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Research Article
Inhibitory Effects of a Novel Nrf2 Activator Rs9 on 2,4,6-Trichlorobenzene-Induced Contact Dermatitis in Mice

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Abstract
Allergic contact dermatitis is an itchy skin condition caused by cell-mediated immune responses due to contact with haptens. The Nrf2 is a transcriptional factor which regulates many antioxidants and it is recently succeeded in obtaining a novel Nrf2 activator, RS9. The efficacy of RS9 in a contact dermatitis model of BALB/c mice was investigated. Topical application of 2,4,6-trichlorobenzene significantly induced ear swelling and the application of RS9 to the ear ameliorated the allergic responses compared to other triterpenoids such as RTA 402 (bardoxolone methyl). These results indicate that RS9 is a promising compound for dermatitis by activating the Nrf2 pathway.

Key words: Nrf2, RS9, contact dermatitis, hepa1c1c 7 cells, triterpenoids

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Allergic contact dermatitis is a chronic cutaneous inflammatory disease, which is characterized by redness, itching, pruritus and eczema, however the exact pathogenesis still remains to be solved. The main reason of contact dermatitis is considered to be delayed-type hypersensitivity reactions in the skin, involving the migration of hapten-specific T cells (Saint-Mezard *et al*., 2004). Topical application of hapten such as 2,4,6-trinitro-1-chlorobenzene (TNCB) is generally used to induce contact dermatitis experimentally (Jung *et al*., 2004). The Nrf2 is a key transcription factor that positively regulates many phase 2 detoxifying enzymes and antioxidant enzymes such as NADPH: quinone oxidoreductase-1 (Nqo1) and heme oxygenase-1 (Hmox1) (Suzuki *et al*., 2013). Triterpenoids are well-known Nrf2 activators, which interact with cysteine residues of an Nrf2 adaptor protein, Keap1 (Sporn *et al*., 2011). This interaction disrupts the Nrf2-Keap1 complex and then liberated Nrf2 are translocated to the nucleus. One of the triterpenoids, bardoxolone methyl [2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) methyl ester/CDDO-Me/RTA 402] was tested for chronic kidney disease with type 2 diabetes; however phase 3 was halted due to cardiovascular mortality (De Zeeuw *et al*., 2013). It is likely that dermatitis is a suitable indication for triterpenoids because focal administration, such as ointment or lotion, would lower the risks observed in the case of systemically administered bardoxolone methyl. Another compound, RTA 408, is currently being tested in clinical trials such as radiation-induced dermatitis with breast cancer (ClinicalTrials.gov Identifier: NCT02142959) (Reisman *et al*., 2014). Obtained a novel Nrf2 activator, RS9 and have demonstrated that RS9 inhibits blood-retinal barrier permeability in rabbits (Nakagami *et al*., 2015). In this study, investigating the effect of RS9 on TNCB-induced contact dermatitis by topical application and compared RS9 to the above two compounds was focused. The TNCB was also applied on the ear of BALB/c mice and contact dermatitis was evaluated by measuring ear thickness.

MATERIALS AND METHODS

Animals and reagents: Female BALB/c mice, aged 7 weeks, were purchased from Japan SLC Inc. (Shizuoka, Japan) and experimental procedures were performed in accordance with the in-house guideline of the Institutional Animal Care and Use Committee of Daiichi Sankyo. All animals received standard laboratory diet and filtered water *ad libitum* under specific pathogen free conditions. The RTA 402, RTA 408 and RS9 were prepared at the in-house chemical laboratories.

***In vitro* assays:** An NQO1 induction assay in hepa1c1c cells and a lactate dehydrogenase (LDH) assay in ARPE-19 cells were conducted according to the method previously reported (Nakagami *et al*., 2015).

Induction of contact dermatitis: The right ear thickness was measured by using a dial thickness gauge (cat. No. G-1A, Ozaki MFG. Co., Ltd., Tokyo, Japan) in millimeter to two decimal places. On day 7, after the measurement of the ear, TNCB (1% in 20 μL acetone) or acetone alone was topically applied once on the right ear as sensitization. On days 4, 2 and 0, triterpenoids dissolved in 20 μL acetone or acetone alone was also applied on the same ear. The TNCB was again applied on day 0 as a challenge and the right ear thickness was measured until day 7. All mice were sacrificed by carbon dioxide and the ear samples were employed for a polymerase chain reaction.

Statistical analysis: Data are shown as Means±SEM, statistical analyses among multiple groups were performed by using one-way analysis ANOVA, followed by the Tukey's test and $p<0.05$ was considered significant.

RESULTS

***In vitro* profiles of RS9:** The RS9 was purified from fermentation products at our laboratory (Fig. 1a) and Nrf2 activity was evaluated by NQO1 induction activity in hepa1c1c 7 cells. The RS9 showed more potent activity than other triterpenoids (Fig. 1b) and the concentration to double the specific activity of RTA 402, RTA 408 and RS9 were 1.1, 2.1 and 0.2 nM, respectively. The RS9 also had lower cytotoxicity compared to others (Fig. 1c).

Effects of RS9 on contact dermatitis: Challenge by application of TNCB induced ear swelling and the ear thickness peaked at 8 h after the challenge (Fig. 2a). The value was significantly larger than that of the group without TNCB (Fig. 2b). After the peak, the ear swelling gradually ameliorated. The ear thickness in the RS9-applied groups also showed a similar pattern; however the values at 8 h after the challenge in the 0.1, 0.3 and 1% RS9-applied groups were significantly lower than that of the vehicle-applied group with

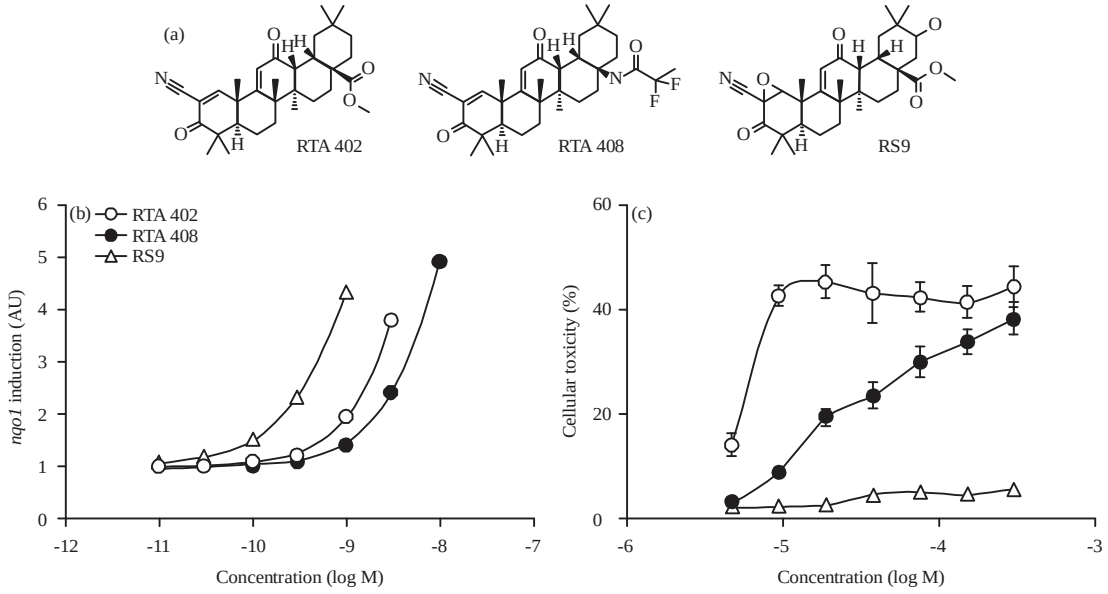

Fig. 1(a-c): *In vitro* Nrf2 activity and toxicity of triterpenoids, (a) Chemical structures of tested compounds, (b) *nqo1* induction activity of Nrf2 activators in hepa1c1c7 cells and (c) An LDH assay in ARPE-19 cells, the experiments were conducted in triplicate

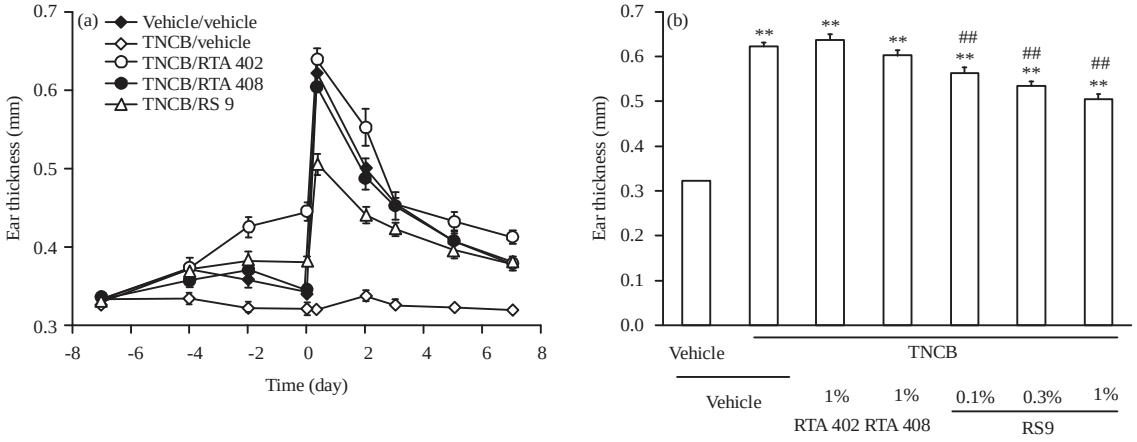

Fig. 2(a-b): Effects of RS9 on TNCB-induced contact dermatitis, (a) Time course of TNCB-induced ear swelling, TNCB was applied on day 7 (sensitization) and day 0 (challenge) except the open diamonds; open diamonds: The vehicle-applied group, closed diamonds: The vehicle-applied group, open circles: The RTA 402-applied group, closed circles: The RTA 408-applied group and open triangles: The RS9-applied group. The compounds were applied on days 6, 4 and 0 and (b) The inhibitory effects of RS9 on TNCB-induced ear swelling. The values indicate the ear thickness measured 8 h after the challenge. **p<0.01 vs. vehicle-applied group without TNCB, ##p<0.01 vs. vehicle-applied group with TNCB, n = 10-20 from two independent experiments

TNCB. The inhibitory effects were not significant in both the RTA 402 and RTA 408-applied groups. The ears were obtained 8 h after the TNCB challenge in all groups and the increases in Nrf2-targeted genes, *nqo1*, were significant in the RS9-applied group (Fig. 3a). The TNCB challenge itself also induced *hmox1* (Fig. 3b), probably reflecting intrinsic protective responses to allergic reactions. Inflammatory genes were also investigated but significant inhibition was not observed in *il1b* and *ccl2* in all of the compounds-applied groups (Fig. 3c and d).

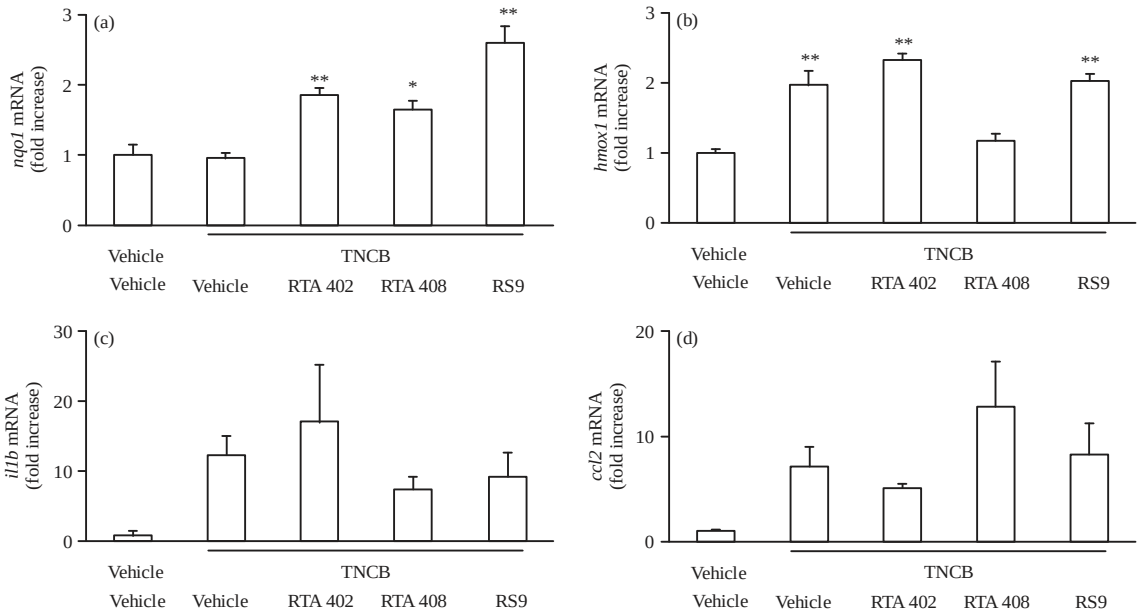

Fig. 3(a-d): Effects of RS9 on Nrf2-targeted genes and inflammatory genes in the skin. Changes of (a) *nqo1*, (b) *hmox1*, (c) *il1b* and (d) *ccl2* genes after topical application of triterpenoids. The samples were collected at 8 h after TNCB challenge. **p<0.05, **p<0.01 vs. vehicle-applied group without TNCB, n = 4

DISCUSSION

We have firstly demonstrated that activation of the Nrf2-Keap1 signaling alleviates TNCB-induced contact dermatitis and RS9 has a superior efficacy to other well-known triterpenoids. The mechanisms of contact dermatitis are complicated; however, in addition to T cells and dendritic cells, it is likely that a variety of cytokines (Harada *et al*., 2005) and the infiltration of neutrophils (Daito *et al*., 2014) are related to the immune responses. The inhibition of inflammatory genes are not significant in the RS9-applied group, therefore further studies are certainly needed to clarify the relationship among Nrf2, inflammation and ear swelling. Allergic contact dermatitis is related to oxidative stress (Kaur *et al*., 2014) and antioxidants such as vitamin E shows some protective effects against the pathology (Kosari *et al*., 2010). In Nrf2-deficient mice, more pronounced responses to haptens are reported compared to wild-type (Van der Veen *et al*., 2013). The level and/or change of Nrf2 in allergic contact dermatitis patients have not been reported yet but it is likely that the activation of Nrf2 is a reasonable strategy considering the above reports. In conclusion, we discovered the effectiveness of RS9 in a contact dermatitis model of BALB/c mice. As mentioned above, RTA 408 is currently being tested for radiation-induced dermatitis. Taken together, the activation of Nrf2 for dermatitis by focal administration seems to be a promising therapy without inducing systemic adverse events. Although detailed studies including toxicological studies are certainly needed regarding RS9, our results raise the possibility that the activation of the Nrf2-Keap1 signaling by RS9 is a novel remedy for contact dermatitis.

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