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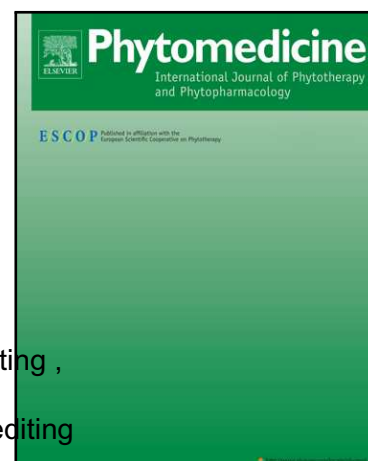
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***Rhodiola rosea* L. modulates inflammatory processes in a CRH-activated BV2 cell model.**Vittoria Borgonetti^{a,†}, Paolo Governa^{b,†}, Marco Biagi^{c,*}, Pasquale Dalia^d, Lorenzo Corsi^d

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Abstract

Background: *Rhodiola rosea* L. (Crassulaceae) has been used for years in the traditional medicine of several countries as an adaptogen drug, able to preserve homeostasis in response to stress stimuli. Currently *R. rosea* roots and rhizome are classified as a traditional herbal medicinal product for temporary relief of symptoms of stress, such as fatigue and sensation of weakness by the European Medicines Agency.

Hypothesis/Purpose: Increasing evidences suggest the involvement of neuroinflammation in response to stress. However, whether the modulation of neuroinflammatory parameters could be involved in the anti-stress effect of *R. rosea* has been barely studied. Thus, the aim of this work is to investigate the possible modulation of molecular inflammatory processes elicited by a *R. rosea* roots and rhizome ethanolic extract in an *in vitro* model of corticotropin releasing hormone (CRH)-stimulated BV2 microglial cells.

Methods: BV2 cells were stimulated with CRH 100 nM and changes in cell viability, cytokines production and heat shock protein 70 (HSP70) levels were evaluated. Intracellular pathways related to inflammation, such as nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) nuclear translocation and mitogen-activated protein kinases (MAPK) activation were also analyzed.

Results: We found that *R. rosea* extract (2.7% m/m rosavin and 1% m/m salidroside) 20 μ g/ml was able to counteract the neuroinflammatory effect of CRH by inhibiting NF- κ B nuclear translocation with a mechanism of action involving the modulation of mitogen-activated protein kinase-activated protein kinase 2

(MKK2), extracellular signal-regulated kinase 1/2 (ERK 1/2) and c-Jun n-terminal kinase (JNK), resulting in a reduction of HSP70 expression.

Conclusion: This work expands the knowledge of the intracellular mechanisms involved in *R. rosea* anti-stress activity and may be useful for the study of other adaptogen drugs.

Keywords

Rhodiola rosea L.; microglia; CRH; HSP70; MAPK; NF- κ B

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Abbreviations

BSA, bovine serum albumin; CCK-8, cell counting kit-8; COX, cyclooxygenase; CRH, corticotropin releasing hormone; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ERK 1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; HCl, hydrochloric acid; HPLC-DAD, high performance liquid chromatography with diode-array detector; HRP, horseradish peroxidase; HSP70, heat shock protein 70; IgG, immunoglobulin G; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; JNK, c-Jun n-terminal kinase; LPS, lipopolysaccharides; MAPK, mitogen-activated protein kinases; MKK2, mitogen-activated protein kinase-activated protein kinase 2; NaCl, sodium chloride; NF- κ B, nuclear factor kappa-light-chain enhancer of activated B cells; NF- κ B/p65, nuclear factor kappa-light-chain enhancer of activated B cells subunit p65; NMDA, N-methyl-D-aspartate; NO, nitric oxide; p38, p38 mitogen-activated protein kinases, PBS, phosphate buffered saline; PBST, phosphate buffered saline with tween 20; PI3K, phosphatidylinositol 3-kinases; PI3KC2G, phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit gamma; PLCB1, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1; RHOD, *Rhodiola rosea* L. root and rhizome extract WS®1375; RIPA, radio immunoprecipitation assay buffer; RPMI, Roswell park memorial institute; RT, retention time; STAT2, signal transducer and activator of transcription 2; TBST, tris-buffered saline with tween 20; TNF- α , tumor necrosis factor- α ; UCN, urocortin; UV, ultraviolet.

Introduction

Rhodiola rosea L., popularly known as arctic root or golden root, belongs to the family of Crassulaceae. The part of the plant used are the dried roots and rhizomes, which mainly contains phenylethanoids and phenylpropanoids, such as salidroside, rosavins and tyrosol, as well as phenolic acids, characteristic flavonoids and essential oil (Chiang et al., 2015). *R. rosea* has been used for years in the traditional medicine of Eastern Europe and Asia, but its actual pharmacologic potential has been more recently and intensively studied in Russia and Scandinavia (Panossian et al., 2010a). In Europe, the dry extract (DER 1.5-5:1) obtained from *R. rosea* roots and rhizome, using ethanol 67-70% V/V as a solvent, is classified as a traditional herbal medicinal product for temporary relief of symptoms of stress, such as fatigue and sensation of weakness, by the European Medicines Agency Committee on Herbal Medicinal Products (European Medicines Agency, 2012). The majority of the clinical studies published have been performed using two standardized extracts: the WS®1375 and the SHR-5, which differ in the content of rosavins and salidroside (European Medicines Agency, 2012). The use of these high-quality standardized extracts instead of other products present in the market allows to gain more clinical reproducibility and a better safety profile (Booker et al., 2016). Although there is the need for more rigorous clinical trials, recent systematic reviews suggested evidence for the use of *R. rosea* for enhancing physical performance and certain mental health conditions, by alleviating mental fatigue (Hung et al., 2011; Ishaque et al., 2012; Panossian, 2013). *R. rosea* acts as an adaptogen, which are drugs able to preserve homeostasis in response to stress stimuli (Panossian, 2003).

A *R. rosea* extract (15 mg/kg), containing 3% rosavins and 1% salidroside, demonstrated adaptogenic activity in the swimming to exhaustion test in mice (Perfumi and Mattioli, 2007), and reduced stress-induced anorexia in rats (Mattioli and Perfumi, 2007). Later, the same research group demonstrated that *R. rosea* is able to inhibit the behavioral and physiological changes induced by chronic mild stress in rats (Mattioli et al., 2009). In 2009, Panossian and colleagues suggested that the adaptogen effects of a fixed combination of herbal drugs, including *R. rosea*, observed in mice, could be linked to the increase of serum Heat Shock Protein 72 levels (Panossian et al., 2009).

CRH and corticosterone are known to be strictly related to physical and psychological stress (Nakatani et al., 2012). Interestingly, Xie and coworkers recently found that *R. rosea* anti-stress activity is related to a reduction of stress-induced CRH in the hypothalamus and corticosterone serum levels, together with a reduction of hypothalamic c-Fos expression, suggesting that modulation of the hypothalamus-pituitary-adrenal axis is necessary for the adaptogenic activity (Xia et al., 2016). This finding led us to study the possible modulation of molecular inflammatory processes elicited by *R. rosea* in an *in vitro* model of CRH-stimulated BV2 microglial cells.

To this aim, we evaluated the changes induced by CRH on microglial activation and cytokines production, and we analyzed some of the classical intracellular inflammatory pathways, such as NF- κ B nuclear translocation pathway, JNK, and MKK2. Finally, as heat shock proteins expression has been related to stress modulation (Asea et al., 2013; Panossian and Wikman, 2009), we measured the levels of HSP70 following CRH stimulation.

Materials and methods

Chemical analyses

The standardized pharmaceutical-grade *R. rosea* roots and rhizome extract WS@1375 (RHOD), furnished by Schwabe Pharma Italia (Egna, Italy), was used in this study.

Salidroside and rosavin content in RHOD was monitored by means of HPLC-DAD analysis.

A Shimadzu Prominence LC 2030 3D instrument equipped with a Bondapak[®] C18 column, 10 mm, 125 Å, 3.9 mm x 300 mm column (Waters Corporation, USA) was used.

Water + 0.1% V/V formic acid (A) and acetonitrile + 0.1% V/V formic acid (B) were used as mobile phase. The following program was applied: A: from 90% at 0 min to 60% in 40 min; flux was set at 1.0 ml/min. Chromatograms were recorded at 224 nm (salidroside) and 254 nm (rosavin).

Analyses were performed using 10 μ l of RHOD solution in ethanol 60% V/V (10 mg/ml) and salidroside and rosavin were used as external standards (Sigma-Aldrich, Milan, Italy). Calibration curves were established using reference standards ranging from 0.008 to 0.500 mg/ml. The correlation coefficient (R^2) of each curve was > 0.99 . The UV spectra of salidroside and rosavin are reported in the supplementary information in Fig. S5.

Cell culture

Murine microglial cells (BV2) were cultured in 75 cm² flasks (Sarstedt, Verona, Germany) in RPMI (Sigma-Aldrich) containing 10% heat-inactivated FBS (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich, Milan, Italy) and 1% penicillin-streptomycin solution (Sigma-Aldrich), until confluence (70-80%). EDTA-trypsin solution (Sigma-Aldrich) was used for detaching cells from flasks and cell counting was performed using a hemocytometer by Trypan blue staining. RHOD was solubilized in ethanol 60% V/V and further diluted in cell culture medium to reach the final working concentration. Based on the available literature (Lee et al., 2013) and according to previously performed dose-response assay (Fig. S1 and S4), treatments were CRH (Sigma-Aldrich) 100 nM, RHOD 20 µg/ml and RHOD 20 µg/ml + CRH 100 nM.

Cell viability

Cell viability was assessed by using CCK-8 (Sigma-Aldrich) assay, following the supplier instructions. 5×10^3 cells/well were seeded into 96-well plates and grown to confluence. Medium was then replaced with 100 µl of fresh RPMI containing treatments to the appropriate well. After the incubation time, 10 µl of CCK-8 was added to each well and incubated for 1 h. Absorbance was measured at 450 nm using a MP96 microplate reader spectrophotometer (Safas, Monte Carlo, Principality of Monaco). Treatments were performed in sextuplicate in three independent experiments and cell viability was calculated by normalizing values to the untreated control.

IL-6 production

The effect of the treatments on IL-6 production was evaluated by using non-competitive sandwich ELISA (Biolegend e-Bioscience DX Diagnostic, Monza, Italy), following the supplier instructions, as previously reported (Governa and Biagi, 2019). 1×10^5 cells/well were seeded into 24-well plates and grown to confluence. Medium was then replaced with 1000 µl of fresh RPMI containing treatments to the appropriate well. After 2 h, cells were lysed by freezing (-80 °C) and thawing (+24 °C) samples three times. Absorbance was measured at 450 nm using a MP96 microplate reader spectrophotometer (Safas). Treatments were performed in triplicate and cytokines production was dosed in duplicate (n=6), by normalizing values to the untreated control.

NF-κB nuclear translocation

The nuclear translocation of the p65 sub-unit of NF-κB was visualized by immunofluorescence indirect labeling. Briefly, 1.5×10^4 cells were seeded in a 8-well polylysine coated slide (Ibidi GmbH, Martinsried, Germany), and treated for 30 min with the respective drugs. Cells were fixed in acetone at -20 °C for 7 min. Antibodies were then diluted in permeabilization wash buffer (PBST) consisting of 1% BSA (Sigma-Aldrich) and 0.1% Triton X-100 (Sigma-Aldrich) in PBS. The primary Rabbit monoclonal NF-κB/p65 antibody (Cell Signaling, Danvers, MA, USA) was diluted 1:300 in PBST and added to the fixed cells. Samples were incubated for 30 min at room temperature. The primary antibody was removed and 1:200 dilution of secondary Alexa Fluor 488 anti-rabbit IgG antibody (ThermoFisher Scientific, Milan, Italy) was

added and incubated at room temperature in the dark for 45 min. Secondary antibody was removed by washing the cells with PBS and the coverslip was mounted on the 8-well glass slides using a mounting medium (Ibidi GmbH, Martinsried, Germany).

In order to confirm the results obtained from immunofluorescence assay, we quantified the nuclear translocation of the p65 sub-unit of NF- κ B by non-competitive sandwich ELISA (Abcam, Cambridge, United Kingdom). Briefly, 1×10^5 cells/well were seeded into 24-well plates and grown to confluence. Medium was then replaced with 1000 μ l of fresh RPMI containing treatments to the appropriate well. After 30, 60 and 120 min, cells were washed three times with PBS and subcellular fractionation were obtained by applying lysis buffers with increasing detergent strength, as previously reported (Baghirova et al., 2015). Total protein content of each sample was evaluated by Bradford (Sigma-Aldrich) method, using albumin (Sigma-Aldrich) as a reference standard. Non-competitive sandwich ELISA assay was performed on both whole cell and nucleus lysates according to the supplier instructions. Absorbance was measured at 450 nm using a MP96 microplate reader spectrophotometer (Safas). Treatments were performed in triplicate and NF- κ B nuclear translocation was calculated as the ratio between the nuclear and the total p65 sub-unit, normalizing values to the untreated control.

MAPK phosphorylation

The phosphorylation of p38, ERK and JNK was also evaluated by using non-competitive sandwich ELISA (Biolegend e-Bioscience DX Diagnostic, Monza, Italy), following the supplier instructions. 1×10^5 cells/well were seeded into 24 multiwell plates and grown to confluence. Medium was then replaced with 1000 μ l of fresh RPMI containing treatments to the appropriate well. After 1 h, cells were washed three times with PBS and lysed using 100 μ l of lysis buffer supplied with the kit. Total protein content of each sample was evaluated by Bradford (Sigma-Aldrich) method, using albumin (Sigma-Aldrich) as a reference standard. Absorbance was measured at 450 nm using a MP96 microplate reader spectrophotometer (Safas). Treatments were performed in triplicate and the phosphorylation rate of p38, ERK and JNK was calculated as the ratio between the phosphorylated and the total form, normalizing values to the untreated control.

Western blotting

The phosphorylation of MKK2 and the total protein levels and HSP70 were evaluated by western blotting. Proteins from control and treated cells were extracted by lysing cells in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Na deoxycholate, 1% Triton X-100, 2 mM PMSF) (Sigma-Aldrich) and quantified using Bradford colorimetric method (Pierce, Rockford, USA) according to the manufacturer's protocol. Equal amount of proteins, 0.5 μ g/ μ l for each sample, was loaded onto a pre-cast 12% SDS-PAGE (Invitrogen, Milan, Italy) and electrophoretically transferred to nitrocellulose membrane (Invitrogen). Membrane was blocked overnight at 4 °C in TBST buffer (20 mM Tris- HCl, 0.5 M NaCl and 0.05% Tween 20) containing 5% non-fat dried milk and incubated with the primary antibody anti-MKK2 (1:1000) and anti-HSP 70 (1:1000) (Cell signaling) at 5 °C overnight under gentle agitation. Membrane was then washed three

times in TBST, incubated for 1h with HRP-conjugated anti-rabbit or anti-mouse antibody (Cell Signaling) and visualized using chemiluminescence method (Amersham, GE Healthcare Europe GmbH, Milan, Italy). The immune-complexes were analyzed using densitometric analysis for determination of relative protein expression, using a BioRad GS 690 Imaging densitometer with molecular analysis software (Life Science, Milan, Italy) with β -actin as loading control.

Statistical analysis

Analysis were performed in duplicate in three independent experiments. The statistic differences were determined by the analysis of the variance (ANOVA). Shapiro-Wilk normality test was used to check the normality distribution of variables. Values are expressed in the range of \pm standard deviation and $p < 0,05$ was considered statistically significant. Graphs and calculations were performed using Graphpad Prism.

Results

Salidroside and rosavin content in RHOD

The used chromatographic method allowed us to separate and identify salidroside (retention time, RT,: 4.45 min) and rosavin (RT = 11.18 min) by comparison with RT and UV spectra of reference standards. Salidroside resulted to be $1.10\% \pm 0.06\%$ m/m, whereas rosavin was $2.71\% \pm 0.09\%$ m/m (Fig. 1).

RHOD counteracts CRH-induced BV2 metabolic activation

It is well known that BV2 microglial cells can be activated by a stressor signal and that this activation leads to a series of cellular and molecular changes (Borgonetti et al., 2019). Fig. 2a shows the changes of cell viability after 2, 6 and 24 h of CRH stimulations. The increase in cell viability, observed after 2 h of stimulation, can be a consequence of cells metabolic activation. RHOD had no effects on cell viability, thus, excluding potential cytotoxic effects. Nevertheless, when cells were pretreated with RHOD, the metabolic activation of BV2 induced by CRH was completely counteracted (Fig. 2b).

RHOD counteracts CRH-induced IL-6 production

Activated BV2 are known to release inflammatory cytokines (Borgonetti et al., 2019). Indeed, as shown in Fig. 3, IL-6 production was increased after CRH stimulation, compared to the untreated control, although no statistical significance was obtained. RHOD alone had no effect on IL-6 production. Interestingly, in the RHOD+CRH cells, IL-6 levels were similar to the untreated control.

RHOD inhibits NF- κ B nuclear translocation

NF- κ B translocation into the nucleus is one of the most studied signals for inflammatory response (Raha et al., 2016; Shabab et al., 2017). The stimulation of BV2 cells with CRH increased the nuclear translocation of NF- κ B p65 sub-unit after 30 min (data not shown), with a maximum translocation observed after 60 min (+

50% compared to the untreated control). RHOD, instead, reduced the CRH-induced increase of NF- κ B nuclear translocation by approximately 78% compared to CRH (Fig. 4).

RHOD modulates MKK2, ERK 1/2 and JNK phosphorylation

CRH stimulation was able to activate MKK2 by 1.3-fold compared to the untreated control, whereas RHOD alone decreased the activation of MKK2. MKK2 activation is related to ERK 1/2 phosphorylation (Ip and Davis, 1998). Consistently with the results obtained on MKK2, CRH stimulation also resulted in an increase of ERK 1/2 phosphorylation and the pretreatment with RHOD counteracted this effect. RHOD alone had no effect on ERK 1/2 phosphorylation. Furthermore, JNK phosphorylation was increased in CRH treated cells compared to control and the pretreatment with RHOD completely abolished the CRH-induced phosphorylation (Fig. 5).

RHOD counteracts the reduction of HSP70 expression induced by CRH

As depicted in Fig. 6, when stimulated with CRH for 1 h, the protein level of HSP70 decreased significantly in comparison to the untreated cells. Interestingly, the treatment of BV2 cells with RHOD in association with CRH was able to counteract the effect of CRH alone, by bringing back the HSP70 protein expression to the control levels. RHOD alone did not alter HSP70 expression.

Discussion

There is increasing evidence suggesting the involvement of inflammation in response to stress (Slavich and Irwin, 2014). Together with peripheral inflammation, an important connection between stress and neuroinflammation has been reported (Liu et al., 2017). The central nervous system reacts to stressors by releasing inflammatory cytokines, and by activating transcription factors and MAPKs (Liu et al., 2017). Moreover, a recent systematic review of preclinical studies highlighted the activation of microglia, expressed as the increase of the ionized calcium binding adaptor molecule 1, as a consequence of stress (Liu et al., 2017). Stress-induced neuroinflammation encompasses the activation of several inflammatory markers, such as NO, iNOS, COX-2, NMDA receptors and NF- κ B (Munhoz et al., 2008).

Whether the modulation of neuroinflammatory parameters could be involved in the anti-stress effect of adaptogens has been barely studied. Nevertheless, a protective effect against neuroinflammation has been observed for other adaptogens, such as *Panax ginseng* C.A. Meyer and its constituent ginsenoside Rb1, has been studied (Ahmed et al., 2016). The anti-inflammatory activity of *R. rosea* has been reported *in vivo* (Bawa and Khanum, 2009) and the neuroprotective effect of a crude extract of *R. rosea* and its main constituents against iNOS, TNF- α , IL-1 β and IL-6 production and has been studied *in vitro* in LPS-stimulated BV2 microglial cells (Bawa and Khanum, 2009; Lee et al., 2013). Moreover, *R. rosea* derivatives were also found to possess neuroprotective activity (Xian et al., 2014). The effect of *R. rosea* on gene expression profile in isolated human brain glioblastoma cells was evaluated by Panossian and coworkers,

who reported the modulation of at least 1062 genes, many of which are involved in neurological, behavioral and psychological disorders (Panossian et al., 2014).

Here, we propose an *in vitro* model of a stress-induced neuroinflammatory condition in BV2 microglial cells, by using CRH as a physiologic stressor instead of the canonical LPS. The study of adaptogen drugs is a fascinating challenge in the field of pharmacology of natural compounds. Indeed, adaptogens are currently represented only by herbal products, which may include mixtures of several constituents, acting on different intracellular pathways. Nevertheless, the lack of a well-known monomolecular adaptogen drug makes it very difficult to compare the mechanistic effects exerted *in vitro* by herbal products with a reference positive control. However, the comparison with the negative control (untreated cells) and the stress stimulation (CRH-treated cells) gave us an interesting indication on the adaptogen mechanism of action of RHOD.

Indeed, CRH stimulation was able to activate cell metabolism, to slightly increase IL-6 production and to promote NF- κ B nuclear translocation. Moreover, the microglial inflammatory response to CRH is mediated by the MAPK pathways, particularly by the phosphorylation of MKK2 and ERK 1/2, but also of JNK. We were not able to observe neither p38 phosphorylation nor STAT2 activation (see supplementary information), which does not seem to participate in the response of BV2 to a stress stimulus. HSP70 expression is thought to be modulated in response to stress, and this has been confirmed in our model, in which CRH reduced HSP70 levels, compared to the untreated control.

Other stress mediators may be involved in the mechanism of action of adaptogens. Indeed, Panossian and co-workers reported that at least 88 out of 3516 genes regulated by adaptogens are closely associated with adaptive stress response. Among them, the most representative include genes encoding CRH, HSPs and genes involved in the MAPK pathway and neuroinflammatory signaling, (Panossian et al., 2018). In particular, the involvement of urocortins (UCNs; Ucn 1, Ucn 2 and Ucn 3) is considered a novel and interesting field of study, due to their stress-coping, cytoprotective and anti-inflammatory effects mediated by corticotropin-releasing hormone receptor 2 (Lawrence et al., 2015). However, the stress-protective effect of *R. rosea* was found to be related to CRH and MAPK modulation, whereas no effects were observed on UCNs gene expression (Panossian et al., 2018). In our work, we observed similar effects on CRH, MAPK, and HSP70, which are consistent with that of Panossian and colleagues (2018), thus, excluding the need to evaluate the modulation of UCNs by RHOD.

Interestingly, in T98G neuroglia cells, adaptogens were found to up-regulate the *PLCB1* gene, which encodes phospholipase C and *PI3KC2G*, encoding phosphatidylinositol 3-kinases (PI3Ks), which is a key upstream regulator of NF- κ B pathway (Panossian et al., 2013). In our work we consistently observe the inhibition of CRH-stimulated NF- κ B activation by RHOD.

The adaptogenic activity of *R. rosea* has been reported to be concentration-dependent and to vary depending on the extract used (Panossian et al., 2010a). Similarly, the modulation of HSPs gene and protein expression was found to be strictly related to the concentration of *R. rosea* extract and salidroside used (Panossian et al., 2014, 2009). Thus, in this work we used a standardized pharmaceutical-grade *R. rosea* extract, which therapeutic doses in human is established by the European Medicines Agency, and we chose the active

concentration based on the available information on *R. rosea* extract pharmacokinetics (Panossian et al., 2010b), and on the concentration-response curve performed on HSP70 modulation analysis (Fig. S4). We found the active concentration of RHOD in our *in vitro* model to be 20 µg/ml which contained approximately 0.22 µg/ml of salidroside and 0.77 µg/ml of total rosavins. This concentration is higher than that reported by Panossian and colleagues (2014, 2009), who, however, used a different *R. rosea* extract (i.e. SHR-5) with different salidroside and rosavins content.

Conclusion

The results obtained in this *in vitro* model, confirm the adaptogenic activity of *R. rosea*. This activity is related to a modulation of the stress-induced microglia activation by CRH. In particular, we found that *R. rosea* might act through the control of two pivotal players of the neuroinflammatory pathway, such as NF-κB nuclear translocation and MAPK phosphorylation, resulting in the modulation of HSP70 levels.

In conclusion, the *in vitro* model of stress-induced neuroinflammation here reported gave information on the molecular mechanism involved in *R. rosea* anti-stress activity and may be useful for the study of other adaptogen drugs.

Authors contribution:

Vittoria Borgonetti:

Investigations, formal analysis, writing - original draft

Paolo Governa:

Investigations, formal analysis, writing - original draft

Marco Biagi:

Conceptualization, validation, resources, writing – review & editing

Pasquale Dalia:

Investigations

Lorenzo Corsi:

Conceptualization, validation, resources, writing – review & editing

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Conflict of interest

The authors declare that there are no conflicts of interest.

References

Ahmed, T., Raza, S.H., Maryam, A., Setzer, W.N., Braidy, N., Nabavi, S.F., de Oliveira, M.R., Nabavi, S.M., 2016. Ginsenoside Rb1 as a neuroprotective agent: a review. *Brain Res. Bull.* 125, 30–43.

- Asea, A., Kaur, P., Panossian, A., Wikman, K.G., 2013. Evaluation of molecular chaperons Hsp72 and neuropeptide Y as characteristic markers of adaptogenic activity of plant extracts. *Phytomedicine* 20, 1323–1329.
- Baghirova, S., Hughes, B.G., Hendzel, M.J., Schulz, R., 2015. Sequential fractionation and isolation of subcellular proteins from tissue or cultured cells. *MethodsX* 2, 440–445.
- Bawa, A.S., Khanum, F., 2009. Anti-inflammatory activity of *Rhodiola rosea*--"a second-generation adaptogen". *Phytother. Res.* 23, 1099–1102.
- Booker, A., Jalil, B., Frommenwiler, D., Reich, E., Zhai, L., Kulic, Z., Heinrich, M., 2016. The authenticity and quality of *Rhodiola rosea* products. *Phytomedicine* 23, 754–762.
- Borgonetti, V., Governa, P., Montopoli, M., Biagi, M., 2019. *Cannabis sativa* L. constituents and their role in neuroinflammation. *Curr. Bioact. Compd.* 15, 147–158.
- Chiang, H.-M., Chen, H.-C., Wu, C.-S., Wu, P.-Y., Wen, K.-C., 2015. *Rhodiola* plants: chemistry and biological activity. *J. Food Drug Anal.* 23, 359–369.
- European Medicines Agency, 2012. EMA Assessment report on *Rhodiola rosea* L., rhizoma et radix [WWW Document]. URL http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-HMPC_assessment_report/2012/05/WC500127861.pdf (accessed 4.5.19).
- Governa, P., Biagi, M., 2019. *Copaifera langsdorffii* Desf. : *in vitro* investigation on anti- *Helicobacter pylori* and anti-inflammatory activities of oleoresin and fruit methanolic extract. *Plant Biosyst. An Int. J. Deal. with all Asp. Plant Biol.*
- Hung, S.K., Perry, R., Ernst, E., 2011. The effectiveness and efficacy of *Rhodiola rosea* L.: A systematic review of randomized clinical trials. *Phytomedicine* 18, 235–244.
- Ip, Y.T., Davis, R.J., 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. *Curr. Opin. Cell Biol.* 10, 205–219.
- Ishaque, S., Shamseer, L., Bukutu, C., Vohra, S., 2012. *Rhodiola rosea* for physical and mental fatigue: a systematic review. *BMC Complement. Altern. Med.* 12, 70.
- Lawrence, K.M., Jackson, T.R., Jamieson, D., Stevens, A., Owens, G., Sayan, B.S., Locke, I.C., Townsend, P.A., 2015. Urocortin--from Parkinson's disease to the skeleton. *Int. J. Biochem. Cell Biol.* 60, 130–138.
- Lee, Y., Jung, J.-C., Jang, S., Kim, J., Ali, Z., Khan, I.A., Oh, S., 2013. Anti-Inflammatory and neuroprotective effects of constituents isolated from *Rhodiola rosea*. *Evid. Based. Complement. Alternat. Med.* 2013, 514049.

- Liu, Y.-Z., Wang, Y.-X., Jiang, C.-L., 2017. Inflammation: the common pathway of stress-related diseases. *Front. Hum. Neurosci.* 11, 316.
- Mattioli, L., Funari, C., Perfumi, M., 2009. Effects of *Rhodiola rosea* L. extract on behavioural and physiological alterations induced by chronic mild stress in female rats. *J. Psychopharmacol.* 23, 130–142.
- Mattioli, L., Perfumi, M., 2007. *Rhodiola rosea* L. extract reduces stress- and CRF-induced anorexia in rats. *J. Psychopharmacol.* 21, 742–750.
- Munhoz, C.D., Garcia-Bueno, B., Madrigal, J.L.M., Lepsch, L.B., Scavone, C., Leza, J.C., 2008. Stress-induced neuroinflammation: mechanisms and new pharmacological targets. *Brazilian J. Med. Biol. Res.* 41, 1037–1046.
- Nakatani, Y., Amano, T., Tsuji, M., Takeda, H., 2012. Corticosterone suppresses the proliferation of BV2 microglia cells via glucocorticoid, but not mineralocorticoid receptor. *Life Sci.* 91, 761–770.
- Panossian, A., Hamm, R., Wikman, G., Efferth, T., 2014. Mechanism of action of *Rhodiola*, salidroside, tyrosol and triandrin in isolated neuroglial cells: an interactive pathway analysis of the downstream effects using RNA microarray data. *Phytomedicine* 21, 1325–1348.
- Panossian, A., Seo, E.J., Efferth, T., 2018. Novel molecular mechanisms for the adaptogenic effects of herbal extracts on isolated brain cells using systems biology. *Phytomedicine* 50, 257–284.
- Panossian, A., Wikman, G., 2009. Evidence-based efficacy of adaptogens in fatigue, and molecular mechanisms related to their stress-protective activity. *Curr. Clin. Pharmacol.* 4, 198–219.
- Panossian, A., Wikman, G., Kaur, P., Asea, A., 2009. Adaptogens exert a stress-protective effect by modulation of expression of molecular chaperones. *Phytomedicine* 16, 617–622.
- Panossian, A., Wikman, G., Sarris, J., 2010a. Rosenroot (*Rhodiola rosea*): traditional use, chemical composition, pharmacology and clinical efficacy. *Phytomedicine* 17, 481–493.
- Panossian, A.G., 2013. Adaptogens in mental and behavioral disorders. *Psychiatr. Clin. NA* 36, 49–64.
- Panossian, A.G., 2003. Adaptogens: tonic herbs for fatigue and stress. *Altern. Complement. Ther.* 9, 327–331.
- Panossian, A.G., Hovhannisyanyan, A., Abrahamyan, H., Gabrielyan, E., Wikman, G., 2010b. Pharmacokinetics of active constituents of *Rhodiola rosea* SHR-5 extract, in: Gupta, V.K. (Ed.), *Comprehensive Bioactive Natural Products, Vol 2. Efficacy, Safety and Clinical Evaluation*. Studium Press, LCC, pp. 307–329.

- Panossian, A.G., Hamm, R., Kadioglu O., Wilkman G., Efferth T., 2013. Synergy and antagonism of active constituents of ADAPT-232 on transcriptional level of metabolic regulation of isolated neuroglial cells. *Front. Neurosci.* 7, 16.
- Perfumi, M., Mattioli, L., 2007. Adaptogenic and central nervous system effects of single doses of 3% rosavin and 1% salidroside *Rhodiola rosea* L. extract in mice. *Phytother. Res.* 21, 37–43.
- Raha, S., Lee, H.J., Yumnam, S., Hong, G.E., Venkatarama Gowda Saralamma, V., Ha, Y.L., Kim, J.O., Kim, Y.S., Heo, J.D., Lee, S.J., Kim, E.-H., Kim, G.S., 2016. Vitamin D2 suppresses amyloid-beta 25-35 induced microglial activation in BV2 cells by blocking the NF-kappaB inflammatory signaling pathway. *Life Sci.* 161, 37–44.
- Shabab, T., Khanabdali, R., Moghadamtousi, S.Z., Kadir, H.A., Mohan, G., 2017. Neuroinflammation pathways: a general review. *Int. J. Neurosci.* 127, 624–633.
- Slavich, G.M., Irwin, M.R., 2014. From stress to inflammation and major depressive disorder: a social signal transduction theory of depression. *Psychol. Bull.* 140, 774–815.
- Xia, N., Li, J., Wang, H., Wang, J., Wang, Y., 2016. *Schisandra chinensis* and *Rhodiola rosea* exert an anti-stress effect on the HPA axis and reduce hypothalamic c-Fos expression in rats subjected to repeated stress. *Exp. Ther. Med.* 11, 353–359.
- Xian, H., Zhao, J., Zheng, Y., Wang, M., Huang, J., Wu, B., Sun, C., Yang, Y., 2014. MADP, a salidroside analog, protects hippocampal neurons from glutamate induced apoptosis. *Life Sci.* 103, 34–40.

Figure legends

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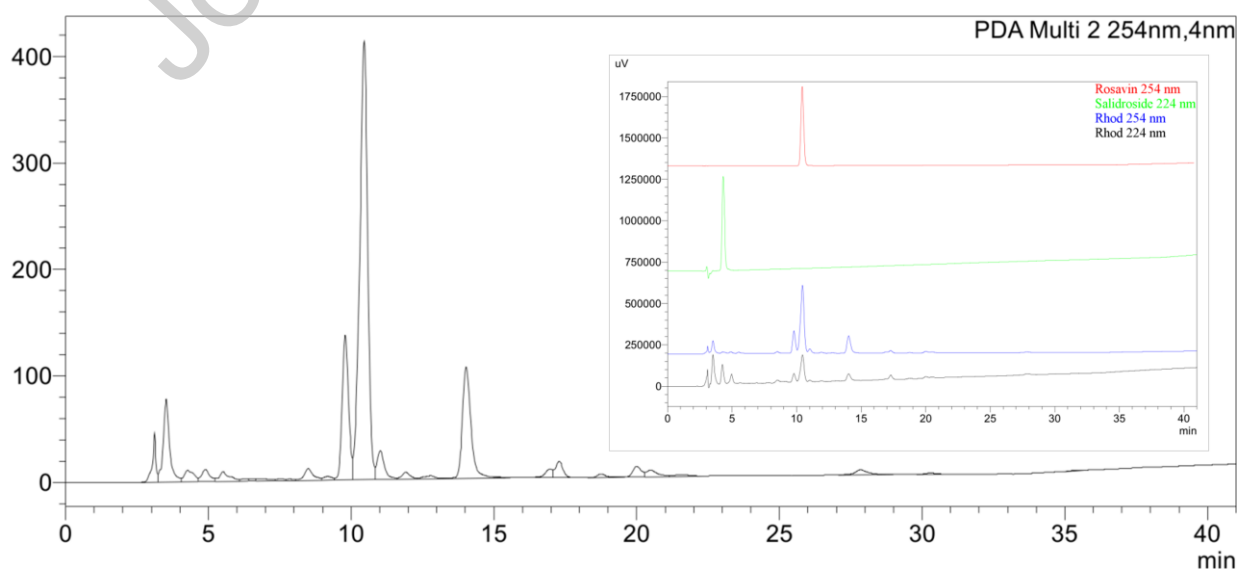


Figure 1. HPLC-DAD chromatogram of RHOD recorded at 254 nm. Rosavin content in the extract resulted to be $2.71\% \pm 0.09\%$ m/m (RT = 11.18 min), whereas salidroside was $1.10\% \pm 0.06\%$ m/m (RT = 4.45 min). The small panel shows the comparison between the chromatograms of RHOD recorded at 254 nm (blue) and 224 nm (black). Chromatograms of rosavin (red) and salidroside (green) are also reported.

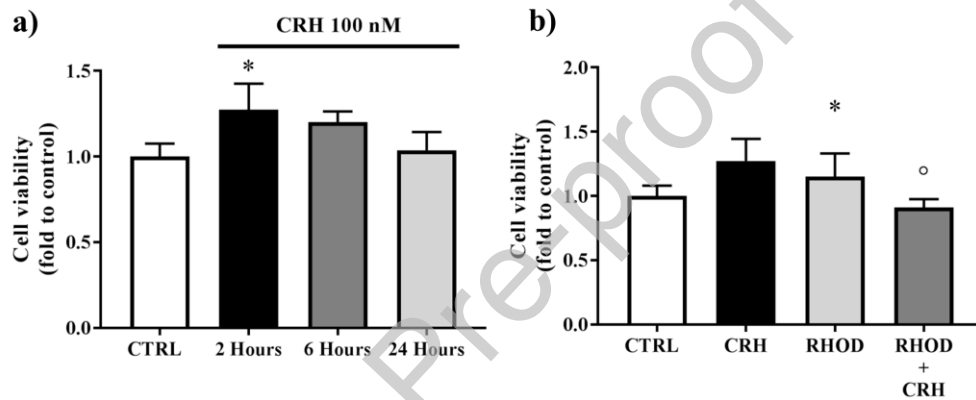


Figure 2. CRH-induced BV2 metabolic activation time course (a) and effect of RHOD pretreatment (30 min) on CRH-activated cells after 2 h of stimulation. * $p < 0.05$ vs control; ° $p < 0.05$ vs stimulus.

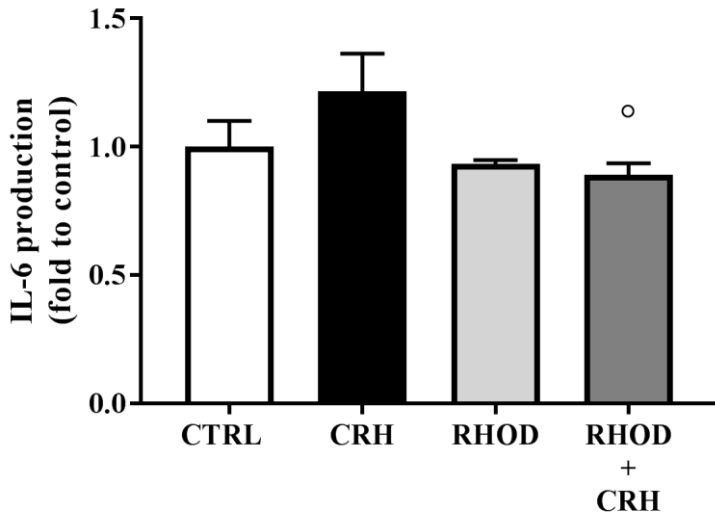


Figure 3. Effect of RHOD pretreatment (30 min) on CRH-induced IL-6 production. ° $p < 0.05$ vs stimulus.

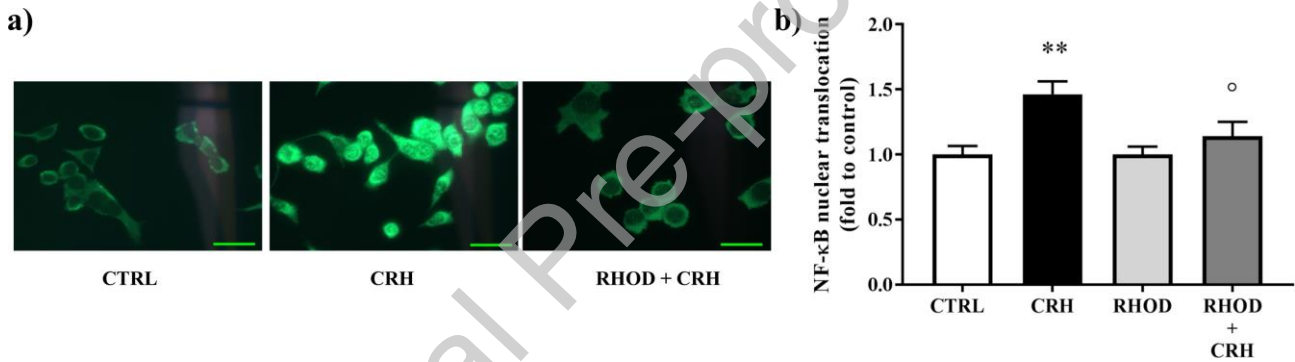


Figure 4. NF-κB nuclear translocation observed by immunofluorescence (a) and by non-competitive ELISA (b), after 60 min of CRH stimulation. ** $p < 0.01$ vs control; ° $p < 0.05$ vs stimulus. Scale bar = 50 μm.

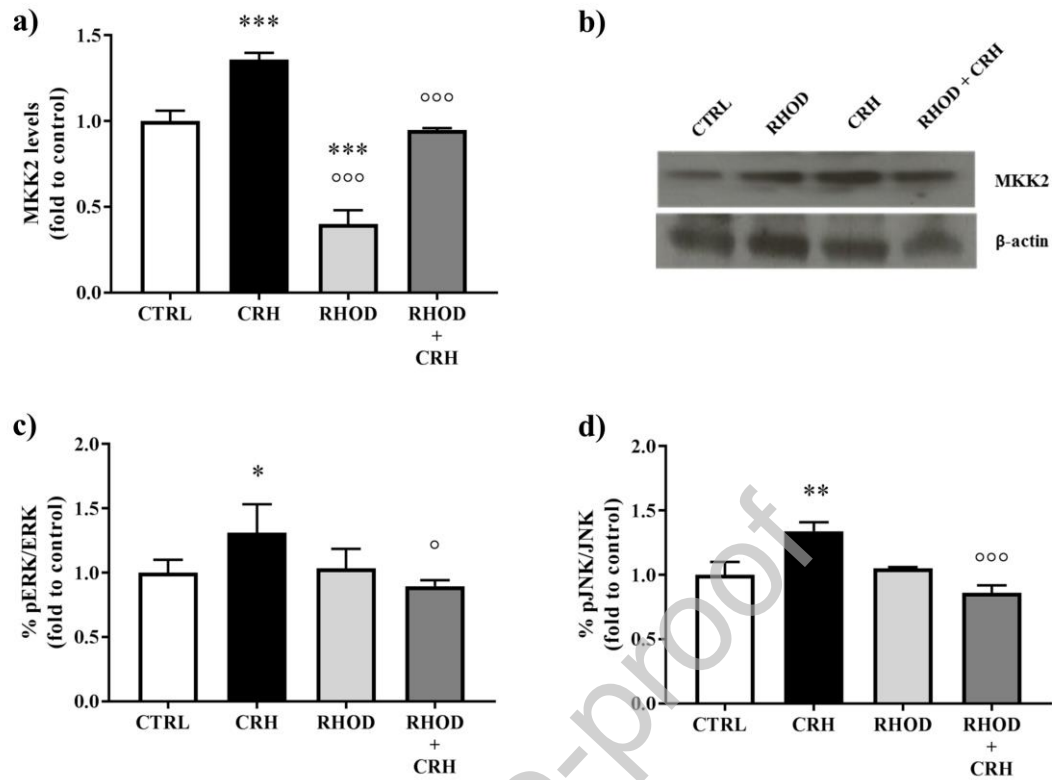


Figure 5. Relative protein quantification, normalized to β -actin (a) and representative western blot bands (b) of MKK2. Modulation of ERK 1/2 (c) and JNK (d) activation quantified by non-competitive ELISA. * $p < 0.05$ vs control; ** $p < 0.01$ vs control; *** $p < 0.001$ vs control; ° $p < 0.05$ vs stimulus; °°° $p < 0.001$ vs stimulus.

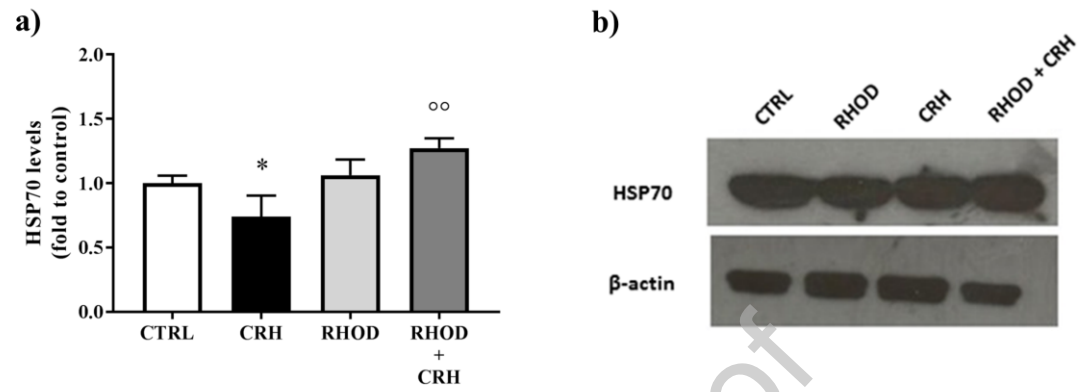
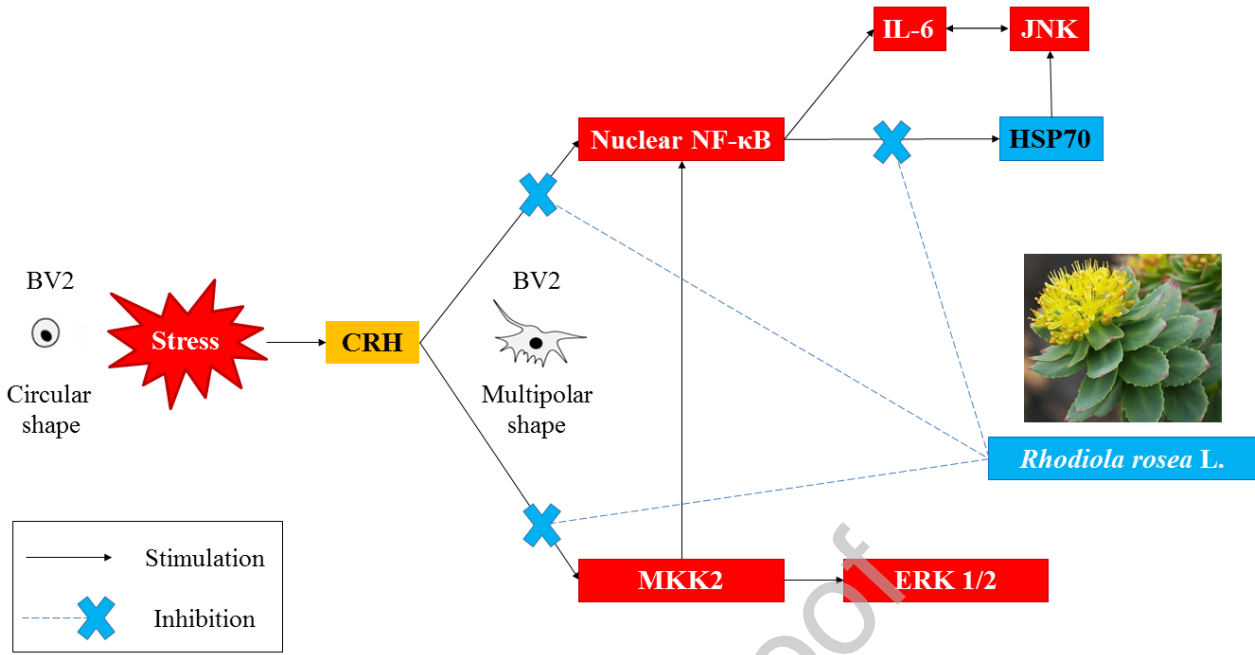


Figure 6. Relative protein quantification, normalized to β -actin (a) and representative western blot bands (b) of HSP70. * $p < 0.05$ vs control; °° $p < 0.01$ vs stimulus.



Graphical_abstract