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ORIGINAL ARTICLE

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Protective Role of (R_S)-glucoraphanin Bioactivated with Myrosinase in an Experimental Model of Multiple Sclerosis

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SUMMARY

Aim: The discovery of new natural compounds with pharmacological properties is a field of interest widely growing. Recent literature shows that *Brassica* vegetables (Cruciferae) possess therapeutic effects particularly ascribed due to their content in glucosinolates, which upon myrosinase hydrolysis release the corresponding isothiocyanates. This study examines the potential neuroprotective and immunomodulatory effects of (R_S)-glucoraphanin from Tuscan black kale (*Brassica oleracea* L. var. acephala sabellica) bioactivated with myrosinase (bioactive R_S-GRA) (10 mg/kg/day intraperitoneally), in an experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis. **Methods:** EAE was induced by immunization with myelin oligodendroglial glycoprotein peptide (MOG_{35-55}) in mice. After immunization, mice were observed daily for signs of EAE and weight loss. Clinical score was evaluated using a standardized scoring system. **Results:** By Western blot analysis of spinal cord tissues, we have demonstrated that treatment with bioactive R_S-GRA significantly decreased nuclear factor (NF)-kB translocation, pro-inflammatory cytokine production such as interleukin-1β (IL-1β), and apoptosis (Bax and caspase 3 expression). **Conclusion:** Our results clearly demonstrate that bioactive R_S-GRA treatment may represent a useful therapeutic perspective in the treatment of this disease.

Introduction

Multiple sclerosis (MS) is a progressive inflammatory and demyelinating disease of the central nervous system (CNS) [1,2] caused by malfunction of the immune system, which affects more than 2.5 million people worldwide every year [3], typically in adults between 20 and 45 years of age; more women than men and Northern European people are shown to be at highest risk for MS [4,5]. MS attacks the myelinated axons in the CNS, destroying the myelin and the axons to varying degrees [6]. According to the traditional literature, MS is believed an inflammatory disease primarily affecting brain and spinal cord white matter [7–9]. The clinical signs of MS are highly variable. Patients with MS often have symptoms of upper motor neuron disease that include hyperreflexia, ataxia, spasticity, and visual defects. In some cases, there is evidence of lower motor neuron disease such as sensory defects and partial or complete paralysis [10].

The etiology of MS is yet unknown, but it appears to involve a combination of genetic susceptibility and a nongenetic trigger, such as a virus, metabolism, or environmental factors, that together result in a self-sustaining autoimmune disorder that leads to recurrent immune attacks against CNS [11–13]. To better understand the etiopathogenesis of MS, researchers use some experimental models and, among these, one of the most used is the experimental autoimmune encephalomyelitis (EAE) [14,15]. EAE is a well-characterized animal model, showing many clinical and pathological features of human MS, such as paralysis, weight loss, demyelination, and CNS inflammation [16].

However, there are great differences between EAE and MS. The first and most obvious is that MS is a spontaneous disease, while EAE is induced by active sensitization with brain tissue antigens. Recently, spontaneous EAE models were developed, but these are dependent on the use of transgenic approaches to ignore the intrinsic mechanisms of regulation that normally suppress tissue-specific autoaggression [17,18]. Furthermore, for inducing the disease, the use of strong immune adjuvants is provided, and it seems unlikely that the same intense "immunological boosts" occur in physiological conditions such as infectious diseases. Also, the adoptive transfer of myelin-specific T cells can induce demyelination, although the extent of primary myelin loss is minimal in

comparison with that seen in patients with MS [19]. Moreover, for the sake of reproducibility, EAE has been studied mainly in inbred animals or in genetically homogeneous populations. Thus, genetic heterogeneity, which is so critical in the multiple sclerosis population, can be considered only when several different models of EAE are studied in parallel.

Although this model shows limitations, it provides a very reproducible disease model to study the main mechanisms involved in the pathogenesis of T-cell-mediated inflammation in the CNS and has offered many insights that have been of great importance for the design of antiinflammatory therapies.

Convincing evidences, supported by both animal and cell line studies, have demonstrated that regular consumption of *Brassicaceae* vegetables (broccoli, cabbage, cauliflower, Brussels sprouts, etc.) can contribute to reduced risk of neurodegenerative disorders and cancer. It is believed that the benefits of these vegetables are due to their high content of specific phytochemicals, known as glucosinolates (GLs) [20].

Their structures share a common core of a β-D-glucopyranose moiety linked via a sulfur atom to a (Z)-N-hydroximinosulfate 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 thiosaccharidic compound found in *Brassicaceae*, notably in Tuscan black kale (*Brassica oleracea* L. var. acephala sabellica).

GLs coexist in the same plant, but in separate cells, with the enzyme β-thioglucoside glucohydrolase, usually known as myrosinase (Myr; EC 3.2.1.147), which have also been found within human bowel microflora [21,22]. After mechanical damage of cells, for example, predation/mastication by humans or animals, freeze–thaw injury, or plant pathogens, GLs are hydrolyzed releasing, apart from glucose and sulfate, several biologically active compounds such as isothiocyanates (ITCs), thiocyanates, and nitriles, depending on the hydrolytic conditions [23–25]. At neutral pH condition, Myr catalyzes the GL hydrolysis (>99%) producing the corresponding ITCs, such as R_S-sulforaphane (R_S-SFN) from R_S-GRA [26].

The mechanism underlying the chemopreventive R_S-SFN effects was interpreted as multipotent and ascribed to its ability to influence carcinogen metabolism, both inhibiting phase I enzyme-mediated activation, by inducing phase-2 enzymes like glutathione-S-transferase (GST) and quinone reductase (QR), and triggering the nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway [27,28].

Recently, CRA-CIN of Bologna (Italy) developed a gram-scale production of natural R_S-GRA starting from Tuscan black kale. The aim of this study was to investigate the possible neuroprotective role of the bioactive R_S-GRA on MS, according to an experimental mouse model of EAE.

Additionally, to gain a better insight into the mechanisms of action of R_S-GRA, we have also investigated its effects on NF-kB translocation and IkB-α degradation, as NF-kB is a transcription factor, which is kept inactive by IkB-α, that plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation, like tumor necrosis factor-α (TNF-α), IL-1β, inducible NO synthase (iNOS), c-Jun N-terminal kinase (JNK), and others.

Moreover, we evaluated the expression of caspase 3 and Bax as marker of apoptosis, as the sequential activation of caspase 3 plays a key role in the execution phase of cell apoptosis, as well as it is known that Bax gene plays an important role in cell death and CNS injury and that neurons lacking Bax are protected against apoptosis [29,30].

All this, to verify whether any therapeutic change due to the bioactive R_S-GRA treatment, is linked to a mechanism involving the modulation of the inflammatory or apoptotic pathways and the subsequent physiological responses.

Materials and Methods

Animals

Male adult C57BL/6 mice (20–25 g; Harlan Nossan, Milan, Italy) were used for all studies. Mice were housed in stainless steel cages and maintained under 12-h/12-h light/dark cycle at 21 $\pm$ 1°C and 50 $\pm$ 5% humidity. The animals were acclimated to their environment for 1 week, and food and water were given *ad libitum*. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (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 $(MOG)_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK; Auspep) was synthesized and purified by Cambridge Research Biochemicals (Billingham, UK).

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

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. All other chemicals were of the highest commercial grade available.

Induction of EAE

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) combined with an equal volume of CFA containing 300 μg heat-killed *M. Tubercolosis* H37Ra. Afterwards, animals have been injected with 100 μL of *B. Pertussis* toxin (500 ng/100 μL, i.p.), that has been also 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.

GLs and Myrosinase Purification, Enzyme Bioactivation of *Rs*-GRA

R_S-GRA was isolated according to a procedure developed at CRA-CIN of Bologna [31]. Seeds of Tuscan black kale, supplied by Suba

Seeds (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) 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-dried [32]. R_S-GRA was characterized by ^{1}H and ^{13}C NMR spectroscopy, and the absolute purity estimated by HPLC analysis of the desulfoderivative, according to the ISO 9167-1 method [33], 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 of 6634 M^1/cm^1 at 225 nm were determined using a Varian Cary 300 Bio UV/vis spectrophotometer).

The enzyme Myr was isolated from seeds of *Sinapis alba* L. as described by Pessina et al. [34], with some modification. The specific activity of the stock solution used in this 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).

Experimental Design

Mice were randomly allocated into the following groups (N = 50 total animals):

1 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 administrated 1 week before EAE induction, and after immunization, the treatment was daily protracted until the sacrifice;
2 EAE group (N = 20): mice subjected to EAE that did not receive bioactive R_S-GRA;
3 Sham group (N = 5): mice that received vehicle (saline) in place of $MOG_{35–55}$;
4 Bioactive R_S-GRA control group (N = 5): mice that received vehicle (saline) in place of $MOG_{35–55}$ and treated with bioactive R_S-GRA (10 mg/kg + 5 μL/mouse Myr, i.p.);

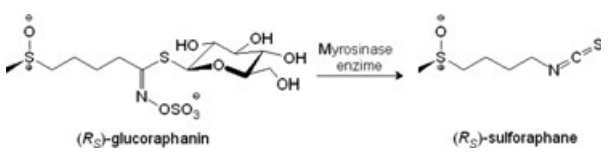

Figure 1 (R_S)-glucoraphanin purified from Tuscan black kale seed, bioactivated with myrosinase enzyme: *in situ* release of (R_S)-sulforaphane.

The experiment provided a housing period of duration of 7 days followed by a period of pretreatment with bioactive R_S-GRA via i.p. injection once a day for 7 days. On the 15th day, the disease was induced according to the following experimental procedure. In the experimental group EAE + bioactive R_S-GRA, the postdrug treatment was continued for a further 7 days after induction of the disease until the 21st day.

At the end of the experiment, the animals were sacrificed, and spinal cord tissues were harvested and processed, to evaluate parameters of disease.

Body Weight and Clinical Score

Mice were observed daily for signs of EAE. Clinical score was evaluated using a standardized scoring system [35]. Briefly, clinical signs were scored as follows: 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 animal.

In addition, the measure of the body weight was daily assessed, and any loss was evaluated as marker of pathology. The graph represents Δ value for each group obtained calculating the difference in body weight between the measure taken the day of sacrifice and that one taken the day of the disease induction (Figure 2).

Western Blot Analysis for IL-1β, JNK, IkB-α, NF-kB p65, Bax, and Caspase 3

All the extraction procedures were performed on ice using ice-cold reagents. In brief, spinal cord 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 NaN_3, 10 mM 2-mercaptoethanol, 50 mM NaF, and protease inhibitor tablets (Roche Applied Science, Monza, Italy), and they were homogenized at the highest setting for 2 min. The homogenates were chilled on ice for 15 min and then centrifuged at 1000 *g* for 10 min at 4°C, and the super-

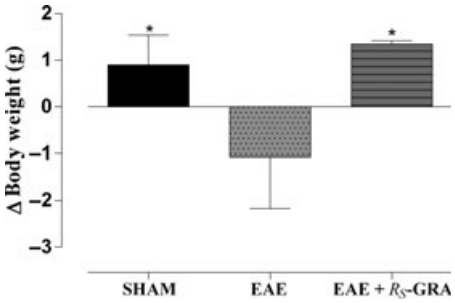

Figure 2 Δ Body weight. C57BL/6 mice were immunized with $MOG_{35–55}$ and CFA and then treated daily with bioactive R_S-GRA at the dose of 10 mg/kg. Mice were monitored daily for weight gain/loss. After EAE induction, significative body weight loss was observed in EAE mice. Also, a significative body weight gain was found in EAE+ bioactive R_S-GRA group and in bioactive R_S-GRA control group mice compared with EAE group and sham group. Graph represents for each experimental group, the Δ body weight calculated by subtracting measures taken the day of animal sacrifice and measures taken the day of immunization. Data are mean value ± SEM. *$P < 0.05$ versus EAE.

natant (cytosol + membrane extract from spinal cord tissue) was collected to evaluate content of IL-1β, JNK, IkB-α, and Bax.

The pellets were suspended in the supplied complete lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EGTA, and 1 mM EDTA protease inhibitor tablets (Roche Applied Science), and then they were centrifuged for 30 min at 15,000 *g* at 4°C, and the supernatant (nuclear extract) was collected to evaluate the content of NF-kB p65 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, Segrate, Milan, Italy) using BSA as standard, and 50 μg of cytosol and nuclear extract from each sample was analyzed.

Proteins were separated on sodium dodecyl sulfate–polyacrylamide 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 IL-1β (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), JNK (1:500; Santa Cruz Biotechnology, Inc.), IkB-α (1:1000; Cell Signaling Technology, Inc., Boston, MA, USA), Bax (1:500; Santa Cruz Biotechnology, Inc.), NF-kB p65 (1:1000; Santa Cruz Biotechnology, Inc.), and caspase 3 (1:1000; Cell Signaling), in 1× PBS, 5% (w/v) nonfat dried milk, and 0.1% Tween-20 (PMT).

Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated with antibody for β-actin protein (1:1000; Santa Cruz Biotechnology, Inc.), α-tubulin (1:250; Santa Cruz Biotechnology, Inc.), and GAPDH HRP conjugated (1:1000; Cell Signaling).

The relative expression of the protein bands of IL-1β (~17 kDa), JNK (~46 kDa), Bax (~23 kDa), IkB-α (~37 kDa), NF-kB p65 (~65 kDa), caspase 3 (~35 kDa) was visualized using an enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent). The protein bands were scanned and quantitated with ChemiDoc™ MP System (Bio-Rad) and a computer program (ImageJ).

Statistical Evaluation

Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used to run all the tests. The results were analyzed by 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 ± SEM of n experiments.

Results

Body Weight Loss as Sign of Disease

Studies on animal models of EAE have demonstrated that the acute phase of the disease coincides with weight loss, probably due to anorexia and deficient fluid uptake. Weight measurement of immunized mice correlated with the severity of the clinical score and showed a significantly reduced weight loss in EAE mice compared with sham mice. A consistent difference between EAE group ($\Delta = -1.066$ g during all the experimental period) and the other groups was observed. After EAE induction, significative body weight gain was found in EAE + bioactive R_S-GRA group ($\Delta = +1.35$ g), compared with EAE group and sham group ($\Delta = +0.9$ g) (Figure 2).

Bioactive R_S-GRA Modulates IL-1β Levels

To test whether pretreatment with bioactive R_S-GRA modulates the inflammatory process through the regulation of secretion of pro-inflammatory cytokines, we analyzed spinal cord tissue levels of IL-1β by Western blot analysis. A basal level of IL-1β production was found in spinal cord samples collected from EAE mice 7 days after EAE induction (Figure 3), while spinal cord levels of IL-1β were attenuated by administration of bioactive R_S-GRA (Figure 3).

Effect of Bioactive R_S-GRA on JNK, IkB-α Degradation, and NF-kB p65

To investigate the cellular mechanisms whereby pretreatment with bioactive R_S-GRA attenuates the development of EAE, c-Jun N-terminal protein kinase (JNK) and nuclear NF-kB expression were evaluated by Western blot analysis in spinal cord tissue. JNK levels were substantially increased in EAE mice (Figure 4A), while bioactive R_S-GRA pretreatment prevented the EAE-induced JNK expression (Figure 4A). In addition, NF-kB p65 levels in the nuclear fractions from spinal cord tissue were significantly increased at 7 days after EAE compared with the sham mice

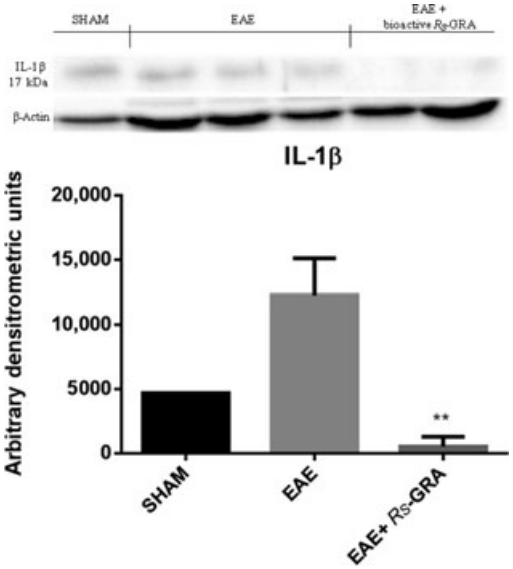

Figure 3 Western blot for IL-1β. By western blot analysis, a basal level of IL-1β was detected in spinal cord samples collected from EAE mice 7 days after EAE, while spinal cord levels of IL-1β were attenuated by the administration of bioactive R_S-GRA. β-actin was used as internal control. $^{**}P < 0.0072$ versus EAE.

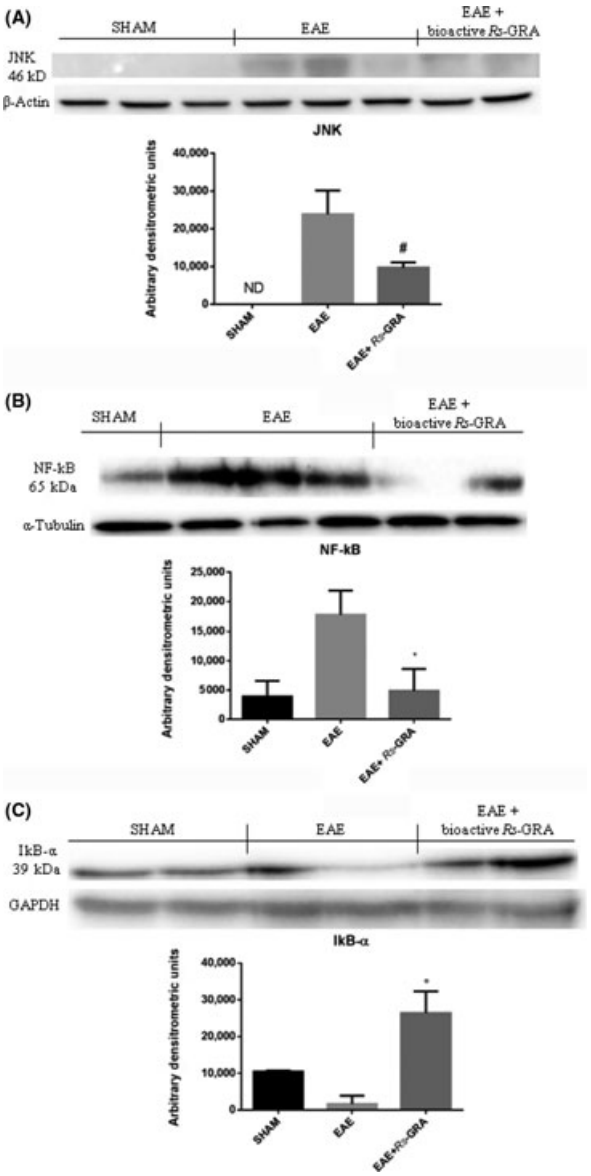

Figure 4 Western blot for JNK, NF-kB p65, and IkB-α. By Western blot analysis, JNK levels were found to be substantially increased in EAE mice (**A**), while bioactive R_S-GRA pretreatment prevented the EAE-induced JNK expression (**A**) In addition, EAE caused a significant increase in nuclear NF-kB p65 compared with the sham mice (**B**). Bioactive R_S-GRA pretreatment reduced the levels of NF-kB p65 (**B**). IkB-α detection following EAE induction shows that EAE mice present a lower IkB-α level (sign of IkB-α degradation) than sham animals. Bioactive R_S-GRA administration prevents the EAE-induced IkB-α degradation. The relative expression of the protein bands was standardized for densitometric analysis to β-actin (**A**), α-tubulin (**B**), and GAPDH (**C**) levels. $^*P < 0.008$ versus EAE, $^{\#}P < 0.0021$ versus EAE, $^{\circ}P < 0.0142$ versus EAE. ND: not detectable.

(Figure 4B). Bioactive R_S-GRA treatment reduced the levels of NF-kB p65 (Figure 4B).

Also, we evaluated IkB-α expression following EAE induction. In spinal cord samples from EAE mice, an IkB-α level lower than sham animals was detected (sign of IkB-α degradation, Figure 4C). Bioactive R_S-GRA administration prevents the EAE-induced IkB-α degradation (Figure 4C).

Effect of Bioactive R_S-GRA on Bax and Caspase 3 Expression

At 7 days after EAE, the appearance of pro-apoptic protein, Bax, in spinal cord homogenates was investigated by Western blot. Bax levels were appreciably increased in spinal cord samples taken from mice subjected to EAE (Figure 5A). Conversely, bioactive R_S-GRA pretreatment prevented the EAE-induced Bax expression (Figure 5A). Sequential activation of caspase plays a central role in the execution phase of cell apoptosis, so that using Western blot analysis, we evaluated the activation of caspase 3. Caspase 3 levels were appreciably increased in the spinal cord from mice subjected to EAE (Figure 5B). On the contrary, pretreatment with bioactive R_S-GRA attenuated the EAE-induced caspase 3 expression (Figure 5B).

Discussion

MS is an inflammatory demyelinating disease of the CNS, culminating in progressive neurological deterioration. Like many other autoimmune diseases, it is initiated by an uncontrolled T-cell response to autoantigens presented in the context of class II major histocompatibility complex (MHC) molecules of antigen-presenting cells (APCs) [3]. The most widely accepted hypothesis suggests a dialogue mediated by T-cell receptors on $CD4^+$ T lymphocytes. These interactions between active $CD4^+$ T cells and myelin antigens apparently provoke a massive destructive inflammatory response and promote continuing proliferation of T and B cells and macrophage activation, which sustains secretion of inflammatory mediators, for example, cytokines/chemokines [11,36,37].

Synthetic sulforaphane (R_S,S_S-SFN) has been extensively studied in recent years, both as chemopreventive agent and as potential novel chemotherapeutic compound [38–42], but this compound currently used is unstable and slightly soluble in water, so the objective of this work was to provide a therapeutic agent of easier availability.

Starting from the idea that R_S-GRA is easily available at CRACIN of Bologna (Italy) together with homogeneous Myr, our goal was to demonstrate the therapeutic effects of bioactive R_S-GRA (Italian patent pending MI2012A001774), as a novel important field of action potentially applicable in inflammatory/autoimmune disease, as EAE.

Astrocytes are the major glial cell within the CNS and have a number of important physiological properties related to CNS homeostasis [43].

Bioactive R_S-GRA can mediate the astroglial response during EAE by modulation of pro-inflammatory cytokines [44], such as IL-1β, that, as many other inflammatory mediators, are controlled by NF-kB. Here, we have demonstrated that therapeutic effects of bioactive R_S-GRA can modulate inflammatory pathway during EAE. It is well known that under normal conditions, NF-kB is present within the cytoplasm in an inactive state, bound to its inhibitory protein IkB-α. In response to a wide

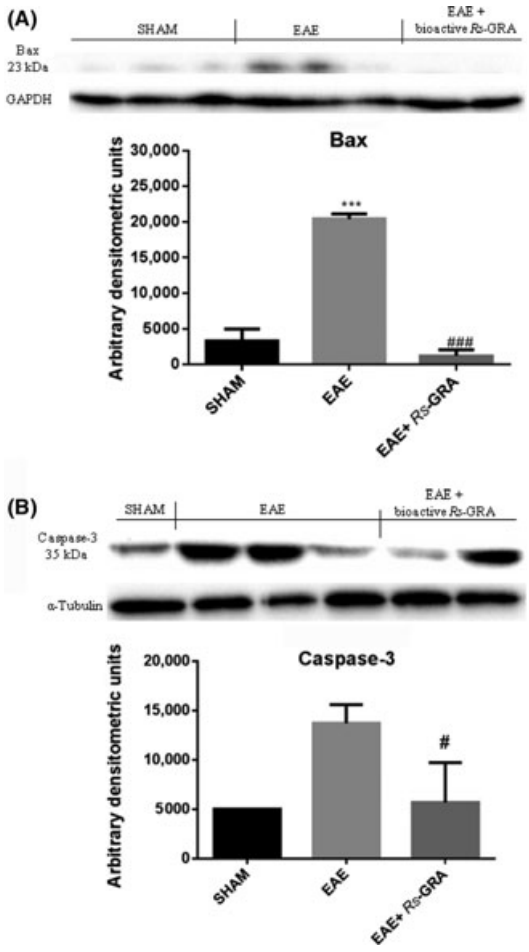

Figure 5 Western blot for Bax and caspase 3. Representative Western blots showing no significant Bax expression in spinal cord tissues from sham mice (**A**). Bax levels were appreciably increased in spinal cord from EAE mice (**A**). Also, spinal cord levels of Bax were attenuated by administration of bioactive R_S-GRA (**A**). $^{***}P < 0.002$ versus SHAM, $^{\#\#\#}P < 0.0020$ versus EAE. Moreover, we also demonstrated caspase 3 activation by Western blot, and EAE caused a significant increase in caspase 3 expression (**B**) compared with the sham mice. Bioactive R_S-GRA reduced caspase 3 levels as shown in figure (**B**). GAPDH (**A**) and α-tubulin (**B**) were used as internal control. $^{\#}P < 0.0269$ versus EAE. ND: not detectable.

range of stimuli including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, IkB-α is phosphorylated by the enzyme IkB-α kinase. Once liberated from its inhibitory protein, NF-kB translocates to the nucleus, where it modulates the transcription of a number of pro-inflammatory genes. Our analysis demonstrated that bioactive R_S-GRA pretreatment inhibits the IkB-α degradation as well as the NF-kB activation. A direct consequence of the inhibitory effect of bioactive R_S-GRA on NF-kB activation is the reduction in pro-inflammatory mediators production under its control, such as IL-1β. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 [45]. IL-1β plays an important role in host protection against infections, but can also promote tissue damage in chronic inflammatory diseases. Moreover, IL-1β is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.

During EAE, IL-1β was demonstrated fundamental in the pathogenesis of inflammatory progression [45,46] implicated in EAE, by activation of T lymphocytes and stimulation of the production of other cytokines in turn [47].

We have clearly confirmed an increase in IL-1β during EAE. On the contrary, attenuated expression of IL-1β was observed in mice that received bioactive R_S-GRA as previously demonstrated. A downregulation of IL-1β in animals treated with bioactive R_S-GRA showed that one of the possible action ways of this treatment is the control of the cytokine production.

Therefore, we have shown that bioactive R_S-GRA administration is able to produce substantial reduction in inflammatory events associated with EAE.

Furthermore, once translocated in the nucleus, NF-kB activates the transcription of genes involved in the progression of the inflammatory pathway, such as JNK.

JNK is a subfamily of the mitogen-activated protein kinase (MAPK) superfamily. JNK was originally identified by its ability to specifically phosphorylate the transcription factor c-Jun on its N-terminal transactivation domain at two serine residues, Ser63 and Ser73. Kinases of the JNK group of MAPKs are primarily activated by pro-inflammatory cytokines, such as IL-1β and TNF-α, and stress stimuli such as UV radiation, pH changes, hypoxia, and genotoxic and oxidative stress [48]. Activation of JNK in response to these stimuli has been linked to several biological responses, including proliferation, differentiation, and apoptosis [48–50]. In normal cells, the activation of JNK is an event that determines the activation of pro-apoptotic proteins of the Bcl-2 family, which cause an induction in the release of cytochrome c from mitochondria.

This study has demonstrated that our pharmacological pretreatment with bioactive R_S-GRA prevents the EAE-induced JNK expression, being that JNK levels substantially decreased in EAE-injected mice. It is conceivable a mechanism mediated by the upstream inhibition of NF-kB p65 translocation and probably the inhibition of JNK phosphorylation.

The results obtained from analysis of all these protein parameters led us to investigate the effect of pretreatment with bioactive R_S-GRA on the apoptosis degree after EAE induction.

Apoptosis is a natural form of cell death, which can be induced by an "intrinsic" mitochondria-mediated pathway [51]. This pathway causes activation of caspases (in particular caspase 3), which, by cleavage of cellular substrates, leads to programmed cell death. 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 process [52]. As cleaved caspase 3 is considered as marker of apoptosis, we evaluated the cleaved caspase 3 expression by Western blot analysis. We found that EAE causes significant increase in cleaved caspase 3 when compared with sham animals, whereas bioactive R_S-GRA pretreatment decreased the level of cleaved caspase 3.

Another important pro-apoptotic factor, playing a pivotal role in developmental cell death and in CNS injury, is Bax. For this reason, we have detected pro-apoptotic transcriptional changes

and upregulation of Bax in EAE. In particular, we have shown that pretreatment with bioactive R_S-GRA reduced Bax expression, leading to a reduction in the pro-apoptotic pathway activation. This finding suggests that the neuroprotective effect of bioactive R_S-GRA is associated with apoptosis regulation.

Finally, Canistro et al. [53] concluded their study reporting that the regular administration of extract containing dietary constituents (120 or 240 mg/kg), including glucosinolates, to healthy humans for chemopreventive purposes should be used with caution. Conversely, in our study, we evaluated the neuroprotective effect (10 mg/kg) of pure glucosinolate R_S-GRA, bioactivated with myrosinase, in an animal model of MS, and we found that the mechanisms involved were both the modulation of the inflammatory pathways and the reduction in the activation of cell death by apoptosis.

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Conflict of Interest

The authors declare no conflict of interest.

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