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Antioxidant properties of 5-hydroxy-4-phenyl-butenolide via activation of Nrf2/ARE signaling pathway

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ABSTRACT

5-Hydroxy-4-phenyl-butenolide (5H4PB) is a bioactive compound with antifungal and anti-obesity properties. Although it has recently been shown that 5H4PB activates peroxisome proliferator-activated receptor-gamma (PPARγ), the effect of 5H4PB on intracellular signaling pathways has not been clarified. In this study, we found that 5H4PB activated the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway, which plays an important role in cellular defense against oxidative stress, and the subsequent upregulation of ARE-dependent cytoprotective genes, including the heme oxygenase-1, catalase, and superoxide dismutase genes, without exhibiting cytotoxicity. In addition, 5H4PB significantly attenuated intracellular ROS generation, glutathione oxidation, and DNA damage induced by hydrogen peroxide (H_2O_2) exposure in mouse fibroblast cells. Furthermore, we demonstrated that pretreatment with 5H4PB confers a significant cytoprotective effect against H_2O_2-induced cell death in mouse cultured fibroblasts and primary hepatocytes. Thus, our study demonstrated that 5H4PB enhanced cellular resistance to oxidative damage via activation of the Nrf2/ARE signaling pathway.

1. Introduction

Oxidative stress, arising from an imbalance between the antioxidant system and the reactive oxygen species (ROS) generation, is usually thought to be the cause of disorders and diseases. Although ROS at low levels act as signaling molecules that promote cell proliferation and survival, a marked increase in ROS levels is associated with damage to a wide range of molecules including lipids, protein, and nucleic acids (McCord, 2000). In addition, oxidative stress plays a major role in the pathogenesis of a variety of disorders and diseases (Giasson et al., 2002; Roberts and Sindhu, 2009). It is therefore widely believed that attenuation of oxidative stress by antioxidants can help prevent various diseases or that antioxidants exert protective effects that lower the risk of various cancers (Uttara et al., 2009).

Antioxidants can be classified into direct and indirect antioxidants on the basis of their mechanisms of action. Direct antioxidants are redox active and directly scavenge ROS (Dinkova-Kostova and Talalay, 2008). On the other hand, indirect antioxidants exert their effects by inducing antioxidant and cytoprotective enzymes, including heme oxygenase-1 (HO-1), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Holtzclaw et al., 2004). The expression of antioxidant enzymes is mainly controlled by the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway (Chen and Kong, 2004). Under normal conditions, Nrf2 is largely bound to

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; ARE, antioxidant response element; CAT, catalase; DAPI, 4,6-diamidino-2-phenylindole; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; 5H4PB, 5-hydroxy-4-phenyl-butenolide; Keap1, Kelch-like ECH-associated protein 1; MI-MAC, multi-integrase mouse artificial chromosome; Nrf2, nuclear factor erythroid 2-related factor 2; PPARγ, peroxisome proliferator-activated receptor-gamma; ROS, reactive oxygen species; SLG, Stable Luciferase Green; SLR3, Stable Luciferase Red3; SOD, superoxide dismutase; TK, thymidine kinase; Trolox, 2,5,7,8-tetramethyl-6-chromanol.

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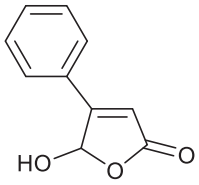

Fig. 1. Chemical structure of 5-hydroxy-4-phenyl-butenolide (5H4PB).

Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm (Ito et al., 1999). During periods of oxidative stress, Nrf2 is released from Keap1, is translocated into the nucleus, and subsequently binds to the AREs in the regulatory regions of target genes and activates the transcription of antioxidant enzymes. The Nrf2/ARE pathway therefore plays a key role in a cellular defense system against oxidative stress, and has been considered as a potential therapeutic target for the prevention of oxidative stress-evoked disorders and diseases (Jeong et al., 2006; Scapagnini et al., 2011). Based on these well-established concepts, many novel inducers of the Nrf2/ARE pathway, which include natural and synthetic products, have been uncovered (Forman et al., 2014).

Butenolides are a class of lactones with a four-carbon heterocyclic ring structure, and their derivatives are widely present in many natural products (Mukku et al., 2000; Beck et al., 2001). Butenolide-ring-containing compounds have attracted much attention owing to their structural diversity and biological activities including antimicrobial (Husain et al., 2010), anti-inflammatory (Ali et al., 2015), and anticancer activities (Wang et al., 2011). 5-Hydroxy-4-phenyl-butenolide (5H4PB) (Fig. 1) was isolated from *Epichloe typhina* on *Phleum pratense* for the first time as an antifungal compound (Koshino et al., 1992). It seems likely that 5H4PB is produced via the interaction between *E. typhina* and *P. pretense*, and the 5H4PB plays a role in the induction of resistance to fungal pathogens (Dickschat, 2017). More recently, it has also been isolated from fragrant vinegars as a peroxisome proliferator-activated receptor-gamma (PPARγ) activator (Tsujino, 2017). In addition, an *in vivo* study suggested that 5H4PB has an anti-obesity effect in mice (Masutani et al., 2016). Thus, although this compound may have beneficial potentials for prevention of disorders or diseases, the underlying mechanism by which 5H4PB modulates intracellular signaling pathways, including the oxidative stress pathway, is unknown.

Here, we elucidated 5H4PB as a new Nrf2/ARE pathway activator that upregulates the expression of antioxidant genes using a cell-based assay system as well as subsequent biochemical studies. Moreover, we also demonstrated that 5H4PB plays a key role in the cytoprotection against cell damage caused by oxidative stress in both cultured mouse fibroblasts and mouse primary hepatocytes. To the best of our knowledge, this is the first report of Nrf2/ARE pathway activation and the antioxidant effect of this butenolide-ring-containing compound.

2. Materials and methods

2.1. 5H4PB

5H4PB was chemically synthesized and provided by Takasago International Co., Ltd. (Tokyo, Japan). 5H4PB was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) at 200 mM as a stock solution and stored at −80 °C until used.

2.2. Cell culture

Mouse fibroblast A9 cells harboring the multi-integrase mouse artificial chromosome (MI-MAC) vector (Takiguchi et al., 2014) (a gift from Dr. M. Oshimura of Tottori University) were grown in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, HyClone, Thermo Scientific, Waltham, MA) at 37 °C in an atmosphere of 5% CO_2.

2.3. Plasmid construction

The following beetle luciferases were used: green-emitting Stable Luciferase Green (SLG; TOYOBO, Osaka, Japan; Nakajima et al., 2005) from *Rhagophthalmus ohbai* (Ohmiya et al., 2000) and red-emitting Stable Luciferase Red3, (SLR3) from *Phrixotrix hirtus* (Viviani et al., 1999; Nakajima et al., 2005). To generate a reporter plasmid carrying ARE, a synthesized oligonucleotide containing five tandem repeats of ARE from the NAD(P)H quinone oxidoreductase gene (5′-TCACAGTGACTCAGCAAAATT-3′) (Dhakshinamoorthy and Jaiswal, 2000) and the herpes simplex thymidine kinase (TK) promoter was ligated into the *Xho*I/*Nco*I site of pNFκB-TK-SLR3 (unpublished construction), from which the NFκB-TK cassette was removed, resulting in pARE-TK-SLR3. An expression cassette containing ARE, the TK promoter, SLR3, and a poly A signal (pA) was excised with *Xho*I and *Nco*I from pARE-TK-SLR3, and ligated into the *Xho*I/*Nco*I site of pNFκB-TK-SLR3-pENTR (unpublished construction) from which an expression cassette was removed, resulting in pARE-TK-SLR3-pENTR. An expression cassette containing the TK promoter, SLG, and pA was PCR-amplified with pTK-SLG (TOYOBO) as a template using 5′-CACCATCAATGTATCTTATCATGTCTGCTCGAG-3′ as the forward primer and 5′-GCGGATACATATTTGAGGATCC-3′ as the reverse primer. The amplified fragment was cloned into pENTR-D-TOPO (Invitrogen, Carlsbad, CA), resulting in pTK-SLG-pENTR. The expression cassettes containing ARE-TK-SLR3-pA and TK-SLG-pA were recombined into pNeo-φC31 attB (Yamaguchi et al., 2011) and pBsd-R4 attB (Yamaguchi et al., 2011) by the LR reaction using LR Clonase II Plus Enzyme Mix (Invitrogen), resulting in pARE-TK-SLR3-φC31-Neo and pTK-SLG-R4-Bsd, respectively. These constructions were inserted by homologous recombination into multi-integration sites of the MI-MAC vector (Takiguchi et al., 2014) as described below.

2.4. Generation of stable cell line

The A9 cells harboring the MI-MAC vector were seeded in six-well plates at 6×10^5 cells per well one day before transfection. Three micrograms of pTK-SLG-R4-Bsd was cotransfected with 1 μg of the R4 recombinase expression plasmid pCMV-R4 (Yamaguchi et al., 2011) (a gift from Dr. T. Ohbayashi) using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. The transfected cells were seeded onto 10 cm dishes three days after transfection, and subcultured for selection with 6 μg/ml blasticidin S (Wako Pure Chemical Indutries) over 2–3 weeks. After that, pARE-TK-SLR3-φC31-Neo was cotransfected into the generated A9 cells with 1 μg of the φC31 recombinase expression plasmid pCMV-φC31 (Yamaguchi et al., 2011) (a gift from Dr. T. Ohbayashi) as described above, and subcultured for selection with 800 μg/ml G418 (Nacalai Tesque, Kyoto, Japan). Integration of the transgene into the corresponding site on the MAC vector was confirmed by genomic PCR.

2.5. Real-time bioluminescence measurement of 5H4PB-induced ARE activation

The generated A9 stable cell lines were seeded on a 24-well plate at 1.5×10^5 cells/well. After one-day incubation, the medium was replaced with DMEM without phenol red (Gibco-BRL,