of EAE

EAE was actively induced in mice using synthetic (MOG)₃₅₋₅₅ as described previously²⁰. After anesthesia, mice were immunized subcutaneously with 300 µl/flank of the emulsion consisting of 300 µg of (MOG)₃₅₋₅₅ in phosphate-buffered saline (PBS) mixed with an equal volume of CFA containing 300 µg heat-killed *M. tuberculosis* H37Ra. Immediately after (MOG)₃₅₋₅₅ injection, the animals received an intraperitoneal injection of 100 µl *B. pertussis* of toxin (500 ng/100 µl, i.p), repeated 48 h later. The disease follows a course of progressive degeneration, with visible signs of pathology consisting of flaccidity of the tail and loss of motion of the hind legs.

Mice were observed daily for signs of EAE to evaluate the degree of disease in each animal. Clinical signs were scored on a six-point scale: 0 = no signs; 1 = partial flaccid tail; 2 = complete flaccid tail; 3 = hind limb hypotonia; 4 = partial hind limb paralysis; 5 = complete hind limb paralysis; 6 = moribund or dead animal²¹. Animals with a score ≤ 5 were sacrificed to avoid animal suffering.

GLs and Myrosinase Purification, Enzyme Bioactivation of *R*_S-GRA

*R*_S-GRA was isolated according to a procedure developed at CRA-CIN of Bologna (Italian patent attending MI2012A001774)²². Seeds of Tuscan black kale, supplied by SUBA & UNICO (Longiano, Italy), were first ground to a fine powder and defatted in hexane. The solvent was removed and the defatted meal was then treated

with boiling 70% ethanol (1:8 w/v) in order to produce a quick deactivation of the endogenous enzyme Myr and to prevent GL hydrolysis. The solid residue was removed by centrifugation and re-extracted using the same w/v ratio. The two solutions were collected and the isolation of *R*_S-GRA from the extract was conducted by one-step anion exchange chromatography, and the purity was further improved by gel-filtration performed using a XK 26/100 column packed with Sephadex G10 chromatography media (GE Healthcare), connected to an AKTA-FPLC System (GE Healthcare). Individual fractions were analyzed by HPLC and those containing pure *R*_S-GRA were pooled and freeze-dried²³. *R*_S-GRA was characterized by ¹H and ¹³C NMR spectroscopy and the absolute purity estimated by HPLC analysis of the desulfoderivative, according to the ISO 9167-1 method²⁴, was 99% (peak purity HPLC) and > 95% weight basis (hydrated salt containing 1-2 equivalents of water. UV spectra and the molar extinction coefficient value 6634 M⁻¹ cm⁻¹ at 225 nm were determined using a Varian Cary 300 Bio UV/Vis spectrophotometer, Palo Alto, CA, USA).

The enzyme Myr was isolated from seeds of *Sinapis alba* L. as described by Pessina et al.²⁵, with some modification. The specific activity of the stock solution used in the present study was about 60 U/mg of soluble protein. The enzymatic activity was 32 U/ml and the solution was stored at 4°C in sterile saline solution at neutral pH until use. One myrosinase unit was defined as the amount of enzyme able to hydrolyze 1 µmol sinigrin per minute at pH 6.5 and 37°C.

*R*_S-GRA (10 mg/kg) was dissolved in PBS solution pH 7.2 and mouse treatment required the enzyme bioactivation of the phytochemical. The *in situ* action of the myrosinase enzyme (5 µl/mouse) for 15 min allowed to have a bioactive *R*_S-GRA quickly, before the i.p treatment (Figure 1).

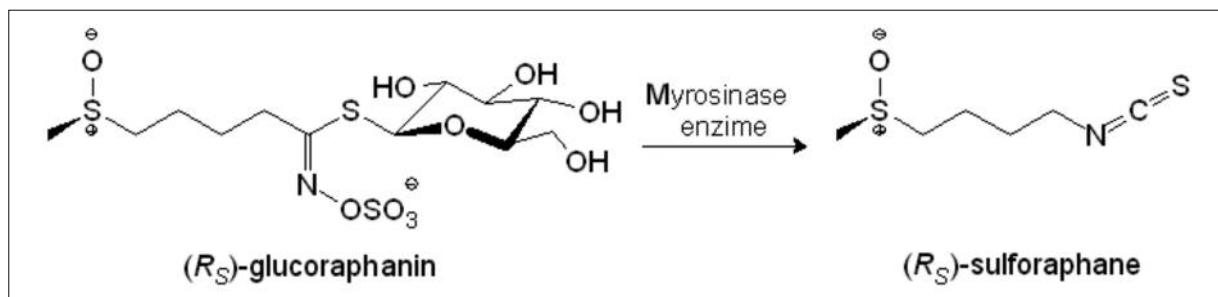


Figure 1. (*R*_S)-glucoraphanin purified from Tuscan black kale seed, bioactivated with myrosinase enzyme: *In situ* release of (*R*_S)-sulforaphane.

Experimental Design

Mice were randomly allocated into the following groups (N = 40 total animals):

- EAE + bioactive *R*_S-GRA group (N = 20): mice subjected to EAE were treated with bioactive *R*_S-GRA (10 mg/kg + 5 µl/mouse Myr, i.p). Bioactive *R*_S-GRA was daily administered 1 week before EAE-induction and, after immunization the treatment was daily protracted until the sacrifice;
- EAE group (N = 20): mice subjected to EAE that didn't receive bioactive *R*_S-GRA.

The experiment provided a housing period of duration of 7 days followed by a period of pre-treatment with bioactive *R*_S-GRA via i.p. injection once a day for 7 days. On the eight day, the disease was induced according to the following experimental procedure. In the experimental group EAE + bioactive *R*_S-GRA, the post-drug treatment was continued for a further 15 days after induction of the disease until the twenty-first day.

At the end of the experiment the animals were sacrificed, and brain tissues were sampled and processed, in order to evaluate the various parameter.

Western Blot Analysis for Claudin-1, Claudin-3, Claudin-5, ZO-1, Phospho-p44/42 MAPK (ERK1/2), FOXP3 and Caspase 3

All the extraction procedures were performed on ice using ice-cold reagents. In brief, brain tissues were suspended in extraction buffer containing 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM NaN₃, 10 mM 2-mercaptoethanol, 50 mM NaF, protease inhibitor tablets (Roche, Basel, Switzerland), and they were homogenized at the highest setting for 2 min. The homogenates were chilled on ice for 15 min and then centrifuged at 1000g for 10 min at 4°C, and the supernatant (cytosol + membrane extract from brain tissue) was collected to evaluate content of claudin-1, claudin-3, claudin-5 and ZO-1.

The pellets were suspended in the complete lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA protease inhibitors tablets (Roche), and then they were centrifuged for 30 min at 15,000g at 4°C, and the supernatant (nuclear extract) was collected to evaluate the content of Phospho-p44/42 MAPK (ERK1/2), FOXP3, ZO-1 and caspase 3.

Supernatants were stored at -80°C until use. Protein concentration in homogenate was estimated by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumine (BSA) as standard, and 50 µg of cytosol and nuclear extract from each sample was analyzed.

Proteins were separated on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) minigels and transferred onto nitrocellulose membranes (Protran nitrocellulose transfer membrane; Whatman Schleicher and Schuell, Dassel, Germany), blocked with PBS containing 5% nonfat dried milk for 45 min at room temperature, and subsequently probed at 4°C overnight with specific antibodies for claudin-1 (1:200 Novus Biologicals, Littleton, CO, USA), claudin-3 (1:200 Novus Biologicals), claudin-5 (1:200 Novus Biologicals), ZO-1 (1:200; Novus Biologicals), Phospho-p44/42 MAPK (ERK1/2) (1:2000; Cell Signaling, Beverly, MA, USA), Foxp3 (1:250; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and caspase 3 (1:1000; Cell Signaling), in 1x PBS, 5% (w/v) non fat dried milk, 0.1% Tween-20 (PMT).

Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody (1:2000; Santa Cruz Biotechnology, Inc), peroxidase-conjugated goat anti-rabbit IgG (1:2000; Santa Cruz Biotechnology, Inc) or peroxidase-conjugated goat anti-guinea pig IgG (1:2000; Santa Cruz Biotechnology, Inc) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of proteic lysates, they were also incubated with antibody for glyceraldehyde-3-phosphate dehydrogenase horse radish peroxidase (GAPDH-HRP) (1:1000; Cell Signaling) and p42 MAP Kinase (Mitogen-Activated Protein Kinase) (ERK2) (1: 1000; Cell Signaling).

The relative expression of the protein bands of claudin-1, claudin-3, claudin-5, ZO-1, ERK1/2, Foxp3 and caspase 3, was visualized using an enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent, Rockford, IL, USA). The protein bands were scanned and quantitated with ChemiDoc™ MP System (Bio-Rad) and a computer program (ImageJ).

Statistical Analysis

Data were analyzed in GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA). The results were statistically analyzed using one-way ANOVA followed by a Bonferroni *post hoc* test

for multiple comparisons. A p value of < 0.05 was considered to be statistically significant. Results are expressed as the mean $\pm$ SEM of n experiments.

Results

Effect of Bioactive R_5 -GRA on Claudin-1, Claudin-3 and Claudin-5 Expression

During EAE, the recruitment of inflammatory cells into CNS parenchyma is accompanied by the breakdown of the BBB. To investigate whether BBB breakdown is also accompanied by the loss or alterations of TJ-associated molecules from the BBB TJs, we investigated the claudin-1, claudin-3 and claudin-5 expression by western blot analysis.

A basal level of claudin-1 (Figure 2A), claudin-3 (Figure 2B) and claudin-5 (Figure 2C) production in brain samples collected from EAE mice 7 days after EAE induction, while brain levels of claudins were increased in animals treated with bioactive R_5 -GRA (Figure 2A, B, C). Visible differences in claudins expression between experimental groups were found and quantified in a graph showing claudin-1,3,5/GAPDH ratio.

Effect of Bioactive R_5 -GRA on ZO-1 Expression

Proteins forming TJs are expressed in endothelial cell of brain vasculature and are crucial component of BBB. Changes in their expression or

localization accompany BBB dysfunction in MS. Specifically, ZO-1 translocation from the membrane to the nucleus during EAE promotes the BBB impairment.

Therefore, we analyzed the expression of ZO-1 by western blot analysis, in both the cytoplasm and nucleus of brain samples collected during EAE, in order to evaluate the BBB integrity following EAE induction.

Obvious differences show a basal level of ZO-1 in the cytoplasm of the brain following EAE (Figure 3A), while ZO-1 expression was increased in animals administered with bioactive R_5 -GRA (Figure 3A). In addition, we also found an increased expression nuclear of ZO-1 in mice subjected to EAE (Figure 3B). However, treatment with bioactive R_5 -GRA prevented the EAE-induced ZO-1 expression in the nucleus of mice (Figure 3B).

Bioactive R_5 -GRA Modulates Production of Treg Cells

Regulatory T (Treg) cells are characterized by the expression of transcription factor Forkhead box P3 (Foxp3). Treg cells play a pivotal role in keeping the inflammatory T cells, e.g. Th1 and Th17, in checking and in maintaining self-tolerance and immune homeostasis. To verify whether the treatment with bioactive R_5 -GRA can modulate the production of Treg cells, we evaluated the expression of the transcription factor Foxp3 by western blot analysis. It was not observed an expression of

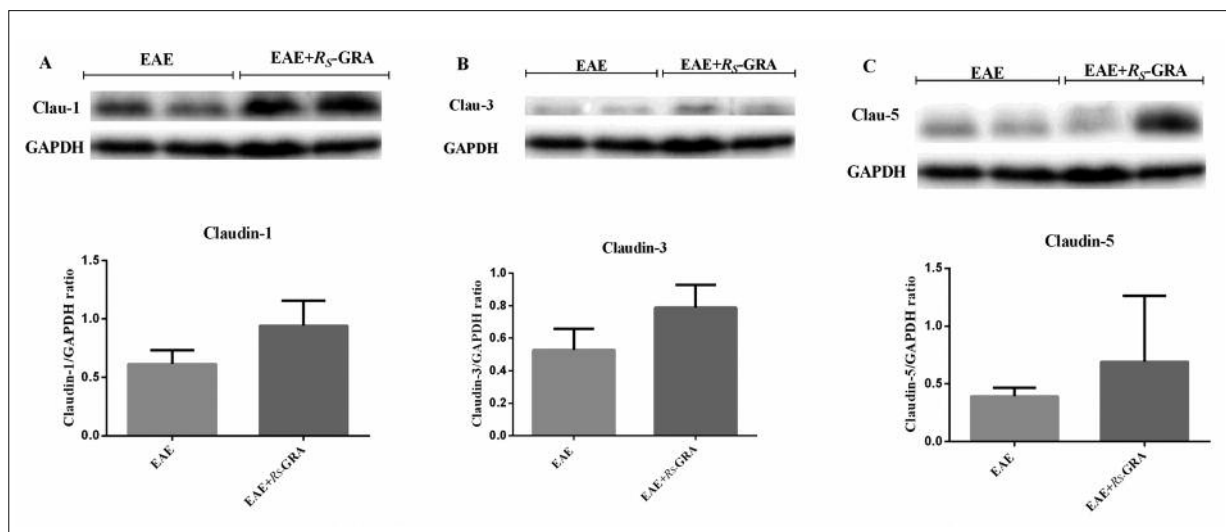


Figure 2. Western blot for claudin-1, claudin-3 and claudin-5. A basal level of claudin-1 (A), claudin-3 (B) and claudin-5 (C) production was found in brain samples collected from EAE mice 7 days after EAE induction, while brain levels of claudins were increased in animals treated with bioactive R_5 -GRA (A, B, C). GAPDH was used as internal control.

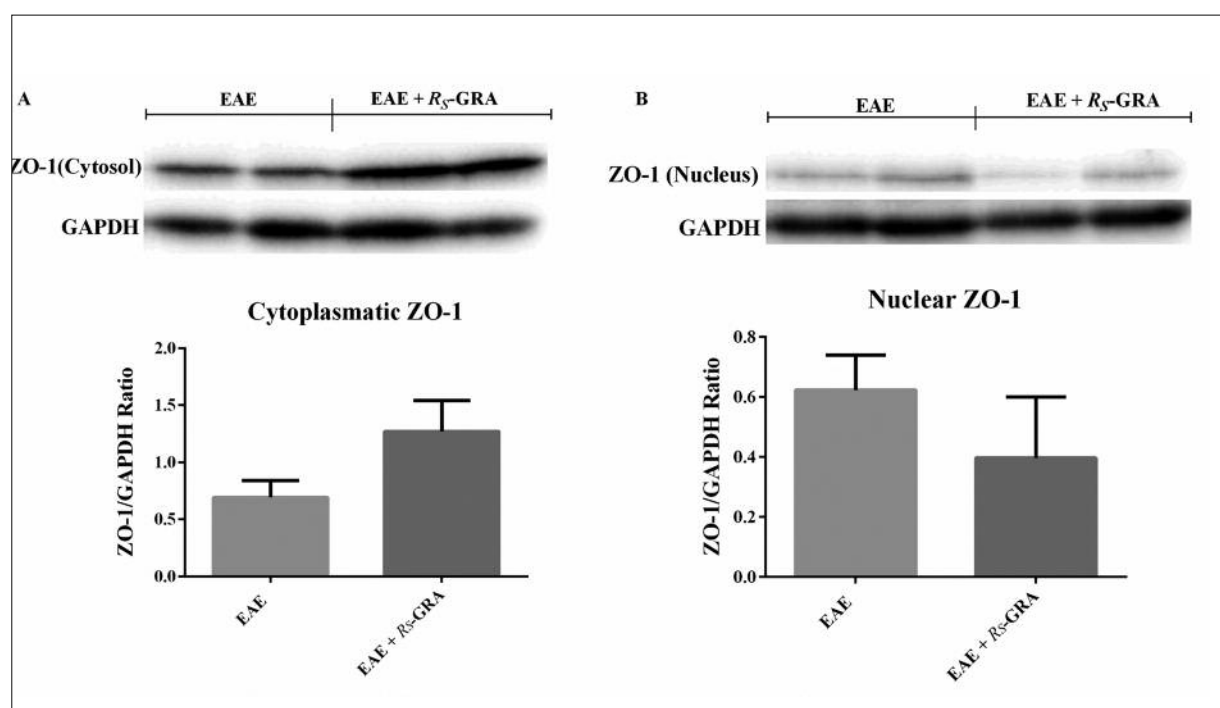


Figure 3. Western blot for ZO-1. A basal level of cytoplasmic ZO-1 was found in brain following EAE (**A**), while ZO-1 expression was increased in animals administered with bioactive *R*_S-GRA (**A**). In addition, a basal level of nuclear ZO-1 was detected in brain samples collected from EAE mice 7 days after EAE (**B**), while brain levels of nuclear ZO-1 were attenuated by administration of bioactive *R*_S-GRA (**B**). GAPDH was used as internal control.

Foxp3 in animals subjected to EAE (Figure 4). While bioactive *R*_S-GRA administration prevents the EAE-induced Foxp3 expression (Figure 4).

Effect of Bioactive *R*_S-GRA on Phospho-p44/42 MAPK (ERK1/2) Expression

To investigate the cellular mechanisms whereby treatment with bioactive *R*_S-GRA attenuates the development of EAE, we also evaluated the level of Phospho-p44/42 MAPK (ERK1/2) which results in expression of pro-inflammatory genes mediating the inflammatory characteristic of EAE. The activation of MAPK pathways in particular the phosphorylation of ERK1/2 expression was investigated by western blot analysis in brain tissue. Obvious differences show that ERK1/2 levels were appreciably increased in brain samples taken from mice subjected to EAE (Figure 5), while the treatment of mice with bioactive *R*_S-GRA reduced levels of ERK1/2 (Figure 5).

Effect of Bioactive *R*_S-GRA on Caspase 3 Expression

By western blot analysis, we evaluated the expression of caspase 3, since, sequential activation

of this protein plays a key role in the execution-phase of cell apoptosis. Caspase 3 levels were appreciably increased in the brain from mice subjected to EAE (Figure 6), conversely, bioactive *R*_S-GRA treatment attenuated the EAE-induced caspase 3 expression (Figure 6).

Visible differences in caspase 3 expression between experimental groups were found and quantified in a graph showing caspase-3/GAPDH ratio.

Discussion

Established evidences, together with the most recent literature, show the beneficial effects of *Brassicaceae* consumption, most likely attributable to the high presence of GLs-derived ITCs, where the *R*_S-SFN was the most evoked protective phytochemical.

Gram-scale production of natural *R*_S-GRA has recently been made available from Tuscan black kale (*Brassica oleracea* L. var. *acephala sabellica*) in line with a procedure developed at CRA-CIN of Bologna, Italy.

Starting from this, in the present paper was investigated the possible neuroprotective role of

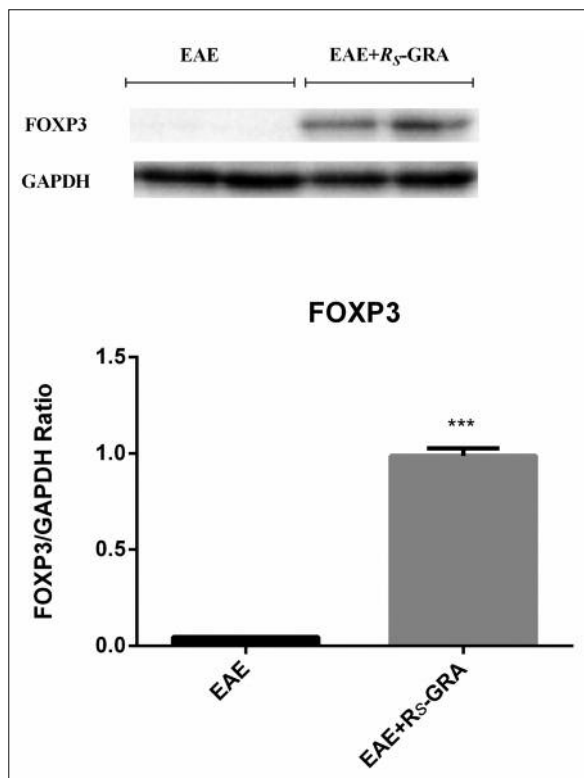


Figure 4. Western blot for Foxp3. Representative western blot showing no significant Foxp3 expression in brain tissues from EAE mice. Foxp3 levels were appreciably increased in brain from EAE mice administered with bioactive R_5 -GRA. GAPDH was used as internal control. *** $p < 0.05$ vs EAE.

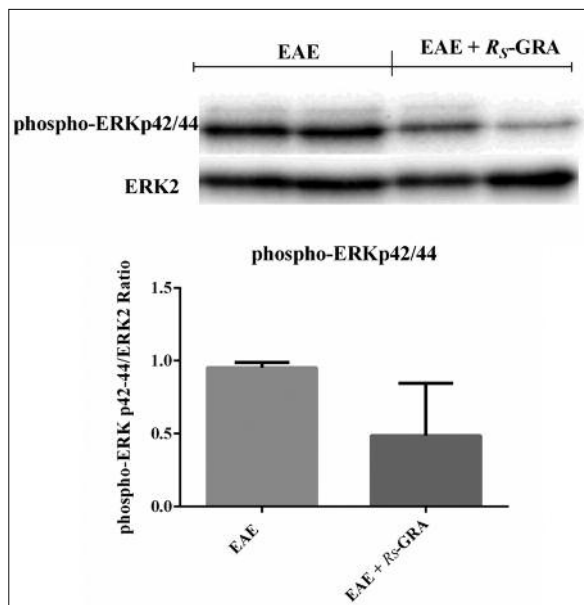


Figure 5. Western blot for ERK1/2. ERK1/2 levels were appreciably increased in brain samples taken from mice subjected to EAE, while the treatment of mice with bioactive R_5 -GRA reduced levels of ERK1/2. ERK2 was used as internal control.

the bioactive R_5 -GRA, as a novel important field of action potentially applicable in preventing dysfunctions of the BBB through a repair mechanism at the level of TJs proteins and, thus, the progression of neurological disorders, according to an experimental model of EAE.

BBB integrity and maintenance of homeostasis in the CNS are critically dependent of TJs between cerebrovascular endothelial cells. Any abnormality in the structure or function of TJs can lead to the BBB dysfunction that consequently may contribute to the development of neurodegenerative diseases, including MS¹⁰.

TJs possess the characteristic molecular architecture of an adhesion complex. They consist of a set of different types of transmembrane proteins that functions in barrier formation and regulation, adhesion, and signal transduction. These transmembrane components are linked to a cytoplasmic plaque that consists of a scaffold of adaptor proteins that anchors the junction to the actin cytoskeleton⁷.

Claudins constitute a large family of membrane proteins expressed in TJs in various cell types (endothelial and epithelial cells)^{5,26,27}, with four membrane-spanning regions, two extracellular and one intracellular loop, and N- and C-terminal cytoplasmic domains²⁸. The extracellular

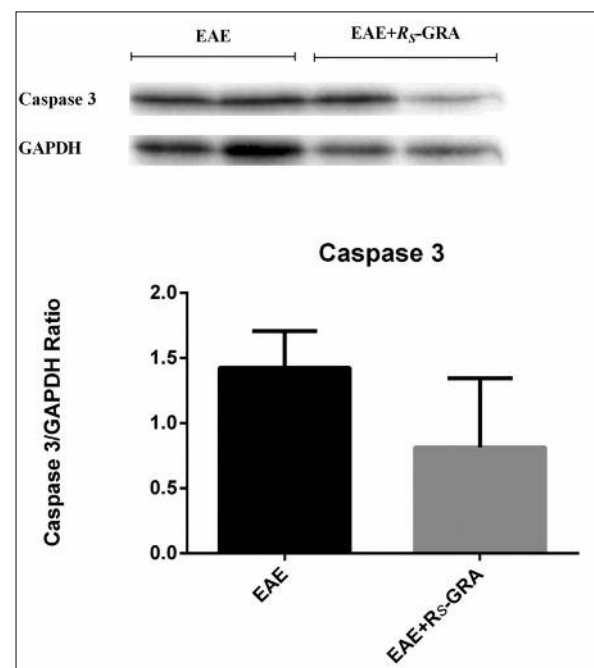


Figure 6. Western blot for caspase 3. By western blot analysis, caspase 3 levels were substantially increased in EAE mice, while bioactive R_5 -GRA reduced caspase 3 expression. GAPDH was used as internal control.

loops show the most remarkable differences between claudin family members. This has led to the speculation that claudins are not only important for the barrier formation but that they are responsible for the selective permeability of the paracellular pathway.

Individual claudins are generally expressed only in a restricted number of specific cell types, suggesting that they are associated with tissue-specific functions of tight junctions²⁹. Brain endothelial cells predominantly express claudin-1, claudin-3 and claudin-5³⁰.

A large corpus of data clearly establishes the key contribution of claudin-1, claudin-3 and claudin-5 to TJs formation and integrity at the BBB, via their capacity of cis- and trans-homodimerization as well as heterodimerization, notably through their second extracellular loop³¹⁻³³. These proteins directly regulate the gate/barrier function as paracellular tight junctions channels^{34,35}.

Changes in the type of claudin expressed, or single amino acid substitutions in claudin protein affect ion selectivity. It was shown that claudin-5 can interact with claudin-3 and the selective loss of the latter during EAE is associated with BBB breakdown³⁶.

The results obtained by western blot analysis, show a basal level of claudin-1, claudin-3 and claudin-5 during EAE. On the contrary, an increased expression of claudins was observed in mice that received bioactive *R*_S-GRA as previously demonstrated. Therefore, one of the possible action ways of this treatment is the control of permeability of TJs.

Additionally, claudins interact with the ZO proteins. ZO-1 is regarded as a major regulatory component of the TJs. It is a member of the membrane-associated guanylate kinase (MAGUK) family of signaling proteins, and has multiple binding sites for protein-protein interactions³⁷. Due to the presence of multiple domains, ZO proteins can function as adapters that connect transmembrane tight junctions protein to actin cytoskeleton and to various signaling molecules³⁸. This interaction is likely critical to the stability and function of TJs, since dissociation of ZO-1 from the junctional complex is often associated with increased permeability³⁹.

ZO-1 may also act as a signaling molecule that communicates the state of the TJs to the interior of the cell, or vice versa. ZO-1 has been shown to colocalize to the nucleus and play a role in transcriptional regulation, in response to bacterial toxins, drugs, growth factors, cytokines, hypoxia and injury⁴⁰⁻⁴⁴.

Also, it has been shown that translocation from the membrane, or lack of ZO-1 in the brain of MS patients promotes the BBB impairment.

Therefore, we analyzed the expression of ZO-1 in both the cytoplasm and nucleus of brain samples collected during EAE. We have clearly confirmed a physiological expression of ZO-1 in the cytoplasm of brain and its increase in mice treated with bioactive *R*_S-GRA. In addition, we also found an increased nuclear expression of ZO-1 in mice subjected to EAE, demonstrating an occurred location of the nucleus. However, treatment with bioactive *R*_S-GRA prevented the EAE-induced ZO-1 expression in the nucleus of mice and increased in animals administered with bioactive *R*_S-GRA, so the bioactive *R*_S-GRA is able to modulate the expression of ZO-1 and its localization probably implementing a repair mechanism at the level of the structural proteins of TJs.

Our findings, therefore, provide direct functional evidence identifying BBB TJs as a critical component in regulating barrier function of the BBB during EAE and confirming the notion that continued BBB TJ alterations are the basis of BBB dysfunction, which contributes to disease pathogenesis in this animal model of EAE.

Although it has been clearly demonstrated that an altered permeability of BBB plays a key role in MS, the underlying mechanisms of alterations of TJs, are not yet known.

Several experimental data showed that inflammation may affect TJ components of BBB through the activation of several pathways⁴⁵.

Regulatory T (Treg) cells expressing the transcription factor Foxp3 play an indispensable role in keeping the inflammatory T cells, e. g. Th1 and Th17, in maintaining peripheral homeostasis and avoidance of autoimmune disease⁴⁶.

Tregs exert their immunosuppressive functions via secretion of inhibitory cytokines, by interfering with the metabolism of T cells and/or contact-dependent manner not yet determined. Furthermore, Treg cells block T cell activation indirectly via their interaction with antigen-presenting cells (APCs): preventing APC maturation and they consequently downregulate the expression of costimulatory molecules and cytokines secretion^{47,48}.

Many studies have investigated the role of Tregs and their suppressive functions with contradictory results, likely due to multiple definitions of Treg subclasses, concluding that their suppressive activities are impaired during disease progression^{49,50}.

It has also been demonstrated that Tregs play a critical role in the protection and recovery of the

animal model of MS. In fact, deficiency in Treg cell numbers and/or function can lead to dominance of inflammatory T cells and progressive breakdown of self-tolerance and perpetuation of inflammation in many human autoimmune diseases, including MS⁵¹.

Rebuilding the balance by boosting the number and/or function of Treg cells have attracted considerable attention as potential treatment of autoimmune diseases^{52, 53}.

Our data demonstrated the selective loss of Foxp3 under pathological conditions, while mice treated with bioactive R_S -GRA clearly demonstrated an increased expression of Foxp3. Therefore, the treatment with bioactive R_S -GRA can play a crucial role in the protection against undesired T cell activation and autoimmune disease, by promoting Tregs production.

Also, so far three major subgroups of MAPKs, involved in inflammation, have been identified: p38 Mitogen-activated protein kinase (MAPK), c-Jun-N-terminal kinase (JNK) and extracellular regulated kinase 1 and 2 (ERK 1/2)⁵⁴.

By phosphorylating specific serines and threonines of target substrates, MAPKs regulate a wide range of processes: cell growth and differentiation, gene expression, mitosis, cell motility, metabolism, cell survival and apoptosis^{55,56}.

ERK1/2 is a cytosolic protein, able to translocate into the cell nucleus following the phosphorylation by other proteins called MEK (MAP Kinases ERK) in response to a variety of cellular stresses, such as hypoxia, UV radiations, oxidative stress and pro-inflammatory cytokines and growth factor stimulation⁵⁷. ERK1/2 once translocated in the nucleus, in turn phosphorylates several protein substrates which include some membrane proteins, cytoskeletal proteins, nuclear substrates and numerous MKs by setting in motion a specific program of gene activation⁵⁸.

Although it has been demonstrated that ERK1/2 is a pro-survival factor in the MAP kinase family and contributes to the regulation of cell proliferation and differentiation, under some circumstances, ERK1/2 can function in a pro-apoptotic manner, in the neuronal system, ERK1/2 has been suggested to be involved in neurodegeneration⁵⁹. Activation of ERK1/2 in response to several stimuli promotes cell apoptosis by enhancing activity of some pro-apoptotic signaling molecules, such as caspase 3⁶⁰.

We have clearly shown an increase of ERK1/2 during EAE. On the contrary, attenuated expression of ERK1/2 was observed in mice that received bioactive R_S -GRA as previously demonstrated.

In addition, caspase 3 is a key regulator of apoptosis, essential for some of the characteristic changes in cell morphology and in some biochemical events associated with the execution and completion of this process⁶¹. As cleaved caspase 3 is considered as marker of apoptosis, we evaluated the cleaved caspase 3 expression by western blot analysis. Our findings demonstrated a significant increase of cleaved caspase 3 after EAE, whereas bioactive R_S -GRA administration attenuated the level of cleaved caspase 3. A protective effect of bioactive R_S -GRA suggests that this treatment could interfere with the EAE-induced neuronal death, preserving cells by the injury.

Taken together, the results of the present study have shown that the bioactive R_S -GRA treatment could have a neuroprotective effect in the preventing BBB dysfunction, through a mechanism that involves a modulation of the inflammatory as well as apoptotic pathway.

Conclusions

The relevance of this investigation consists in the possible use of (R_S)-glucoraphanin, a glucosinolate that never before now between other compound of the same category was found ready to use thanks to the exogenous bioactivation by myrosinase enzyme. This new formulation has proved a therapeutic agent in preventing dysfunction of the BBB.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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