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, as well as in disease processes such as pathological aging, arthritis, and metastasis. Oxidative conditions generate reactive oxygen species (ROS) (e.g., hydrogen peroxide \(\text{H}_2\text{O}_2\)) 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 \(\text{H}_2\text{O}_2\)-induced damage using a human skin keratinocyte model. Baicalein pretreatment significantly inhibited \(\text{H}_2\text{O}_2\)-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

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 \(\text{H}_2\text{O}_2\) 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). \(\text{H}_2\text{O}_2\) 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-acetylcysteine (NAC), a precursor of glutathione (Kheradmand et al., 1998; Cho et al., 2006; Zaw et al., 2006). Previous work from our group demonstrated that triphlorethol-A, 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 oxidant damage in an ischemia-reperfusion model (Shao et al., 1999; Shao, 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., 2011). 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 kinase [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 1.5×10^5 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 in 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 1.5×10^5 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 Fluorokine® E human 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 1.5×10^5 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 in 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).

Fig. 1. Chemical structure of baicalein (5,6,7-trihydroxyflavone).

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