pISSN 1225-4339 eISSN 2287-4992
Korean J. Food Nutr. Vol. 28. No. 4, 579~585 (2015)
http://dx.doi.org/10.9799/ksfan.2015.28.4.579

THE KOREAN JOURNAL OF
한국식품영양학회지
FOOD AND NUTRITION

Rutin Suppresses Neoplastic Cell Transformation by Inhibiting ERK and JNK Signaling Pathways

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Rutin의 ERK 및 JNK 신호전달체계 억제를 통한 암예방 효능

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국문요약

Rutin은 메밀에 함유되어 있는 것으로 잘 알려져 있는 flavonoid 물질로서, 최근 연구들에서 rutin의 항염증 및 암예방 활성이 보고되어져 왔다. 그러나, rutin의 암예방 활성과 관련된 분자생물학적 기전에 대한 연구는 아직까지 미비한 실정이다. 따라서, 본 연구에서는 발암 과정 중 하나인 세포의 악성 변형을 EGF로 유도하여 rutin이 이를 억제하는지 여부를 확인하는 실험을 진행하였으며, 그 분자생물학적 기전을 규명하고자 하였다. Soft agar assay 실험 결과, rutin은 EGF로 유도된 세포의 악성 변형을 25 μM, 50 μM 100 μM에서 농도별로 감소시켰다. 또한 EGF로 유도된 MEK/ERK 및 MKK4/JNK 신호전달체계의 인산화를 저해하였다. 그러나 이와는 대조적으로 rutin은 EGF로 유도된 MKK3/6/p38 신호전달체계 인산화는 감소시키지 못하는 것으로 확인되었다. 이상의 연구결과들은 rutin이 암화 과정 중 발생되는 세포의 악성변형 과정을 촉진시킨다고 잘 알려져 있는 MEK/ERK 및 MKK4/JNK 신호전달체계의 활성화를 억제함으로써 암예방 활성을 나타낸다는 것을 제시하고 있으며, 이는 메밀의 생리활성 성분인 rutin의 암예방 생리활성 소재로서의 이용 가능성을 보여주는 중요한 연구 결과라 할 수 있겠다. 또한 위 연구결과는 MEK/ERK 및 MKK4/JNK 신호전달 체계를 표적으로 하는 생리활성 소재 탐색에도 활용 가능할 것으로 생각되어진다.

검색어: rutin, 세포 변형, ERK, JNK, 암예방

Introduction

Neoplastic cell transformation, which entails the conversion of a tissue with a normal growth pattern into a malignant tumor, represents a major event associated with the carcinogenic process (Balkwill & Mantovani 2001; Coussens & Werb 2002). Previous studies have shown that epidermal growth factor (EGF) receptors are overexpressed in colon, lung, kidney, and skin carcinomas (Stanton et al. 1994; Lage et al. 2003) and that EGF induces neoplastic transformation in JB6 P+ mouse skin epidermal cells. The JB6 P+ mouse epidermal cell line is a well-established model used for screening cancer chemopreventive agents and elucidating their molecular mechanisms of action (Dong & Cmarik 2002).

It is also well known that EGF activates mitogen-activated protein kinases (MAPKs). MAPKs influence a wide range of intracellular signaling molecules that are involved in a number of biological processes such as proliferation, differentiation, and apoptosis (Bode & Dong 2003, PT review). In mammalian cells, the three major MAPK pathways are the extracellular signal-regulated kinase (ERK), stress-activated protein kinase-1/c-Jun N-terminal kinase (JNK), and stress-activated protein kinase-2/

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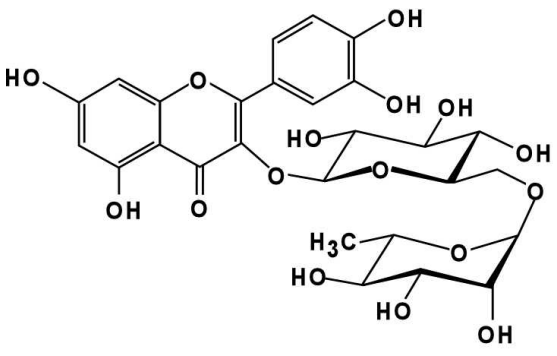
p38 α and β (p38) pathways (Kyriakis & Avruch 2001). The ERK signaling cascade, which is primarily activated by EGF, plays a critical role in transmitting the signals initiated by this growth factor, and most tumor phenotypes are linked to the degradation of this pathway (Cowley et al. 1994; Minden et al. 1994; Dhillon et al. 2007; Kang et al. 2011). JNK and p38 are also sensitive to growth factors and alterations in the expressions of JNK, and p38 proteins are frequently observed in human tumors and cancer cells (Wagner & Nebreda 2009; Kang et al. 2011). Furthermore, it is known that MAPKs are activated following phosphorylation by MAPK kinases (MAPKKs) such as mitogen-activated protein-ERK kinase (MEK) and MAPK kinase (MKK) 4, MKK7, MKK3, and MKK6 (Bode & Dong 2003; Zarubin & Han 2005; Kang et al. 2011). Therefore, these pathways are attractive targets for the development of novel anti-cancer agents.

Rutin (3-O-rhamnosyl-glucosyl quercetin; Fig. 1) is a well-known flavonoid present in buckwheat, and its major effects in the body include anti-inflammatory (Lin et al. 2012; Wang et al. 2012) and anti-carcinogenic (Lapa Fda et al. 2011) effects. Rutin exhibited anti-tumor effects in nude mice bearing SW480 tumors (Alonso-Castro et al. 2013) and inhibited human leukemia tumor growth in an *in vivo* model (Lin et al. 2012). Additionally, rutin was recently shown to suppress ultraviolet B (UVB)-induced cyclooxygenase-2 (COX-2) expression and inducible nitric oxide synthase (iNOS) activity in hairless mouse skin (Choi et al. 2014). However, little is known about the underlying molecular mechanisms of rutin or its specific targets of action. Thus, the present study investigated whether rutin would inhibit EGF-induced neoplastic cell transformation via the suppressions of the MEK/ERK and MKK4/7/JNK pathways in JB6 P+ mouse skin epidermal cells. The results revealed that rutin may be an effective agent for the chemoprevention of cancer.



Rutin
(3-O-Rhamnosyl-glucosyl quercetin)

Fig. 1. The chemical structure of rutin.

Materials And Methods

1. Chemicals

The present study used Eagle's minimum essential medium (MEM) obtained from Cellgro (Manassas, VA), penicillin-streptomycin and 0.5% Trypsin-EDTA purchased from GIBCO® Invitrogen (Auckland, NZ), EGF and an antibody against p38 MAPK from BD Sciences (Franklinlakes, NJ), and fetal bovine serum (FBS) and rutin purchased from Sigma-Aldrich (St. Louis, MO). The antibodies against phosphorylated ERK, total ERK, phosphorylated JNK, total JNK, phosphorylated p38, total p38, goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and phosphorylated JNK (T183/Y185), phosphorylated MEK1/2 (S217/221), total MEK1/2, phosphorylated MKK4 (S257/T261), and total MKK4 were purchased from Cell Signaling Technology (Beverly, MA). The protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA).

2. Cell culture

The JB6 P+ mouse epidermal cell line was cultured in monolayers and incubated at 37℃ in a 5% CO_2 incubator. The cells were maintained in MEM containing 5% FBS and penicillin-streptomycin.

3. Anchorage-independent transformation assay

The effects of rutin on EGF-induced cell transformation were investigated in JB6 P+ cells. The cells (2.4×10^4 cell/mL) were exposed to EGF in combination with rutin (25, 50, 100 μM) in 1 mL of 0.33% basal medium Eagle's (BME) agar containing 10% FBS or in 3 mL of 0.5% BME agar containing 10% FBS. The cultures were maintained at 37℃ in a 5% CO_2 incubator for 10 days, after which the cell colonies were counted under a Nikon phase-contrast microscope (Tokyo, Japan) with the aid of NIS-Elements 3.0 and Image-Pro Plus software programs (v. 5, Media Cybernetics; Silver Spring, MD).

4. Western blot analysis

The JB6 P+ cells (1×10^5 cell/mL) were cultured in a 6-cm dish for 24 hours and then starved in MEM with 0.1% FBS for

an additional 24 hours to eliminate the influence of FBS on the activation of the kinases. The cells were treated with rutin (25, 50, 100 μM) for 1 hour before being exposed to EGF (10 ng/mL) for 30 minutes. The harvested cells were disrupted, and then the supernatant fractions were boiled for 5 minutes. The resultant protein concentrations were determined using a dye-binding protein assay kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The lysate proteins (20－40 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore; Milford, MA). After blotting, the membranes were incubated with specific primary antibodies overnight at 4℃. After hybridization with the appropriate horseradish peroxidase-conjugated secondary antibodies, the protein bands were visualized by an ECL plus Western blotting detection system (AmershamTM, UK). The relative amounts of the proteins associated with the specific antibodies were then quantified using an ImageJ program.

5. Statistical analysis

All data are expressed as means ± standard deviations (SD), and a Student's *t*-test was used for single statistical comparisons. In all analyses, a *p*-value <0.05 was considered to indicate statistical significance.

Results And Discussion

1. Effects of rutin on EGF-induced neoplastic cell transformation in JB6 P+ cells

The neoplastic transformation of cells is considered a major event associated with carcinogenic processes (Dong et al. 1994); thus, the inhibition of neoplastic transformation could be an effective strategy for delaying carcinogenesis. Thus, the present study examined the inhibitory effects of rutin on EGF-induced neoplastic transformation in JB6 P+ cells (Fig. 2). Based on the number of cell colonies, rutin significantly inhibited EGF-induced neoplastic transformation in JB6 P+ cells at 25, 50 and 100 μM based on the number of cell colonies without any cytotoxic effects (Fig. 2A), suppressing EGF-induced cell transformation by 59% and 68%, respectively (Fig. 2B). These findings indicate that rutin possesses potent anti-tumor promoting effects.

2. Effects of rutin on EGF-induced MEK/ERK signaling in JB6 P+ cells

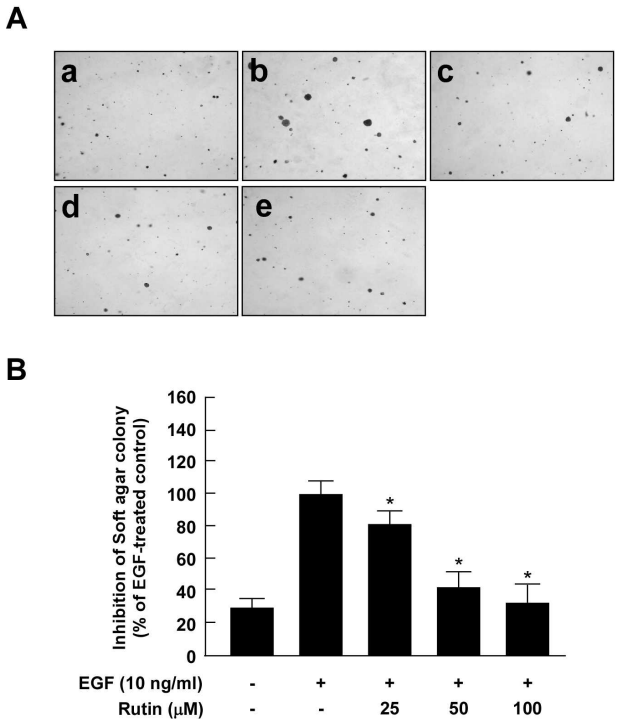
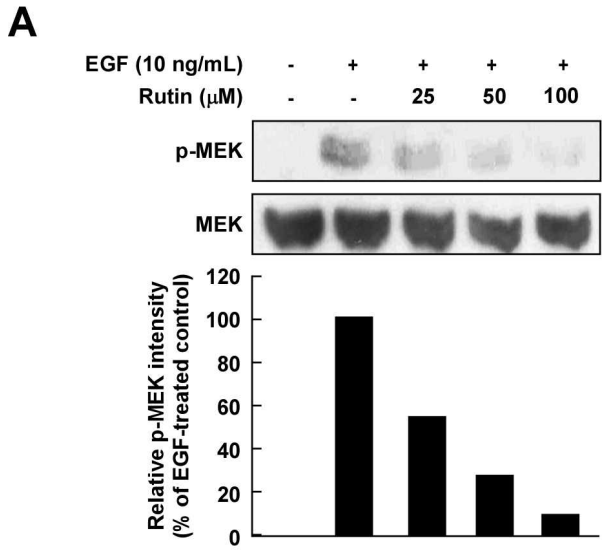


Fig. 2. Effects of rutin on EGF-induced neoplastic cell transformation in JB6 P+ cells. (A) JB6 P+ cells were treated without or with rutin for 10 days, after which the colonies were counted; the groups are as follows: (a) untreated control, (b) EGF alone, (c) EGF and 25 μM rutin, (d) EGF and 50 μM rutin, and (e) EGF and 100 μM rutin. (B) The cell colonies were counted using a microscope with the aid of Image-Pro Plus software. The numbers of colonies in the soft agar are presented as percentages relative to EGF-stimulated cells. The data are presented as means ± standard deviations of the number of colonies determined from three independent experiments. An asterisk (*) indicates a significant difference between a group treated with combined EGF and rutin and the group treated with EGF alone ($p<0.05$).

MEK and ERK play critical roles in the transmission of the signals initiated by EGF, and a majority of tumor phenotypes are linked to a deregulation of the MEK/ERK pathway (Cowley et al. 1994; Minden et al. 1994; Dhillon et al. 2007; Kang et al. 2011). Therefore, this pathway is an attractive target for the development of novel anti-cancer agents. Rutin inhibited the EGF-induced phosphorylation of MEK and also strongly suppressed the phosphorylation of MEK downstream kinases such as ERK (Fig. 3). These findings suggest that the rutin attenuated activity in the MEK/ERK pathway, leading to the suppression of neoplastic cell transformation.



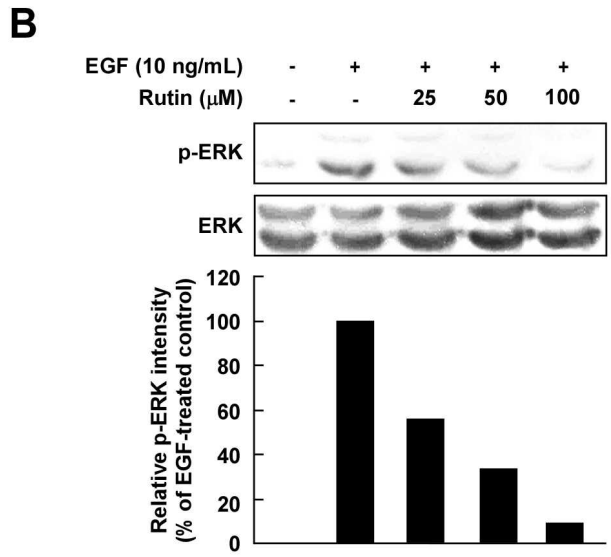
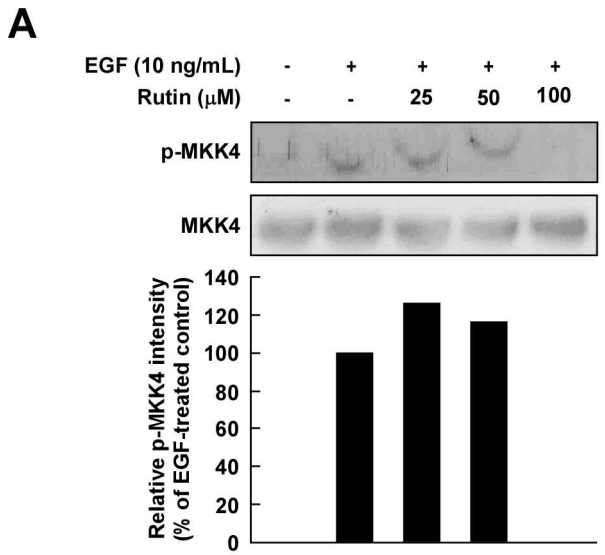


Fig. 3. Effects of rutin on the EGF-induced phosphorylations of MEK and ERK in JB6 P+ cells. The cells were pretreated with rutin at the indicated concentrations (25, 50, 100 μM) for 1 hour, stimulated by EGF, and then harvested 30 minutes later. The level of phosphorylated and total MEK1/2 and ERK proteins were determined by Western blot analyses. The data represent three independent experiments, which produced similar results.



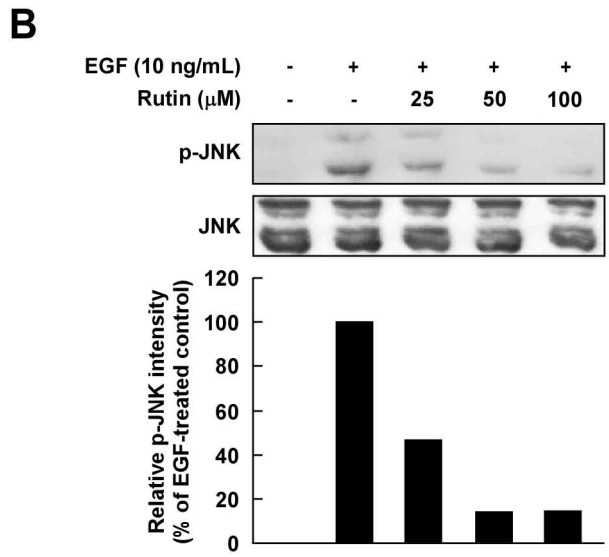


Fig. 4. Effects of rutin on the EGF-induced phosphorylations of MKK4 and JNK in JB6 P+ cells. The cells were pretreated with rutin at the indicated concentrations (25, 50, 100 μM) for 1 hour, stimulated by EGF, and then harvested 6 hours later. The levels of phosphorylated and total MKK4 and JNK proteins were determined by Western blot analyses. The data represent three independent experiments, which produced similar results.

3. Effects of rutin on EGF-induced MKK4/JNK signaling in JB6 P+ cells

JNK is upregulated in many types of cancers (Sakurai et al. 2006; Ouyang et al. 2008; Chang et al. 2009; Kang et al. 2011) and MAP2 kinases, such as MKK4 and MKK7, are the best-known upstream proteins of the JNK pathway. Activated JNK targets the activation of activator protein 1 (AP-1) and increases the expression levels of oncogenic proteins (Kang et al. 2011). Therefore, the MKK4/7/JNK pathway is also considered an attractive target for cancer prevention or treatment. Similar to the

above findings, the EGF-induced phosphorylations of MKK4 and JNK, which are MKK4 downstream kinases, were decreased by rutin in a dose-dependent manner (Fig. 4). These findings suggest that rutin attenuated activity in the MKK4/JNK pathway, leading to the suppression of neoplastic cell transformation. However, increased MKK4 phosphorylation at 25 or 50 μM is hard to explain. Therefore, further study must reveal target proteins of rutin which control the EGF-induced MKK4/JNK pathway.

4. Effects of rutin on EGF-induced MKK3/6/p38 signaling in JB6 P+ cells

MKK3/6 and p38 induce the expression of the COX-2 enzyme, which is a pro-inflammatory mediator that contributes to the progression of breast cancers and gliomas (Timoshenko et al. 2006; Xu & Shu 2007). Thus, the MKK3/6/p38 pathway is thought to play a critical role in cell transformation and carcinogenesis. To determine whether rutin affected MKK3/6/p38 phosphorylation, its effects on the EGF-induced phosphorylation of MKK3/6 and p38 were examined. In contrast to the above findings, rutin did not inhibit the EGF-induced phosphorylation of MKK3/6 and p38 in JB6 P+ cells in a dose-dependent manner (Fig. 5).

Collectively, the present findings demonstrate that the inhibition of cell transformation by rutin was mainly due to the suppression of the MEK/ERK and MKK4/JNK pathways, but not of the MKK3/6/p38 pathway. Single polyphenol can bind and inhibit many signaling molecules (Kang et al. 2011). Because numerous signaling proteins are correlated with each other in the promotion stage of cancer, polyphenols can act as multiple signaling inhibitors in cancer chemoprevention (Kang et al. 2011). With the consideration in these aspects, further studies to determine the target proteins of rutin would elucidate the exact molecular mechanisms of rutin as chemopreventive natural compound.

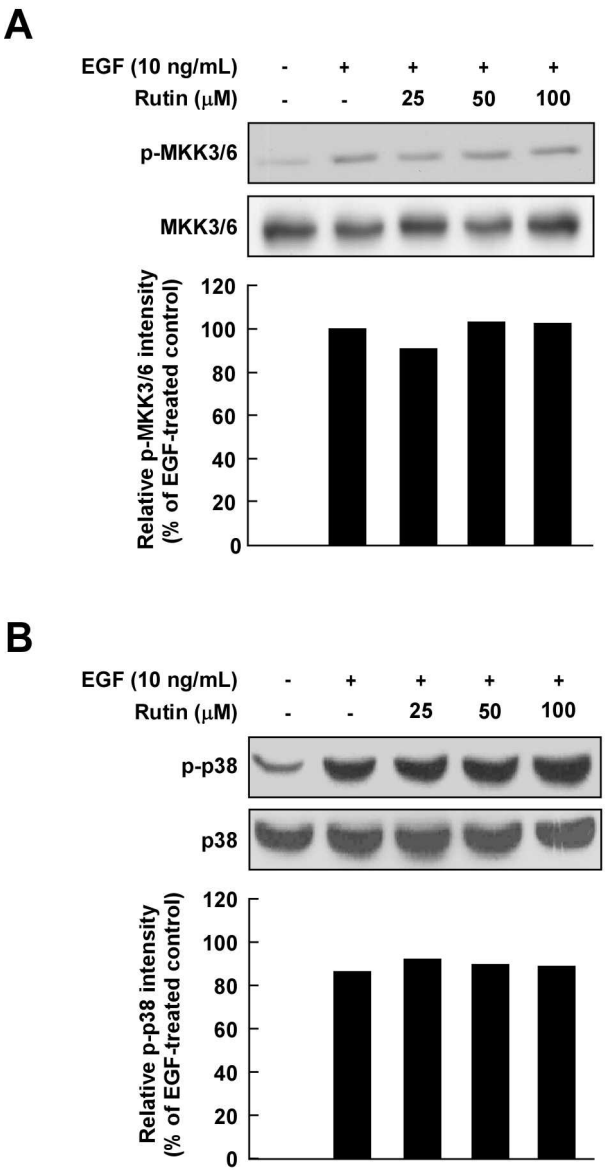


Fig. 5. Effects of rutin on the EGF-induced phosphorylations of MKK3/6 and p38 in JB6 P+ cells. The cells were pretreated with rutin at the indicated concentrations (25, 50, 100 μM) for 1 hour, stimulated by EGF, and then harvested 6 hours later. The levels of phosphorylated and total MKK3/6 and p38 proteins were then determined by Western blot analyses. The data represent three independent experiments, which produced similar results.

Summary

Rutin is a well-known flavonoid found in buckwheat. Recent studies have demonstrated that the biological actions of rutin include anti-inflammatory and anti-cancer effects, but the underlying molecular mechanisms of these actions are not yet fully understood. Neoplastic cell transformation is considered a major event that contributes to carcinogenesis, and the present study aimed to determine whether rutin would exert anti-tumor effects via the inhibition of EGF. The present findings demonstrate that rutin inhibited EGF-induced neoplastic cell transformation in JB6 P+ cells primarily by suppressing activity in the MEK/ERK and MKK4/JNK pathways. On the other hand, rutin had no effect on the phosphorylation of MKK3/6 and p38. Collectively, these

results suggest that rutin exerted a potent inhibitory influence on the molecular activity of the MEK/ERK and MKK4/JNK pathways and strongly attenuated EGF-induced neoplastic cell transformation. These findings provide insight into the biological actions of rutin and the molecular basis for the development of new chemoprotective agents.

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Received 17 July, 2015
Revised 27 July, 2015
Accepted 11 August, 2015