INTRODUCTION

The degradation of the extracellular matrix (ECM) is essential for embryonic development, morphogenesis, reproduction, and tissue remodeling. The family of matrix metalloproteinases (MMPs) in general, and matrix metalloproteinase-1 (MMP-1) in particular, play a central role in these processes. MMP-1, or interstitial collagenase, is a secreted protein that contributes to the etiology of many age-related degenerative diseases (Jacob, 2003; Kähäri and Saarialho-Kere, 1997). MMP-1 is prominently involved in the proteolytic release and activation of growth factors, cytokines, and signaling peptides, which also have the potential to modulate the senescent microenvironment (Dasgupta et al., 2010). Reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) readily undergo reactions with thiol groups and may, thus, participate in a common mechanisms underlying the activation of several different MMPs, including MMP-1 (Rajagopalan et al., 2003). $H_2O_2$ regulates the activity of critical signaling molecules, leading to augmented MMP-1 expression in human skin cells (Brenneisen et al., 1997). Furthermore, the redox activation of c-Jun-N-terminal kinase (JNK) controls the activity of the activator protein-1 (AP-1) transcription factor, resulting in an age-dependent increase in MMP-1 expression (Dasgupta et al., 2010). Moreover, oxidative stress stimulates the activity of extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK), which are upstream of the AP-1 transcription factor. The results of this study suggest that baicalein is involved in the inhibition of oxidative stress-induced expression of MMP-1 via inactivation of the ERK/JNK/AP-1 signaling pathway.

Abstract

The matrix metalloproteinase (MMP) family is involved in the breakdown of the extracellular matrix during normal physiological processes such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes such as pathological aging, arthritis, and metastasis. Oxidative conditions generate reactive oxygen species (ROS) (e.g., hydrogen peroxide [$H_2O_2$]) in cells, which subsequently induce the synthesis of matrix metalloproteinase-1 (MMP-1). MMP-1, an interstitial collagenase, in turn stimulates an aging phenomenon. In this study, baicalein (5,6,7-trihydroxyflavone) was investigated for its in vitro activity against $H_2O_2$-induced damage using a human skin keratinocyte model. Baicalein pretreatment significantly inhibited $H_2O_2$-induced up-regulation of MMP-1 mRNA, MMP-1 protein expression and MMP-1 activity in cultured HaCaT keratinocytes. In addition, baicalein decreased the transcriptional activity of activator protein-1 (AP-1) and the expression of c-Fos and c-Jun, both components of the heterodimeric AP-1 transcription factor. Furthermore, baicalein reduced phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK), which are upstream of the AP-1 transcription factor. The results of this study suggest that baicalein is involved in the inhibition of oxidative stress-induced expression of MMP-1 via inactivation of the ERK/JNK/AP-1 signaling pathway.

Key Words: Baicalein, Matrix metalloproteinase, Oxidative stress, Reactive oxygen species, Hydrogen peroxide, Signal transduction

Baicalein Attenuates Oxidative Stress-Induced Expression of Matrix Metalloproteinase-1 by Regulating the ERK/JNK/AP-1 Pathway in Human Keratinocytes

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Abstract

The matrix metalloproteinase (MMP) family is involved in the breakdown of the extracellular matrix during normal physiological processes such as embryonic development, reproduction, and tissue remodeling. Oxidative conditions generate reactive oxygen species (ROS) (e.g., hydrogen peroxide [$H_2O_2$]) in cells, which subsequently induce the synthesis of matrix metalloproteinase-1 (MMP-1). MMP-1, an interstitial collagenase, in turn stimulates an aging phenomenon. In this study, baicalein (5,6,7-trihydroxyflavone) was investigated for its in vitro activity against $H_2O_2$-induced damage using a human skin keratinocyte model. Baicalein pretreatment significantly inhibited $H_2O_2$-induced up-regulation of MMP-1 mRNA, MMP-1 protein expression and MMP-1 activity in cultured HaCaT keratinocytes. In addition, baicalein decreased the transcriptional activity of activator protein-1 (AP-1) and the expression of c-Fos and c-Jun, both components of the heterodimeric AP-1 transcription factor. Furthermore, baicalein reduced phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK), which are upstream of the AP-1 transcription factor. The results of this study suggest that baicalein is involved in the inhibition of oxidative stress-induced expression of MMP-1 via inactivation of the ERK/JNK/AP-1 signaling pathway.

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INTRODUCTION

The degradation of the extracellular matrix (ECM) is essential for embryonic development, morphogenesis, reproduction, and tissue remodeling. The family of matrix metalloproteinases (MMPs) in general, and matrix metalloproteinase-1 (MMP-1) in particular, play a central role in these processes. MMP-1, or interstitial collagenase, is a secreted protein that contributes to the etiology of many age-related degenerative diseases (Jacob, 2003; Kähäri and Saarialho-Kere, 1997). MMP-1 is a prominently involved in the proteolytic release and activation of growth factors, cytokines, and signaling peptides, which also have the potential to modulate the senescent microenvironment (Dasgupta et al., 2010). Reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$) readily undergo reactions with thiol groups and may, thus, participate in a common mechanisms underlying the activation of several different MMPs, including MMP-1 (Rajagopalan et al., 2003). $H_2O_2$ regulates the activity of critical signaling molecules, leading to augmented MMP-1 expression in human skin cells (Brenneisen et al., 1997). Furthermore, the redox activation of c-Jun-N-terminal kinase (JNK) controls the activity of the activator protein-1 (AP-1) transcription factor, resulting in an age-dependent increase in MMP-1 expression (Dasgupta et al., 2010). Moreover, oxidative stress stimulates the activity of extracellular signal-regulated kinase (ERK), which are also important for the regulation of MMP-1 expression. Blockade of the ERK pathway was found to abrogate the Ras- and serum-induced stimulation of the MMP-1 promoter, indicating a role for ERK in the transcriptional regulation of MMP-1 (Frost et al., 1994). These studies suggest that the ERK/JNK/AP-1 pathway may be the major activator of MMP-1 gene and protein expression.

A number of studies demonstrate inhibition of MMP-1 up-
regulation by antioxidants (Brenneisen et al., 2002; Nelson and Melendez, 2004), including N-acetylcyesteine (NAC), a precursor of glutathione (Kheradmand et al., 1998; Cho et al., 2006; Zaw et al., 2006). Previous work from our group demonstrated that triphenylthiohydantoin, an antioxidant, participates in the modulation of MMP-1 level in cultured cells (Kang et al., 2008). These data provide further support for the ability of ROS to initiate signaling pathways that lead to MMP-1 induction.

Baicalein (5,6,7-trihydroxyflavone) is a flavonoid derived from the roots of Scutellaria baicalensis. Baicalein attenuates oxidative stress and protects cardiomyocytes from lethal oxidative damage in an ischemia-reperfusion model (Shao et al., 1999; Shao et al., 2002). In addition, our recent work showed that baicalein ameliorated mitochondrial oxidative stress by activating nuclear factor (erythroid-derived 2)-like 2-mediated induction of manganese superoxide dismutase (Lee et al., 2011) and protected cellular components against oxidative damage by scavenging ROS and inhibiting apoptosis (Kang et al., 2012). On the other hand, the protective effect of baicalein against ROS-associated stimulation of MMP-1 expression has not been investigated. Therefore, the current study focused on the ability of baicalein to safeguard cultured human keratinocytes against H2O2-mediated MMP-1 induction and investigated the possible underlying molecular mechanisms.

MATERIALS AND METHODS

Cell culture

Human keratinocytes (HaCaT cells) were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 U/ml). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Reagents

Baicalein (Fig. 1) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The primary MMP-1 antibody was purchased from Epitomics, Inc. (Burlingame, CA, USA). Primary antibodies against phospho MEK1 (mitogen-activated protein (MAP) kinase kinase1), MEK1, phospho ERK1/2, ERK2, phospho SEK1 (stress-activated protein kinase (SAPK)/ERK kinase1), SEK1, phospho JNK1/2, JNK1/2, c-Fos, and phospho c-Jun were purchased from Cell Signaling Technology (Beverly, MA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were seeded in a 96-well plate at a density of 1.5×105 cells/well. Sixteen hours after plating, the cells were treated with baicalein at a concentration of 5 μg/ml. After 30 min, H2O2 (1 mM) was added to the plate. The cells were incubated for an additional 48 h at 37°C. Cells were then lysed in lysis buffer (100 μl: 120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% NP-40). Aliquots of the lysates (40 μg protein) were boiled for 5 min and electrophoresed on a 10% SDS-polyacrylamide gel. The proteins in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), and the membranes were subsequently incubated with the primary antibodies. The membranes were further incubated with secondary anti-IgG-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA) followed by exposure to X-ray film. The protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, UK).

Determination of MMP-1 activity

Cells were seeded in a 96-well plate at a density of 1.5×105 cells/well. Sixteen hours after plating, the cells were treated with baicalein at indicated concentrations or pretreated with 1 mM of N-acetyl cysteine (NAC) for 1 h. After 30 min, H2O2 (1 mM) was added to the plate. The cells were incubated for an additional 48 h at 37°C. MMP-1 activity was determined using a Fluorokinin™ E homolog active MMP-1 fluorogenic assay (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer’s instructions, which uses a quenched fluorogenic substrate. Production of the fluorescent cleavage product was determined using a fluorescence plate reader set (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 320 nm and emission wavelength of 405 nm.

Transient transfection and AP-1 luciferase assay

Cells were transiently transfected with a plasmid harboring the AP-1 promoter using Lipofectamine™ 2000, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Following an overnight transfection, cells were treated with baicalein (5 μg/ml). After an additional incubation for 1 h, cells were treated with H2O2 (1 mM). After 6 h, the cells were washed twice with PBS and then lysed with a passive lysis buffer (Promega, Madison, WI, USA). Following vortex-mixing and centrifugation at 12,000×g for 30 sec at 4°C, the supernatant was stored at −70°C until use in the luciferase assay. After mixing the cell extract (20 μl) with the luciferase assay substrate reagent (100 μl) at room temperature, the mixture was solved by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

Western blot analysis

Cells were seeded in a 96-well plate at a density of 1.5×105 cells/well. Sixteen hours after plating, the cells were treated with baicalein at a concentration of 5 μg/ml. After 30 min, H2O2 (1 mM) was added to the plate. The cells were incubated for an additional 48 h at 37°C. Cells were then lysed in lysis buffer (100 μl: 120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% NP-40). Aliquots of the lysates (40 μg protein) were boiled for 5 min and electrophoresed on a 10% SDS-polyacrylamide gel. The proteins in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA), and the membranes were subsequently incubated with the primary antibodies. The membranes were further incubated with secondary anti-IgG-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA) followed by exposure to X-ray film. The protein bands were detected using an enhanced chemiluminescence Western blot detection kit (Amersham, Little Chalfont, UK).

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were seeded in a 96-well plate at a density of 1.5×10^5 cells/well. Sixteen hours after plating, the cells were treated with baicalein at indicated concentrations or pretreated with 1 mM of N-acetyl cysteine (NAC) for 1 h. After 30 min, H2O2 (1 mM) was added to the plate. The cells were incubated for an additional 48 h at 37°C. MMP-1 activity was determined using a Fluorokinin™ E homolog active MMP-1 fluorogenic assay (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer’s instructions, which uses a quenched fluorogenic substrate. Production of the fluorescent cleavage product was determined using a fluorescence plate reader set (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 320 nm and emission wavelength of 405 nm.

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placed in an illuminometer to measure the light produced. The amount of light produced provided a measure of AP-1 luciferase activity, and hence, AP-1 transcriptional activity.

Statistical analysis
All measurements were performed in triplicate, and all data represent the mean ± the standard error. The results were subjected to an analysis of variance (ANOVA) using Tukey’s test to analyze differences between the means; \( p < 0.05 \) was considered to be significant.

RESULTS

Reduction of \( \text{H}_2\text{O}_2 \)-induced MMP-1 expression and activity by baicalein treatment
\( \text{H}_2\text{O}_2 \), and other ROS induce oxidative stress in many types of cells, including human keratinocytes (O’Toole et al., 1996; Shvedova et al., 2003). In particular, excessive amounts of ROS can stimulate mRNA and protein expression of MMP-1, a hallmark of oxidative stress (Brenneisen et al., 1997). Baicalein treatment at 1, 5, and 10 \( \mu \text{g/ml} \) significantly prevented the production of active MMP-1 in a concentration-dependent manner (Fig. 2A), and 5 \( \mu \text{g/ml} \) of baicalein was determined as the optimal concentration for further study. In the current study, \( \text{H}_2\text{O}_2 \) treatment markedly increased the levels of MMP-1 mRNA and protein in cultured HaCaT cells compared with those in control cells (no treatment), as evidenced by RT-PCR (Fig. 2B) and Western blot (Fig. 2C) analyses. However, baicalein pretreatment inhibited the increased transcription of MMP-1 mRNA in \( \text{H}_2\text{O}_2 \)-treated cells (Fig. 2B). Consistent with the RT-PCR results, baicalein also partially inhibited the \( \text{H}_2\text{O}_2 \)-induced increase in MMP-1 protein expression (Fig. 2C). Moreover, \( \text{H}_2\text{O}_2 \) treatment increased the amount of active MMP-1 in HaCaT cells, whereas pretreatment with baicalein or an antioxidant NAC significantly prevented this increase (Fig. 2D). These results suggest that baicalein blocked \( \text{H}_2\text{O}_2 \)-induced MMP-1 expression and activity.

Attenuation of \( \text{H}_2\text{O}_2 \)-induced activation of AP-1 by baicalein
The AP-1 transcription factor exists as either a c-Jun/c-Jun homo-dimer or a c-Jun/c-Fos hetero-dimer and is involved in the activation of MMP genes (Glover and Harrison, 1995; Farrell et al., 1989). \( \text{H}_2\text{O}_2 \) treatment stimulated the expression of both c-Fos and phospho c-Jun, while baicalein pretreatment attenuated the \( \text{H}_2\text{O}_2 \)-mediated increase in AP-1 transcriptional activity, as assessed by the AP-1 luciferase assay (Fig. 3B).

Reduction of \( \text{H}_2\text{O}_2 \)-induced phosphorylation of MEK-ERK and SEK-JNK by baicalein
Members of the Fos and Jun families of transcription factors are regulated by members of the MAP kinase family, particularly ERK and JNK (Cano et al., 1994; Murphy et al., 2002; Bogoyevitch et al., 2010). As shown in Fig. 4A, phospho ERK1/2 and phospho MEK1, which is upstream of ERK1/2, were markedly increased by \( \text{H}_2\text{O}_2 \) treatment. Baicalein reduced the amount of phospho ERK1/2 and phospho MEK1 induced by \( \text{H}_2\text{O}_2 \) (Fig. 4A). Furthermore, baicalein pretreatment attenuated the \( \text{H}_2\text{O}_2 \)-mediated up-regulation of phospho JNK1/2 and
phospho SEK1, which is upstream of JNK1/2 (Fig. 4B). Taken together, the results of this study suggest that baicalein inhibits oxidative stress-induced expression and activity of MMP-1 by inactivating AP-1 and its associated signaling kinases.

**DISCUSSION**

ROS, including H$_2$O$_2$, hasten aging of the skin and contribute to processes such as ultraviolet B-induced photaging, loss of elasticity, and wrinkling. These processes result, in large part, from the deterioration of the connective tissue matrix of the skin and, in particular, its collagen component. ROS produced in skin cells may contribute to the biological changes that are observed in the aging organ by accelerating the MMP-related ECM degradation system (Kawaguchi et al., 1996). Furthermore, ROS are important intermediates in downstream signaling pathways that culminate in the induction of increased steady state levels of MMP-1 (Brenneisen et al., 1997), an interstitial collagen-degrading enzyme. In this study, H$_2$O$_2$ caused a pronounced increase in MMP-1 mRNA and protein expression in cultured human HaCaT keratinocytes. Importantly, H$_2$O$_2$ also increased the amount of active MMP-1. Pretreatment with baicalein, a flavonoid component of *Scutellaria baicalensis*, partially suppressed the actions of H$_2$O$_2$. Furthermore, H$_2$O$_2$ augmented the activity of the AP-1 transcription factor, which is formed by a c-Fos/c-Jun heterodimer, as well as the expression of c-Fos and phospho c-Jun. AP-1 binds to the 5’ residues of the MMP-1 promoter, stimulating the expression of the MMP-1 gene (Mackay et al., 1992; Lin et al., 1993). Notably, baicalein pretreatment also reduced H$_2$O$_2$-induced c-Fos/phospho c-Jun expression and AP-1 transcriptional activity.

In conclusion, the results of the present study suggest that baicalein can safeguard HaCaT cells against oxidative stress-induced senescence-associated MMP-1 expression and activation via inhibition of the ERK/JNK/AP-1 pathway. These results have important implications for therapies designed to protect against premature aging of the skin and age-related skin disorders.

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