food & nutrition research

ORIGINAL ARTICLE

Resveratrol derivative production by high-pressure treatment: proliferative inhibitory effects on cervical cancer cells

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Popular scientific summary

- Yields of RK4, a conjugate of resveratrol and caffeic acid, were significantly increased under high-pressure conditions.
- RK4 was more effective against cancer cells than resveratrol.
- The present study also suggested that high-pressure treatment is an effective method to produce novel functional food ingredients.

Abstract

***Background*:** In recent years, functional food components have attracted considerable attention. Resveratrol, a food polyphenol, has been widely studied due to its various physiological activities. Previously, we identified a novel resveratrol derivative, named RK4, in food, which is formed by a chemical reaction involving resveratrol and caffeic acid. Furthermore, it was suggested that high-pressure treatment is an important factor in RK4 production.

***Objectives*:** The purpose of this study was to clarify relationships between high-pressure processing and component production and to compare RK4 with the known functional ingredient resveratrol to examine the physiological value of RK4. Through this research, we aimed to develop high-pressure treatment technology that adds new usefulness for food.

***Methods*:** Resveratrol and caffeic acid were reacted under high-pressure treatment and in various conditions of concentration and temperature. RK4 levels in the reaction solution were quantitatively analyzed using liquid chromatography-mass spectrometry. In addition, HeLa cervical cancer cells were exposed to RK4 and resveratrol, and survival rates were measured using the methyl thiazolyl tetrazolium (MTT) method after culturing for 24 h. Activation of an apoptosis-inducing marker was detected by western blotting of cells cultured for 48 h after addition of the test compounds.

***Results*:** By reacting resveratrol and caffeic acid under high-pressure conditions (~100 MPa), the amount of RK4 produced was significantly increased. It was also found that the reaction temperature and time contributed to this reaction. RK4 exhibited stronger cytotoxicity to HeLa cells than resveratrol. It was also shown that RK4 activated p38, cleaved poly ADP ribose polymerase, and induced apoptosis.

***Conclusions*:** RK4 is a valuable component for further research as a novel compound with wider functionality than that of resveratrol. High-pressure treatment may substantially contribute to the production of novel food ingredients. Further elucidation of the relationships between high-pressure treatment and production of new ingredients has promising potential to guide development of new applications in food processing.

Keywords: *food processing; caffeic acid; apoptosis; HeLa; PARP; p38*

To access the supplementary material, please visit the article landing page

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Prevention of lifestyle-related diseases, such as arteriosclerosis and diabetes, is known to be highly linked to eating habits. Therefore, interest in health promotion based on specific foods or bioactive food components is rapidly increasing, and research in this area is at present actively undertaken. Among such areas, intake of fruits, vegetables, and grains has been proven to offer protective effects against lifestyle-related diseases (1–4). Such protective role is mainly attributed to the phytochemical contents of certain foods, which are classified as bioactive non-nutrient compounds in fruits, vegetables, grains, and other plants (5). Approximately 10,000 plant-derived chemical substances have been identified to date, and among them, polyphenols are one of the most interesting components because they possess various physiological effects on humans such as lowering blood pressure and reducing inflammation (6).

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a well-known polyphenol found in nuts, grape skins, and wine. Resveratrol is also one of the most widely studied polyphenols, with >10,000 reports of it in the literature. This stilbene polyphenol has attracted interest in popular culture over many years because of its potential biological and health benefits. Since a number of epidemiological studies have revealed the cardioprotective benefits of wine (7, 8), a field investigating the role of resveratrol has flourished, and a wealth of scientific research has followed (9). Resveratrol has since been identified as a compound that exerts cancer chemoprotective (10) and anti-inflammatory effects (11), improves vascular function (12), extends lifespan, ameliorates aging-related phenotypes (13, 14), opposes the effects of a high-calorie diet (15), mimics the effects of calorie restriction (16), and improves cellular function and metabolic health in general (17).

We previously found that resveratrol is partly polymerized with organic acids in foods during their processing to produce new derivatives (18). Among these resveratrol derivatives, we have focused on the component RK4, in which resveratrol and caffeic acid (3,4-dihydroxy cinnamic acid) are polymerized (Fig. 1) because it has been reported to have certain bioactivities stronger than those associated with resveratrol (19). Nuclear magnetic resonance (NMR) assignment of RK4 was summarized in Fig. S1 and Table S1. We have been studying the relationship between RK4 production and food processing, particularly high-pressure processing. High-pressure processing is expected to be a futuristic method of food processing because it does not involve the loss of food flavors and often generates novel proteins and starches with new physical properties that are different from those generated by heat processing (20). In this research, we aimed to determine important relationships involving high-pressure treatment and RK4 production and those mechanisms involved in RK4 production. Regarding the physiological function of RK4, only the growth inhibitory effect on colorectal cancer cells has been reported (18), while other functions are unknown.

During the past few decades, it has been shown that resveratrol could be a good candidate as a therapeutic drug for several disorders such as heart disease, diabetes, obesity, neurological disorders, and cancer (21, 22). Jang et al. reported the antitumor effects of resveratrol using a mouse skin cancer model (23), and many studies have shown that resveratrol inhibits cell proliferation and induces apoptosis in cancer cell lines (24, 25). Human cervical cancer is the most common form of cancer in women and accounts for a small percentage of all cancer cases diagnosed worldwide. Resveratrol has also been demonstrated to have antiproliferative effects and induces apoptosis in HeLa cells, a cervical cancer cell line (26). Based on these reports, we hypothesized that RK4 may also be expected to have similar bioactivities, and we, hence, decided to measure the antiproliferative effects of RK4 on HeLa cells.

It has been shown that p38 is one of the targets for the induction of apoptosis in cancer cells by various anticancer substances (27) and is a mitogen-activated protein kinase (MAP kinase) that responds to stress stimuli such as cytokines and ultraviolet irradiation. Poly ADP ribose polymerase (PARP) is also one of the targets used to induce apoptosis by resveratrol (28) and is a zinc-dependent eukaryotic DNA-binding protein. It is known that 113 kDa PARP is cleaved into fragments of 89 kDa and 24

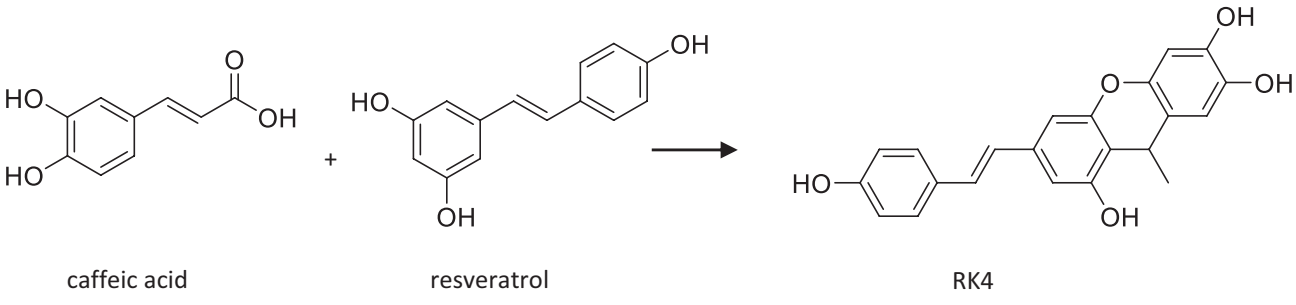

Fig. 1. Formation of RK4 from caffeic acid and resveratrol reaction.

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kDa, which are considered to be specific markers of the early stages of apoptosis (29). In this study, these two proteins were mainly used to detect the induction of apoptosis by RK4 in HeLa cells.

The purpose of this study was to determine the relationships between high pressure treatment and RK4 production by reacting resveratrol and caffeic acid and to evaluate the potential usefulness of RK4 in cancer prevention by comparing its effects on cervical cancer cells with those of resveratrol. Moreover, through this research, we sought to establish RK4 as a functional food component with beneficial effects.

Materials and methods

Reagents and NMR analysis

Resveratrol was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). Caffeic acid-adducted resveratrol (RK4) was synthesized by UHA Mikakuto Co., Ltd. (Osaka, Japan) (18). Dimethyl sulfoxide (DMSO) (Kanto Chemical Co., Inc., Tokyo, Japan) was used to dissolve these reagents. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. NMR spectra were measured on a Bruker AVANCE III 400 instrument (Bruker BioSpin, Billerica, MA, USA). Chemical shifts (δ) are reported in ppm, and coupling constants (*J*) are reported in hertz. The chemical shifts in the ^{1}H and ^{13}C NMR spectra were corrected using the residual solvent signals.

Preparation of high-pressure processed samples

Preparation of high-pressure treated samples was performed by UHA Mikakuto Co., Ltd. Resveratrol and caffeic acid at various concentrations were dissolved in 10% EtOH (50 mM boric acid) to prepare reaction solutions. These reaction solutions were treated using a high-pressure enzyme reactor TOTAL EXTRACTION (Toyo Koatsu Co., Ltd., Hiroshima, Japan) under different temperatures (50 and 90°C), time periods (1, 3, 5, 6, and 18 h), and pressure settings (25, 75, and 100 MPa).

Quantitative analysis using LC-MS/MS

High-pressure processed samples were diluted in MeOH and filtered through a 0.22 μm pore size syringe filter (Hawach Scientific, Shaanxi, China) for HPLC analysis. Chromatographic separation was performed using an HPLC instrument with an ACQUITY UPLC® BEH C18 column (1.7 μm, φ2.1 × 100 mm) (Waters Corporation, MA, USA). The eluent was solvent A, H_2O (0.1% formic acid), and solvent B, MeCN (0.1% formic acid) (Thermo Fisher Scientific K.K., Tokyo, Japan). A gradient flow program was performed with solvent B: 2% (0 min), 2% (1 min), 39% (4 min), 41% (8 min), and 98% (9 min) at a flow rate of 0.4 mL/min. The injection volume was 20 μL, and three injections were performed for each sample. Mass spectrometry was performed on a TSQ Quantum™ Access MAX (Thermo Fisher Scientific K.K.) triple quadrupole mass spectrometer in selected reaction monitoring (SRM) scan mode (negative mode). The analytical conditions for RK4 were determined by the infusion method and were performed with collision energy −29 V, parent ion *m/z* 361.14, product ion *m/z* 345.12, and tube lens voltage −142.66 V. In addition, based on the quantitative results, we sought to analyze relationships involving the amount of RK4 produced and the reaction time physicochemically using the following formula:

$$C_S = C_A \left(1 + \frac{k_2}{k_1 - k_2} e^{-k_1 t} + \frac{k_1}{k_2 - k_1} e^{-k_2 t}\right) \quad (1)$$

Cell line and cell culture

The human HeLa cervical cancer cell line was purchased from CH3 BIOSYSTEMS LLC (Amherst, NY, USA). Cells were cultured in RPMI-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (Moregate, Brisbane, Australia) and penicillin-streptomycin solution (×100) (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Cells were maintained in a humidified environment with 5% CO_2 at 37°C.

Cell viability

The effects of RK4 and resveratrol on the viability of HeLa cells were determined using methyl thiazolyl tetrazolium (MTT) assays. Briefly, logarithmically growing HeLa cells were seeded in 96-well plates at a density of 2×10^3 cells per well in 100 μL of culture medium. After 24 h of pre-culture, cells were exposed to either RK4 (0, 3.13, 6.25, 12.5, 25, and 50 μM) or resveratrol (0, 12.5, 25, 50, 100, and 200 μM) for 24 h. Next, 5.0 mg/mL MTT (Fujifilm Wako Pure Chemical Corporation) was added, and the cells were incubated at 37°C for an additional 3 h. After discarding the medium, DMSO (200 μL) was added, and the spectrophotometric absorbance at 535 nm was measured using a SPECTRA MAX 190 microplate reader (Molecular Devices, Osaka, Japan). The concentrations of RK4 and resveratrol, at which cell growth was inhibited by 50% (IC_{50}), were calculated using the following formula:

$$\text{Cell viability (\%)} = 1 - \left(\frac{A_{\text{Sample}} - A_{\text{Blank}}}{A_{\text{Contlol}} - A_{\text{Blank}}}\right) \times 100 \quad (2)$$

Western blot analysis

HeLa cells were seeded in 6 cm cell culture dishes at 6.0×10^5 cell/dish. After 24 h of pre-culture, cells were treated with RK4 (final concentrations 0, 0.2, 0.78, 3.13,

and 12.5 μM) and resveratrol (final concentrations 0, 0.2, 0.78, 3.13, and 12.5 μM) for 48 h. Then, the cells were dissolved in sodium dodecyl sulfate (SDS) sampling buffer (Nacalai Tesque, Inc.) containing 2-mercaptoethanol, protease inhibitor cocktail, phosphatase inhibitor cocktail I (Sigma-Aldrich), phosphatase inhibitor cocktail II (Sigma-Aldrich), 1 mM β-glycerophosphate, and 2.5 mM sodium pyrophosphate, and boiled for 10 min. Samples were electrophoresed on 5–20% SDS-polyacrylamide gels (Atto Corporation, Tokyo, Japan) and then transferred to 0.1 μm nitrocellulose (NC) membranes (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween-20 containing 5% skim milk (Morinaga Co., Ltd., Tokyo, Japan) and treated with various (p38, PARP, and β-actin) antibodies. Immunoreactive protein bands were visualized using ECL Pro (PerkinElmer, Inc., MA, USA) detection reagent using a C-DiGiT blot scanner (LI-COR Corporate, NB, USA). The protein band intensities were quantified using software tools (Image Studio Digits 5.2.5) (LI-COR Corporate). Values were obtained from at least three independent experiments. All antibodies used in this experiment were obtained from Cell Signaling Technology (MA, USA).

Statistical analysis

All results are presented as means ± SD of three independent experiments. Differences between control and RK4 or resveratrol treatments were performed using one-way analysis of variance (ANOVA). Control and treatment outcomes were compared using Student's unpaired *t*-test (*$P < 0.05$, **$P < 0.01$).

Results

RK4 yields under various reaction conditions

To investigate the reaction processes involved in RK4 formation, caffeic acid and resveratrol were reacted together under pressure conditions of 25, 50, 75, and 100 MPa at 90°C for 18 h. Quantified results for RK4 abundance identified using LC-MS/MS are shown in Fig. 2. Concentrations of caffeic acid and resveratrol in reaction solutions A, B, and C were different, whereas the molar ratios were equivalent (caffeic acid:resveratrol = 2.53:1). The substrate content of each solution was as follows: solution A: caffeic acid 0.024 g, resveratrol 0.012 g; solution B: caffeic acid 0.12 g, resveratrol 0.06 g; solution C: caffeic acid 0.6 g, resveratrol 0.3 g. Values are expressed as means ± SD ($n = 3$). The production rate of RK4 at 25 MPa was 0.2 ± 0.02% for reaction solution A, 0.7 ± 0.02% for B, and 1.2 ± 0.04% for C. At 50 MPa, the production rate of the reaction solution A was 0.3 ± 0.02%, B was 0.6 ± 0.01%, and C was 1.5 ± 0.06%. At 75 MPa, the rate of production of reaction solution A was 0.4 ± 0.03%, B was 1.2 ± 0.02%, and C was 2.2 ± 0.1%. At 100 MPa, the production rate of reaction solution A was 1.0%, B was 4.1%, and C was 10.2%.

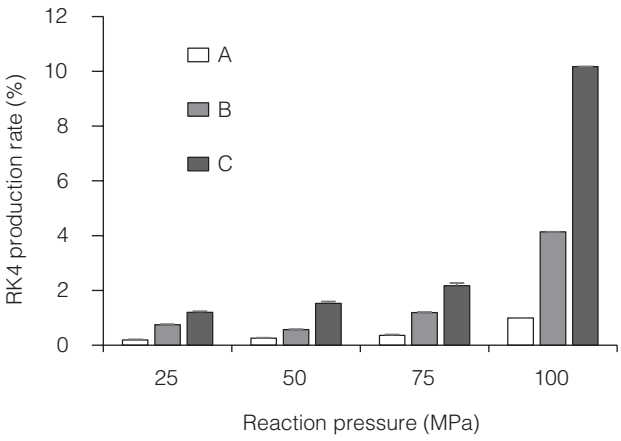

Fig. 2. RK4 production rates under various pressure conditions. The production rate of RK4 (the quantity of RK4 produced divided by the substrate content in the reaction solution) was quantified using LC-MS/MS. The reactants, including caffeic acid and resveratrol, were reacted at various pressures (25, 50, 75, and 100 MPa) for 18 h at 90 °C. Concentrations of the substrates (caffeic acid and resveratrol) in the solutions of A, B, and C were different, whereas the molar ratios were equivalent (caffeic acid:resveratrol = 2.53:1). The substrate content of each solution was as follows: solution A: caffeic acid 0.024 g, resveratrol 0.012 g; solution B: caffeic acid 0.12 g, resveratrol 0.06 g; solution C: caffeic acid 0.6 g, resveratrol 0.3 g. Values are expressed as means ± SD ($n = 3$).

Chemical kinetic analysis of RK4

To investigate the relationship between the amount of RK4 produced and the reaction time under high-pressure conditions, reactions involving caffeic acid and resveratrol were carried out at 100 MPa and 90°C for 1–18 h. The substrate content of each solution was as follows. Solution A: caffeic acid 0.024 g, resveratrol 0.012 g; solution B: caffeic acid 0.12 g, resveratrol 0.06 g; solution C: caffeic acid 0.6 g, resveratrol 0.3 g. Values are expressed as means ± SD ($n = 3$). The results are shown in Fig. 3a. The production quantity at the reaction time of 1 h was 0.455 ± 0.015 ppm for reaction solution A, 1.23 ± 0.016 ppm for B, and 1.23 ± 0.031 ppm for C. The production amounts for reaction times of 3, 5, 6, and 18 h were increased under all reaction conditions. In particular, at the reaction time of 18 h, yields were remarkably increased in reaction solutions B and C. It was found that the amount of production was approximately 20 ppm or less at 1–6 h and 18 h (reaction solution A), whereas the yield increased significantly to 60.2 ± 0.479 ppm in B and 108.6 ± 1.56 ppm in C at 18 h. Furthermore, to perform a physicochemical analysis from the quantitative results of Fig. 3a, the relationship

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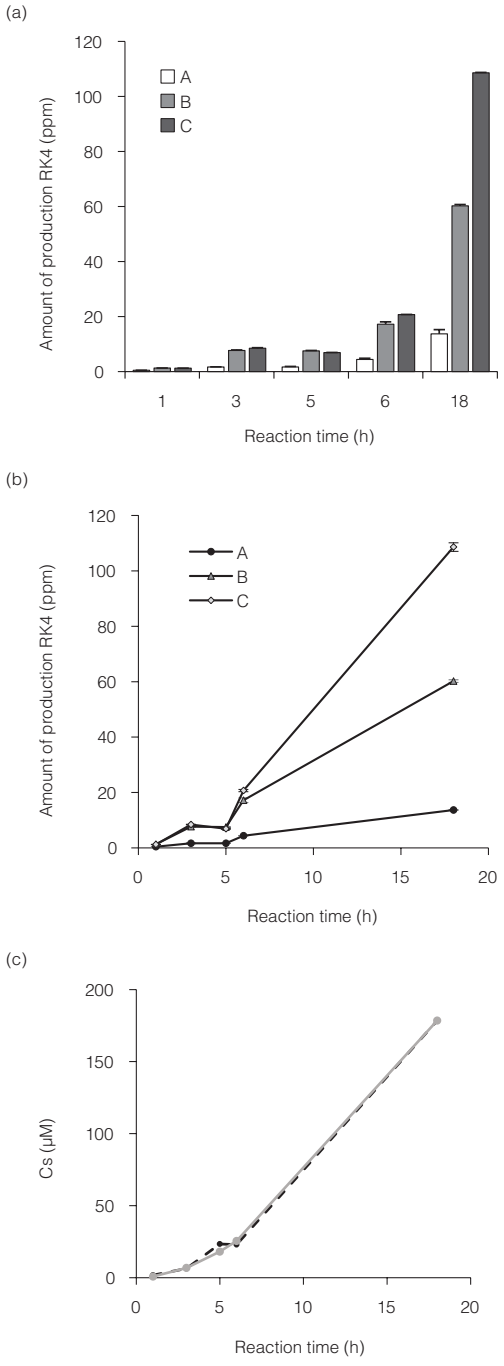

Fig. 3. Amounts of RK4 produced after various reaction times. (a) and (b) A solution of caffeic acid and resveratrol was reacted at 100 MPa and 90 ºC for 1–18 h to generate and subsequently quantify the amount of RK4. Quantitative results are shown as the amount produced (ppm). The substrate concentrations were different among A, B, and C. The substrate content of each solution was as follows. Solution A: caffeic acid 0.024 g, resveratrol 0.012 g; solution B: caffeic acid 0.12 g, resveratrol 0.06 g; solution C: caffeic acid 0.6 g, resveratrol 0.3 g. Values are expressed as means ± SD (n = 3). (c) A graph edited to identify rate coefficients. The dotted line represents measured values, and the solid line is the simulated graph from which the rate coefficients were calculated.

between the production amount and the reaction time was represented as a line graph, as shown in Fig. 3b. From the results shown in Fig. 3b, the sequential reactive relationship between the production amount of RK4 and the reaction time was observed. Furthermore, the rate coefficient in the production reaction of RK4 was determined using Equation 1, which expresses the relationship between substrate and product concentrations in the sequential reaction. Specifically, by substituting the expected values of k_1 and k_2 into Equation 1, the shape was used as a line graph depicting the relationship between reaction time and the production of RK4 (Cs) (Fig. 3c). The reaction rate constant (k_1) involving caffeic acid and resveratrol at intermediate formation stages was approximately k_1 = 0.02, and the rate coefficient (k_2) from the intermediate to the formation of RK4 was approximately k_2 = 0.046.

RK4 inhibits HeLa cell viability

The antiproliferative effects of RK4 and resveratrol on cancer cell viability were measured using the MTT method to compare the effects of the two food factors *in vitro*. HeLa cells were exposed to various concentrations of reagents (RK4 or resveratrol) for 24 h. Both RK4 and resveratrol exerted significant growth inhibitory effects on HeLa cells in a concentration-dependent manner (Fig. 4). Cell survival rates after RK4 treatment were 97.9 ± 8.08, 66 ± 5.53, 58.5 ± 4.6, 46.9 ± 5.84, and 8.2 ± 3.44% at 3.13, 6.25, 12.5, 25, and 50 μM, respectively, and the IC_{50} value was 23.6 μM. Cell survival rates after resveratrol treatment were 106.2 ± 6.26, 108.1 ± 10.8, 111.1 ± 2.19, 77.8 ± 2.07, and 16.9 ± 11.5% at 2.5, 25, 50, 100, and 200 μM, respectively, and the IC_{50} was 145.3 μM.

RK4 induces apoptosis in HeLa cells

To investigate the mechanism by which RK4 causes cytotoxicity to HeLa cells, the cells were treated with various concentrations of RK4 and resveratrol for 48 h (Fig. 5). Activation of p38 was observed in cells treated with RK4, whereas no activation was observed in cells treated with resveratrol (Fig. 5a and b). In addition, in cells treated with RK4, a gradual decrease in ADP-ribose polymerase (PARP) was observed in a concentration-dependent manner, and a marked increase in its cleaved form was also noted. In cells treated with resveratrol, PARP levels tended to decrease, while its cleavage form could not be detected (Fig. 5a, c, and d).

Discussion

Relationship between RK4 production and high-pressure processing

We previously reported that temperature and pressure are important factors in RK4 formation (19). However, the

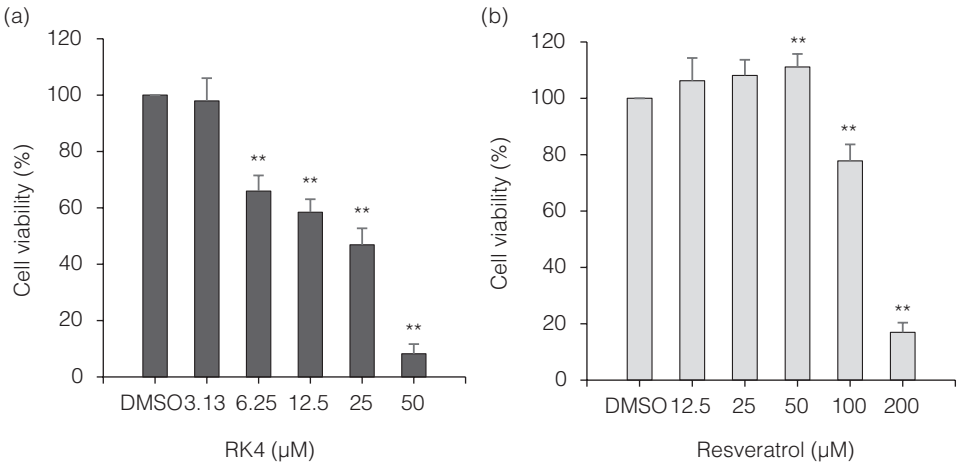

Fig. 4. Growth inhibitory effects of RK4 and resveratrol on HeLa cells. (a) HeLa cells were treated with RK4 (0, 3.13, 6.25, 12.5, 25, and 50 μM) for 24 h. (b) HeLa cells were treated with resveratrol (0, 12.5, 25, 50, 100, and 200 μM) for 24 h. Values are expressed as means ± SD (n = 3). Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.001$, compared with control (DMSO).

contribution of pressure in the formation of RK4 and its relationship with other conditions such as temperature and reaction time have not been clearly identified.

In particular, high-pressure treatment of foods is one of the attractive treatments available in food processing methods because of its convenience. Therefore, in this study, to understand the relationship between the formation of a novel resveratrol derivative (RK4) and high-pressure treatment, resveratrol and caffeic acid were reacted under various conditions, and then quantitative analysis of RK4 by LC-MS/MS was performed. The experiments were conducted by changing the pressure, temperature, and time. It was found that pressure treatment significantly contributed to the formation of RK4, and that reaction time and concentration of the reactant solution were also important, as shown in Figs. 2 and 3.

As presented in Fig. 2, the RK4 production rate under pressure conditions of 75 MPa or lower was small, and difference in the production rate between the reaction solutions A, B, and C was <2%. However, a high production rate was confirmed in reaction solutions B and C at 100 MPa. Compared to the production rate under the condition of 75 MPa or less, the rate of reaction solution B was approximately twice as fast, and the rate of solution C was four or more times faster. In particular, reaction solution C at 100 MPa exhibited a remarkably high reaction rate of 10.2%, suggesting a strong relationship between production efficiency and reaction conditions such as pressure and substrate concentration. This result suggested that a high-pressure condition of 100 MPa is effective for the production of RK4.

Furthermore, in order to investigate potentially more efficient reaction conditions, we focused on the relationship between the amount of RK4 produced and reaction times. The yield of RK4 tended to increase when the reaction time was prolonged (Fig. 3a). Moreover, from the results of Fig. 3b, it was suggested that the RK4 formation reaction is sequential in that it proceeds via an intermediate. Next, we analyzed possible mechanisms involved using Equation 1, which expresses the relationship between substrate concentration (resveratrol) and product concentration (RK4). Using Equation 1, the RK4 production reaction was assumed to be sequential. Additionally, it was found that there was an approximate two-fold difference between the reaction rate coefficient (k_1) for resveratrol in intermediate formation and the reaction rate coefficient (k_2) from the intermediate to the formation of RK4 ($2k_1 \fallingdotseq k_2$). This result indicated that there is an approximate two-fold difference in the progress rate of the reaction in the two-step reaction sandwiching the intermediate until RK4 formation. For more efficient production of RK4, it is important to increase the reaction rate at the reaction time of approximately 1–5 h, as shown in Fig. 3b, which is assumed to be the first stage of the reaction.

Effects of RK4 on cancer cells

RK4, which is the main subject of this study, is a newly discovered derivative of resveratrol. Thus far, it has been suggested that RK4 has stronger effects than resveratrol on various bioactivities. Cancer cell growth inhibitory activity is one such activity, and RK4 has been suggested as a potential alternative to resveratrol in cancer research (19). In this study, we investigated the growth inhibitory activity of RK4 and its mechanism of action in HeLa cells. We confirmed that RK4 has a greater antiproliferative inhibitory effect than resveratrol and identified a number of the target molecules involved in induction of apoptosis by RK4.

Currently, food factors that can selectively induce apoptosis in cancer cells have received much attention

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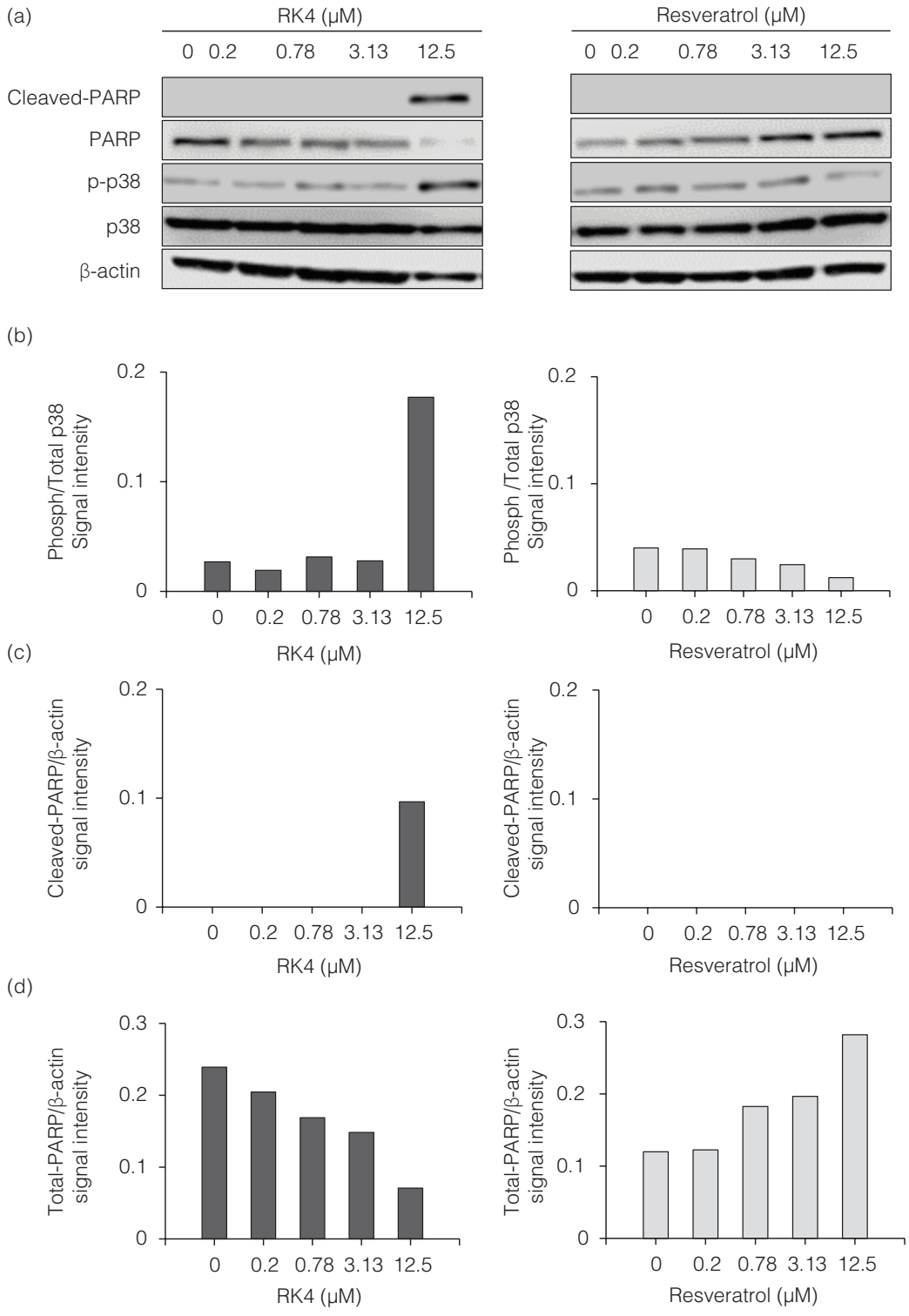

Fig. 5. RK4 activated p38 and cleaved PARP at lower concentrations than resveratrol. Cellular proteins were isolated from HeLa cells treated with various concentrations of RK4 or resveratrol (0, 0.2, 0.78, 3.13, and 12.5 μM) for 48 h. (a) Changes in p38 and PARP abundance were analyzed by western blotting. Each experiment was repeated at least three times, and representative data are shown. (b–d) Protein-level densitometric analysis of apoptosis-related proteins isolated from Hela cells treated with RK4 and resveratrol. The values shown represent the detection intensities of each target protein divided by the detection intensity of the precursor or β-actin. This experiment was performed at $n = 3$, and only representative values are displayed.

in the development of new approaches to cancer prevention. Many natural substances already employed in cancer chemotherapy also have apoptosis-inducing activity, and the anticancer properties of these substances are thought to be partly due to induction of apoptosis (30–32). Resveratrol, which is a precursor of RK4 and was used as a molecule for comparison in this study, is a compound that has high bioactivities and is highly anticipated as a functional phytochemical (2, 3, 10–17). Caffeic acid is a natural phenolic acid product found in fruits (33), wine (34, 35), and coffee (36). It has been reported to exert diverse biological activities such as antibacterial (36), antioxidative (37), and anti-inflammatory (34) effects, which are similar to induction by resveratrol. These two compounds, which are precursors of RK4, have been reported to induce apoptosis in various cancer cells, and some of the mechanisms involved have been elucidated (24, 25, 38, 39).

The IC_{50} values were 23.6 μM for RK4 and 145.3 μM for resveratrol. These values indicated that RK4 exerts greater growth inhibitory effects on HeLa cells than resveratrol, and our results strongly suggested its potential as a functional food component. Western blotting analysis revealed that RK4 strongly induced p38 activation. In addition, there was no activation of p38 by resveratrol. These results suggested that RK4 may induce stress-responsive apoptosis in HeLa cells. In addition, it was shown that RK4 induced the cleavage of PARP, and this result supported the effect of RK4 in inducing apoptosis in HeLa cells. A schematic diagram of a proposed pathway by which RK4 acts on cancer cells is presented in Fig. 6. This study showed that RK4 is a more potent inducer of apoptosis than that of resveratrol, and further supports the idea that it represents a promising candidate for an antitumor agent. However, since only a small number of protein markers of apoptotic induction were successfully detected in this study compared with previous reports, it will be necessary to investigate other such apoptotic markers in order to further elucidate the mechanisms of action of RK4.

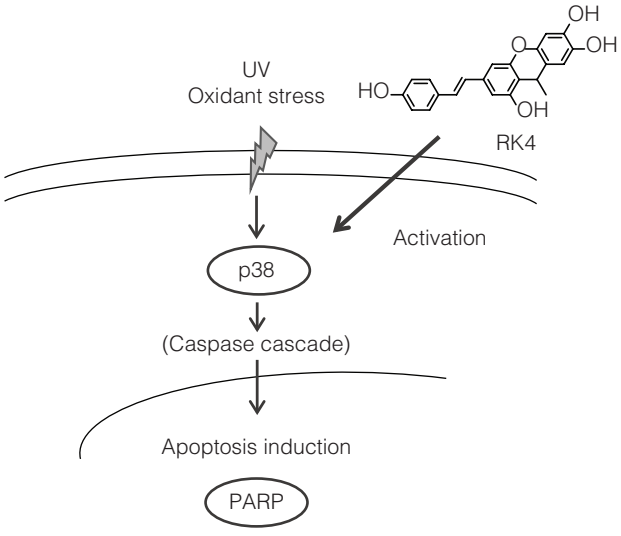

Fig. 6. Schematic diagram of apoptosis induction by RK4. Extracellular environments involving UV and oxidative stress are known to promote the production of intracellular reactive oxygen species and induced stress-responsive apoptosis. RK4 may increase such extracellular or internal stress, activate the p38 MAPK cascade, and induce apoptosis.

Conclusions

In this study, the quantification of RK4 in high-pressure-treated sample indicated that the component composition of foodstuffs is significantly modified by high-pressure treatment, and that such alterations may yield components other than RK4 with similar high bioactivities. High-pressure processing has been reported to be an innovative non-thermal food preservation method because it can inactivate microorganisms with minimal influence on the physicochemical and sensory properties of foods (40–42). However, there are few studies on the increase of the functional component(s) by high-pressure processing as far as we know. Thus, we have demonstrated the potential importance of high-pressure processing in future food production and the value of RK4 as a functional food ingredient.

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Conflicts of interests

The authors declare no potential conflicts of interest.

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