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Beneficial effects of (R_S)-glucoraphanin on the tight junction dysfunction in a mouse model of restraint stress

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ABSTRACT

Aims: The purpose of this work is to evaluate the effects of (R_S)-glucoraphanin, a glucosinolate present in *Brassicaceae*, notably in Tuscan black kale, and bioactivated with myrosinase enzyme (bioactive R_S-GRA) (10 mg/kg intraperitoneally), and to assess its capacity to prevent the dysfunction of the blood–brain barrier (BBB), a fundamental structure for brain homeostasis, in a mouse model of restraint stress.
Main methods: CD1 mice were subjected to restraint stress by blocking the body with a tape on a table for 150 min at the four extremities. After the sacrifice of the animals, stomachs and brains were collected to perform histological evaluation, Evan's blue dye, immunohistochemistry and western blotting analysis, to evaluate whether immobilization stress leads to alterations of tight junction (TJ) components, such as claudin-1, claudin-3 and ZO-1.
Key findings: Immobilization causes considerable damage to BBB as shown by detection of Evan's blue dye, indicating a high level of extravasation due to stress. BBB alterations were accompanied by an enhancement of GFAP expression, IkB-alpha degradation followed by increased NF-kBp65 nuclear translocation, as well as caspase 3 overexpression. Conversely, our results revealed that bioactive R_S-GRA treatment significantly counteracts the changes in all these parameters and preserves TJ integrity reducing the production of pro-inflammatory cytokines, such as TNF-α and IL-1β, and increasing the production of IL-10, an anti-inflammatory cytokine. Additionally, bioactive R_S-GRA shows antioxidant properties modulating iNOS and nitrotyrosine expression.
Significance: Our results clearly show that bioactive R_S-GRA could represent a possible treatment during pharmacological therapy of stress.

Introduction

Selye (1950) defined stress as a state characterized by altered homeostasis with several physiological and behavioral changes. Recently, it was reported that the protective function of the blood–brain barrier (BBB), fundamental for the homeostasis in normal neuronal activity, can be altered during stress conditions (Esposito et al., 2002). BBB, a highly selective barrier able to separate the brain parenchyma from the systemic circulation, is made up of brain microvessel endothelial cells, astroglia, pericytes, perivascular macrophages and basal lamina. Brain microvessel endothelial cell sheets form a semipermeable barrier through a circumferential seal around the apical pole of the cells called tight junctions (TJs). They permit the paracellular transport of ions, water, and various macromolecules between endothelial cells (gate function) and establish a fence function by limiting the free movement of lipids and proteins between the apical and the basolateral cell surface, then creating a cell polarity.

Changes in BBB permeability are due to the dislocation of the TJ components, important for the maintenance of structural integrity of TJs and for the permselectivity of the barrier (Kuge et al., 2006; Mazzon et al., 2009; Mazzon et al., 2002). For this reason, TJs have been extensively studied at the morphological, functional, and molecular levels.

They are composed of trans-membranous proteins, namely claudins, occludin, junctional adhesion molecules (JAM-A, JAM-B, JAM-C), endothelial cell-selective adhesion molecule (ESAM) and intracellular proteins, such as zonula occludens (ZOs). Among the members of the claudin family, claudin-1, -2, -3, -5, -11 and -12 were recently detected in BBB endothelium.

Inflammation, oxidative stress, toxin, drugs and hormones have been shown to affect the TJ barrier and fence functions (Esposito et al., 2002; Fukui et al., 2012). In oxidative stress conditions, reactive oxygen species (ROS) overproduction causes TJ modulation and structural

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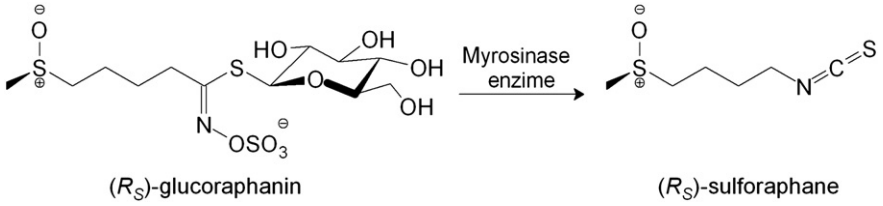

Fig. 1. Chemical structure and enzymatic reaction for bioactivation of R_S-GRA by Myr. (R_S)-glucoraphanin purified from Tuscan black kale seed, bio-activated by myrosinase enzyme: *in situ* release of (R_S)-sulforaphane.

changes (Kumar et al., 2012; Yu et al., 2012). Moreover, elevated glucocorticoid and glutamate levels trigger oxidative stress through the production of ROS, resulting into lipid peroxidation, protein oxidation, DNA damage, and cell death (Madrigal et al., 2006; Richier et al., 2006). Therefore, an antioxidant therapy strategy has been used to develop drugs for various degenerative disorders in the brain. Actually, glucosinolates (GLs), phytochemical components found in Brassicaceae, for example in Tuscan black kale, were investigated for their antioxidant and anti-inflammatory properties in several fields, principally in cancer and cardiovascular diseases (Abdull Razis et al., 2011b; Barillari et al., 2005). To explicate biological activities, they are bioactivated through hydrolysis reaction catalyzed by myrosinase (Myr; beta-thioglucosidase glucohydrolase; EC 3.2.1.147) and transformed into isothiocyanates (ITCs).

Therefore, the aim of this study is to test the protective effects of bioactive R_S-GRA in a mouse model of restraint stress. In order to gain a better insight into the mechanisms of action of this molecule, we investigated iNOS, nitrotyrosine, NF-kBp65, pro-inflammatory cytokines, Nrf2, GFAP and caspase 3 as main mediators responsible of BBB disruption during the processes triggering restraint stress.

Material and methods

Animals

CD mice (4–5 weeks old, 20–22 g) were purchased from Harlan (Italy). Animals were housed in a controlled environment and provided with standard rodent chow and water *ad libitum*. Animal care was in compliance with regulations in Italy (Ministerial Declaration 116/92) as well as with European Economic Community regulations (O.J. of European Commission L 358/1 12/18/1986). Experimental design was approved by the Ministry of Health, Labour and Social Affairs, Department of Veterinary Public Health, Nutrition and Food Safety, Directorate General of Animal Health and the Veterinary Medicine of the former Ministry of Health, Office VI-Animal Welfare.

Experimental procedures did not cause any significant animal suffering.

GLs and myrosinase purification, enzyme bioactivation of R_S-GRA

R_S-GRA was isolated by CRA-CIN of Bologna, Italy (Italian Patent Attending MI2012A001774) together with homogeneous Myr according to a procedure previously described (Abdull Razis et al., 2010). Seeds of *Brassica oleracea* L. var. acephala sabellica (Tuscan black kale) were ground to a fine powder and subsequently defatted with hexane. The solvent was removed and the defatted seed meal was treated with boiling 70% ethanol in order to quickly deactivate the endogenous enzyme Myr. R_S-GRA was extracted using an Ultraturrax homogenizer at medium speed for 15 min. The resulting homogenate was centrifuged at 17,700 $\times g$ for 30 min. The isolation of the R_S-GRA from the extract was carried out by one-step anion exchange chromatography. GLs purity was further improved by gel-filtration performed using a XK 26/100 column packed with Sephadex G10 chromatography media (GE Healthcare, Italy), connected to an AKTA-FPLC System (GE Healthcare, Italy). Individual fractions were analyzed by HPLC and those containing pure R_S-GRA were pooled and freeze-dried (Wagner et al., 2010). R_S-GRA was characterized by ^{1}H and ^{13}C NMR spectrometry and the purity was assayed by HPLC analysis of the desulfo-derivative according to the ISO 9167-1 method (EEC Regulation 1864/90, Enclosure VIII1990). The purity was 99% (HPLC peak purity) 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.

The Myr enzyme was isolated from seeds of *Sinapis alba* L. according to a study by Pessina et al. (1990) although with some modifications. The specific activity of the stock solution used in the present study was 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 Myr unit was defined as the amount of enzyme able to hydrolyze at pH 6.5 and 37 °C 1 μmol/min of sinigrin (Abdull Razis et al., 2011a).

Enzyme bioactivation of R_S-GRA and animal treatment

For GL treatment, a lyophilized R_S-GRA compound was dissolved in PBS solution pH 7.2 at room temperature to administrate a final concentration of 10 mg/kg. Mouse treatment required the enzyme bioactivation of the phytochemical. The *in situ* action of the Myr enzyme (5 μl/mouse) for 15 min allowed having a bioactive R_S-GRA quickly, before the i.p. treatment (Fig. 1).

Experimental protocol

Mice (n = 45) were randomly divided into three groups:

- sham group: animals did not suffer restraint stress: they have been just briefly anesthetized as the other groups and received an equal volume of saline solution *via* i.p. as treatment;
- stressed mice group: animal body was immobilized with a tape on a table for 150 min at the four extremities;
- stressed mice + bioactive R_S-GRA group: stressed mice were pretreated 1 h before the immobilization with a single dose of bioactive R_S-GRA, obtained as above described.

Before the induction of restraint stress, all animals were anesthetized with isoflurane. At the end of the immobilization-time they were sacrificed with an intraperitoneal injection (i.p.) of Tanax (5 ml/kg body weight).

Mice were fasted 24 h before the induction of stress. Since fecal pellet output is known to increase under stress conditions, the feces were collected to evaluate the stress effect during the stress period. At the end of the observation-time, mice were sacrificed and the stomach and cerebral hemispheres were collected to perform histological evaluation, immunohistochemistry (IHC) and expression analysis by western blotting.

Albumin Evan's blue determination

Ten animals for each group were used to assess BBB permeability, quantitatively evaluated by measuring the amount of extravasated Evan's blue dye in brain tissue according to a procedure previously described (Hawkins and Egleton, 2006).