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THE EFFECTS OF RESVERATROL AND SULFORAPHANE ON THE REGULATION OF INOS/NRF2 PATHS IN THE OROFACIAL INFLAMMATION-INDUCED MODEL

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ABSTRACT

This study aimed to identify the effect of resveratrol and sulforaphane on the regulation of inflammation in the orofacial inflammation-induced model. Orofacial inflammation was induced by injecting 5% formalin (50 μl) under the skin of the right side of the face of rats using an insulin syringe. Resveratrol and sulforaphane were found to suppress the secretion of IL-6 or IL-1β and regulate the expression of Nrf2 (or NFE2L2: Nuclear factor (erythroid-derived 2)-like 2), iNOS (inducible nitric oxide synthases) or Nox4 (NADPH oxidase 4). Resveratrol and sulforaphane seem to regulate pro-inflammatory cytokines (IL-6 or IL-1β) and iNOS/Nrf2, and thus to reduce and protect against orofacial inflammation.

KEYWORD: Resveratrol, Sulforaphane, Cytokine, iNOS, Nrf2.

INTRODUCTION

Resveratrol is a stilbenoid (molar mass: 228.25) produced by a stilbene synthase (STS) in plants,[1,2,3] and is found in various plants such as mulberries, peanuts, grapes, raspberries and cranberries. Resveratrol is a type of phytoalexin produced when plants are under stress.[4,5] and grapes produce resveratrol, a strong antibacterial substance, to defend themselves from the attack of fungi. That is why more resveratrol is found in grapes than other plants. In addition, resveratrol is known to have strong anticancer and antioxidant effects and to reduce the level of serum cholesterol.[6] It is also reported to have antiviral, neuroprotective, anti-inflammatory and anti-aging effects and extend lifespan.[7,8,9,10]

Sulforaphane is a compound within the isothiocyanate group[11] and its molar mass is 177.29. The glucosinolates, known to have a strong antioxidant effect, are hydrolyzed by myrosinase produced by enterobacteria in the process of digestion,[12] and thus produce isocyanate (ITC), indole-3-carbinol (I3C), allycyanate and sulforaphane.[13] Sulforaphane is found in cruciferous vegetables such as cabbage, broccoli, cauliflower, kale and red radish.[14] It is known to have an anticancer effect and inhibit the growth of Helicobacter pylori as well as the activity of pro-inflammatory factors, and earlier studies that used a rat model also reported that sulforaphane is very effective in inhibiting the growth of skin cancer.[15,16,17]

Orofacial inflammation is the pain caused by inflammation in the mouth and face that are densely innervated and psychologically sensitive, and pain disorders associated with orofacial inflammation include trigeminal neuralgia, deafferentation pain and burning mouth syndrome. In particular, trigeminal neuralgia is a neuropathic disorder that is commonly found in the mouth and facial area. Patients with trigeminal neuralgia experience sudden and electric shock-like pain in one side of the face even due to normal stimuli that normally do not cause any pain, such as washing the face, brushing the teeth or eating food. They mostly feel the pain only in one side of the face. They do not feel any pain without any stimulus, but stimuli applied to the area trigger severe pain again. In this regard, this study aimed to analyze the effects of sulforaphane and resveratrol on the regulation of inflammation using an orofacial inflammation-induced animal model.

METHODS

Laboratory Animals

In this study, Sprague-Dawley white male rats (240~280 g) purchased from Hyochang Science (Daegu, Korea) were used. Water and feed were freely provided, and the 12-hour day and night shift and a constant temperature (23-24℃) were maintained. This study was conducted according to the ethical standards of the Korean Pain Research Society for experiments on conscious animals.

Preparation of Reagents

Resveratrol and sulforaphane were purchased from Sigma Aldrich (St. Louis, MO, USA), and 100% DMSO

and 2.5% DMSO were used as a solvent for resveratrol and sulforaphan respectively. The concentration of resveratrol used in this study was 15 µg/ 50µl (0.3 mg/ml) or 150 µg/50µl (3 mg/ml), and that of sulforaphan was 20µg/ 50µl (0.4 mg/ml) or 200 µg/50µl (4 mg/ml).

Orofacial Inflammation-induced Model

In this study, 5% formalin (50 µl) was injected under the skin of the right side of the face of laboratory animals using an insulin syringe. Formalin was used after diluting it with normal saline solution.

Sacrifice of Laboratory Animals and Collection of Samples

All the laboratory animals in this study were fasted for 24 hours prior to sacrifice. After measuring their final weight, they were anesthetized with Avertin and 7-8 mL of blood was collected from their vena cava. After checking that the blood collected in serum separator tubes was coagulated, the samples were centrifuged at 3000 rpm for 15 minutes, and the centrifuged serum was moved to E-tubes and was stored at –70℃ for cytokine analysis.

Cytokine Analysis

Each cytokine (IL-6, IL-1β) was measured using ELISA kits purchased from Koma Biotech (Seoul, South Korea). The test was carried out on 96-well flat-bottom ELISA plates according to the procedures stated in the manual of the kit, and the absorbance of the samples was measured at 450 nm using a microplate reader. The concentration of IL-6 and IL-1β within the collected serum was calculated using the calibration curve of standard solution (18).

Tissue Extraction and Protein Quantification

The medulla oblongata of laboratory animals was removed and tissues were collected for protein quantification analysis. The removed medulla oblongata was minced with a surgical blade, and a proper amount of a lysis buffer (PRO-PrepTM, protein Extraction solution) was added to the collected tissues depending on their amount. They were homogenized at 4℃ using a homogenizer. After analyzing them using a western blot quantification method, primary antibodies (iNOS, Nrf2, NOX4, β-actin) were incubated at 4℃ overnight. They were washed 5 times, and secondary antibodies were added for 2 hours at room temperature. The expression of antibodies was analyzed using Amersham ECL Prime detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK), and was quantified using VisionWorks Analysis Software (UVP, Cambridge, UK).

Statistical Analysis

The statistical significance of data was expressed as mean value±standard deviation using SPSS Version 18. Differences between the control group and the experimental groups were analyzed using One-way ANOVA. When the p-value is lower than 0.05, the difference was regarded to be statistically significant.

RESULTS

Changes in Pro-inflammatory Cytokines Caused by Resveratrol and Sulforaphane

Changes in the IL-6 concentration in the serum of orofacial inflammation-induced animals caused by resveratrol and sulforaphane were measured, and the IL-6 concentration in the serum of the naive group was 132.50±3.1; the formalin-treated group, 402.54±11.5; the resveratrol-treated group, 198.83±8.5; the sulforaphane-treated group, 218.54±6.2; and the resveratrol and sulforaphane-treated group (Res.+Sul.), 148.10±4.8 pg/ml (Figure 1). That is, the IL-6 concentration of the Res.+Sul. group was similar to that of the naive group, indicating that resveratrol and sulforaphane are effective in regulating orofacial pain.

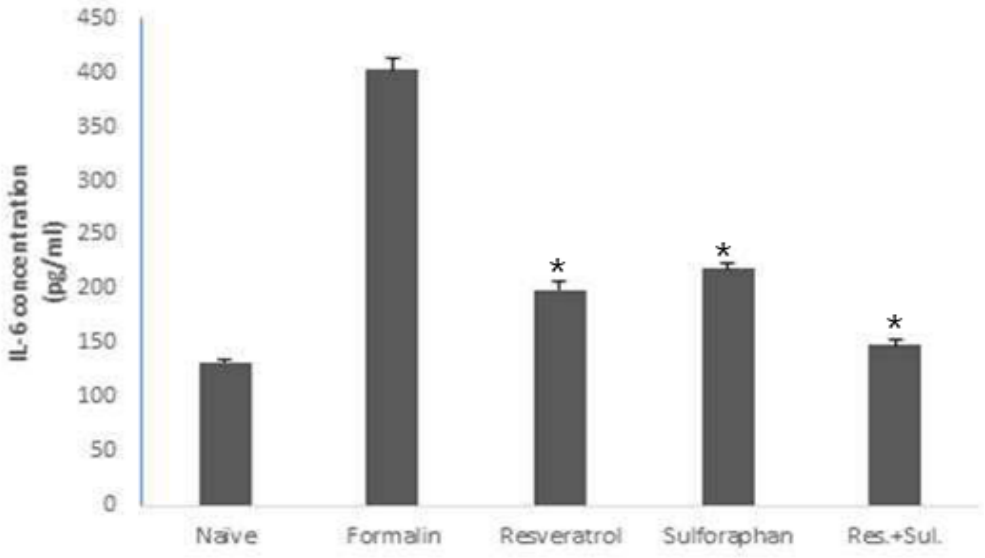

Figure 1: Changes in IL-6 in the serum of rats caused by resveratrol and sulforaphane.

***, p 〈0.05 (compared with the formalin-treated group).**

The IL-1β concentration in the serum of the naive group was 42.32±0.9; the formalin-treated group, 271.40±8.8; the resveratrol-treated group, 123.86±6.3; the sulforaphane-treated group, 140.46±5.9; and the resveratrol and sulforaphane-treated group (Res.+Sul.), 88.7±3.8 pg/ml (Figure 2). That is, the IL-1β concentration of the Res.+Sul. group was statistically significantly lower than that of the formalin-treated group.

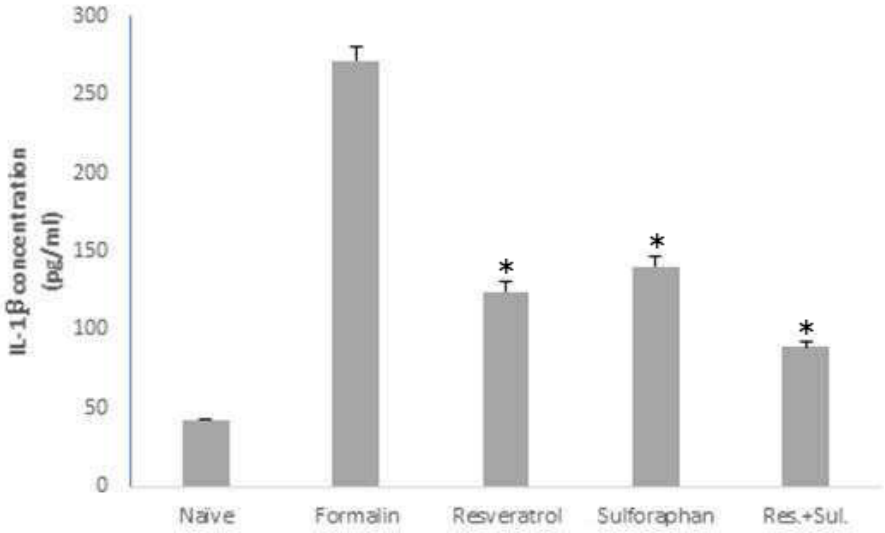

Figure 2: Changes in IL-1β in the serum of rats caused by resveratrol and sulforaphane.

***, p 〈0.05 (compared with the formalin-treated group).**

Changes in the Expression of Inflammatory Signal Transduction Factors Caused by Resveratrol and Sulforaphane

Changes in the expression of Nrf2 (or NFE2L2: Nuclear factor (erythroid-derived 2)-like 2), iNOS (inducible nitric oxide synthases) and Nox4 (NADPH oxidase 4), known as inflammatory signal transduction factors, in the brain of orofacial inflammation-induced rats were measured in order to identify the effects of resveratrol and sulforaphane on the level of inflammation. The expressed level of Nrf2 of the control group (formalin-treated) was 1.62±0.18; the resveratrol-treated group, 1.18±0.21; the sulforaphane-treated group, 0.89±0.08; and the resveratrol and sulforaphane-treated group (Res.+Sul.), 0.69±0.09 (Figure 3). The expressed level of Nrf2 of the Res.+Sul. group was statistically significantly lower than that of the control group.

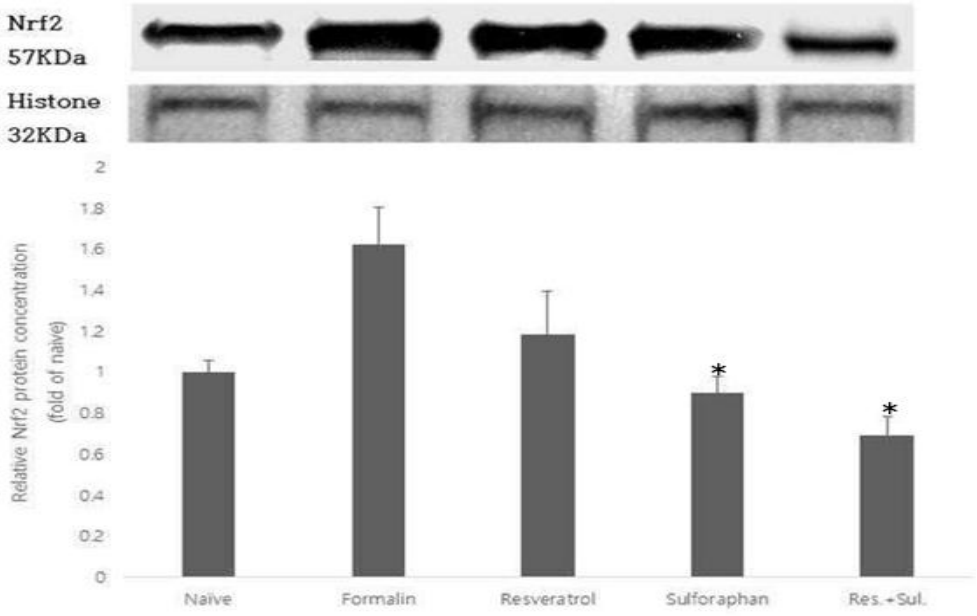

Figure 3: Differences in the expression of Nrf2 in the oblongata medulla of rats.

***, p〈0.05 (compared with the formalin-treated group).**

The expressed level of iNOS of the control group (formalin-treated) was 1.85±0.35; the resveratrol-treated group, 2.14±0.19; the sulforaphane-treated group, 1.66±0.218; and the resveratrol and sulforaphane-treated group (Res.+Sul.), 0.87±0.16 (Figure 4). The expressed level of iNOS of the Res.+Sul. group was statistically significantly lower than that of the control group.

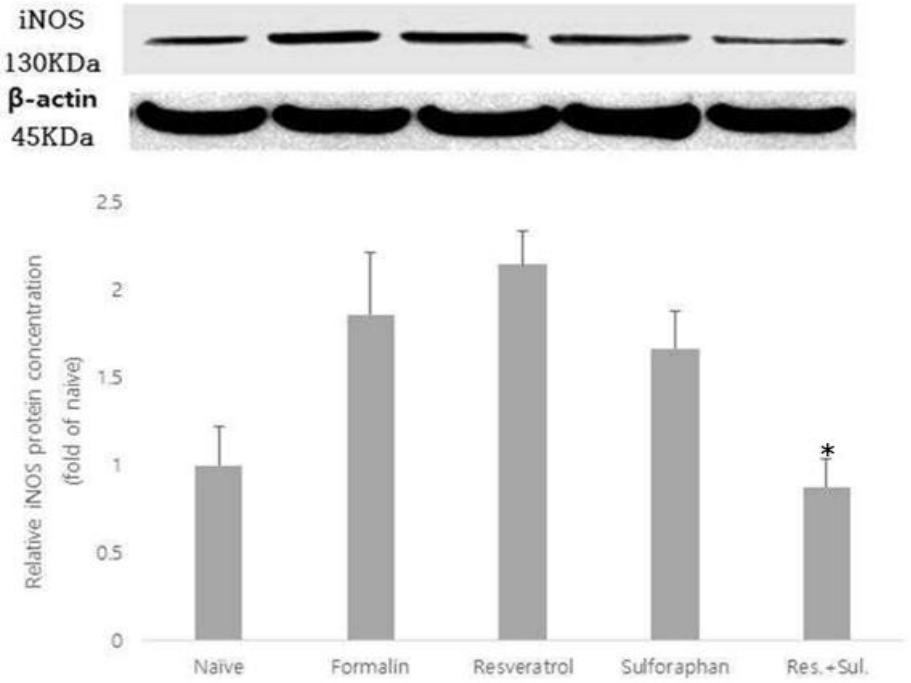

Figure 4: Differences in the expression of iNOS in the oblongata medulla of rats.

***, p〈0.05 (compared with the formalin- treated group).**

The expressed level of Nox4 of the control group (formalin-treated) was 3.15±0.18; the resveratrol-treated group, 3.42±0.20; the sulforaphane-treated group, 2.17±0.15; and the resveratrol and sulforaphane-treated group (Res.+Sul.), 0.89±0.106 (Figure 5). The expressed level of Nox4 of the Res.+Sul. group was statistically significantly lower than that of the control group, which indicates that treating with both resveratrol and sulforaphane is effective in regulating pain, and thus relieving, preventing or treating orofacial pain.

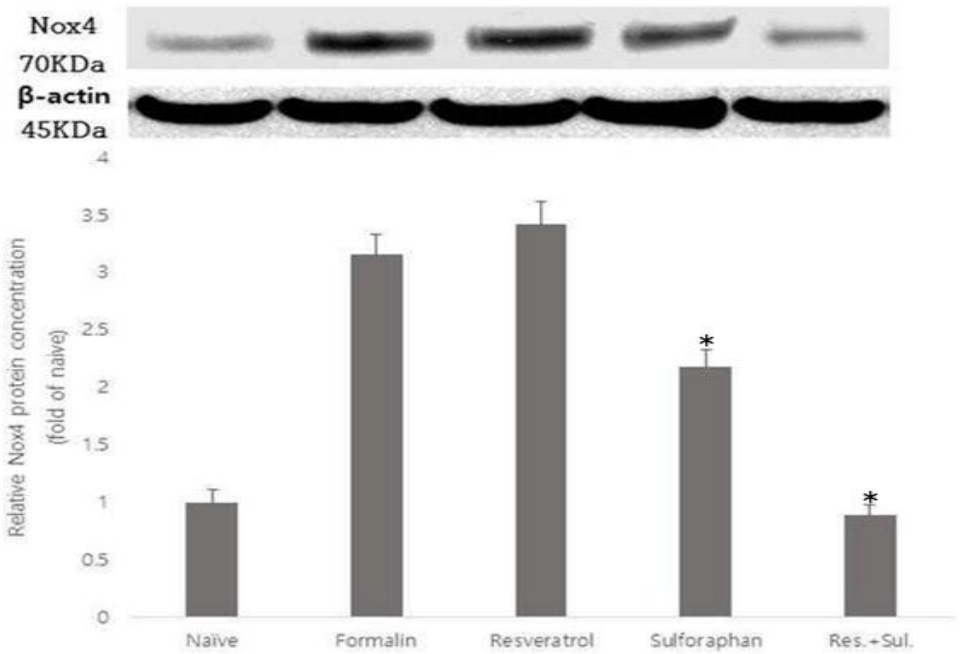

Figure 5: Differences in the expression of NOX4 in the oblongata medulla of rats.

***, p〈0.05 (compared with the formalin-treated group).**

DISCUSSION

In this study, the effects of resveratrol and sulforaphane on pain regulation were examined using an orofacial inflammation-induced model.

Pro-inflammatory cytokines are very important to promoting inflammation, and they are produced by macrophages, increasing white blood cells in blood and activating inflammatory signal transduction proteins such as NF-kB (19, 20). It was reported that the intraperitoneal injection of montelukast to inflammation-induced rats associated with neuropathic disorders caused by sciatic nerve compression statistically significantly reduced the level of IL-1β and IL-6 in the spinal cord, indicating that montelukast was effective in regulating inflammation caused by the neuropathic disorders (21). These results show that it plays an important role in regulating pro-inflammatory cytokines. In this study, the concentration of IL-1β and IL-6, pro-inflammatory cytokines, was reduced in the groups treated with sulforaphane and resveratrol, and, in particular, the secretion of cytokines in the group treated with the two substances together was suppressed, reducing inflammatory reactions. The expression of signal transduction proteins including Nrf2, iNOS and NOX4 was also reduced in the groups treated with sulforaphane and resveratrol, and, in particular, the expression of the proteins in the group treated with the two substances together was suppressed, showing similar results to those of the secretion of pro-inflammatory cytokines. The combination of the two substances seems to have a synergic effect in regulating inflammation. Therefore, it can be concluded that the two substances prevent the deformation and destruction of tissues caused by inflammation, and reduce and suppress the symptoms of inflammation, indicating that resveratrol and sulforaphane have an anti-inflammatory effect in the orofacial inflammation-induced model.

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