JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Davallialactone from Mushroom Reduced Premature Senescence and Inflammation on Glucose Oxidative Stress in Human Diploid Fibroblast Cells

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ABSTRACT: Mushrooms are both food and a source of natural compounds of biopharmaceutical interest. The purpose of this study was to clarify whether davallialactone from mushroom extract affected the pathogenesis of hyperglycemia oxidative stress and the aging process in human diploid fibroblast (HDF) cells. The high-glucose state with glucose oxidase resulted in glucose oxidative stress, induction of inflammatory molecules, dysfunction of antioxidant molecules, and activation of mitogen-activated protein kinase (MAPKs) and its downstream signaling in old HDF cells. The exposure of glucose oxidative stress in middle-stage cells led to stress-induced premature senescence (SIPS) via senescence-associated β-galactosidase (SA β-gal) activity and displayed replicative senescence phenomena. However, davallialactone reduces the pathogenesis of glucose oxidative stress and the aging process through down-regulation of SA β-gal activity. These results strongly suggest that natural compounds, especially mushroom extract davallialactone, improve the pathogenesis of glucose oxidative stress and the aging process. Hence, davallialactone has potential in the treatment of diabetes mellitus or age-related disease complications.

KEYWORDS: *davallialactone, aging, diabetes mellitus, mushroom, glucose oxidative stress, inflammation*

INTRODUCTION

Mushroom-derived compounds possess a nutritionally functional food and a source of biopharmaceuticals. Some mushrooms have established traditional oriental therapies. More recently, scientific and medical studies have reported that the natural extracted compounds demonstrated potent and unique properties of therapeutic treatment such as antimicrobial, antiallergic, anticancer, immunosuppressive, and anti-inflammatory activities.[1] In particular, the mushroom *Inonotus xaranticus* (Berk) Imaz. Et Aoshi (Hymenochactaceae) is widely distributed in East Asia. We identified that davallialactone, a hispidin analogue from the mushroom *I. xeranticus* belonging to the family Hymenochaetaceae, has potent antioxidant and anti-inflammatory properties.[2,3] However, the effect of davallilactone on aging-related inflammation under high glucose oxidative stress is not completely understood.

Diabetes mellitus (DM) is a complex multisystem disorder characterized by hyperglycemia due to the insufficiency of insulin secretion or action, leading to disease complicated by chronic oxidative stress.[4] Increased oxidative stress is a major cause of hyperglycemia-induced diabetic complications. Hyperglycemia in an organism stimulates reactive oxygen species (ROS) formation from a variety of sources such as glucose autoxidation, NAD(P)H oxidase, and lipooxygenase.[5] The aging process is linked to the development of chronic inflammation and disease complications by physical malfunctions.[6,7] Pathological conditions of metabolic disorder can stimulate cellular ROS generation and oxidative stress.[8,9]

In normal physiological conditions, endogenous ROS helps to maintain proliferation and differentiation in cell growth. However, when ROS accumulate over prolonged periods, they cause chronic oxidative stress and inflammation. The enzyme glucose oxidase catalyzes glucose to hydrogen peroxide (H_2O_2) and D-glucono-δ-lactone. In cells, it aids in breaking sugar down into its metabolites. In addition, this enzyme induces insulin resistance in vitro and in vivo and plays a central role in glucose oxidative stress.[10] We hypothesized that the state of diabetes generates continuous glucose oxidase secretion for management of glucose levels that then reacts with D-glucose substrate to yield H_2O_2. H_2O_2 produced by glucose oxidase may have harmful effects such as hyperglycemia stress.

Aging cells are characterized by permanent growth arrest, the expression of senescence-associated β-galactosidase (SA β-gal) activity, and accumulation of ROS formation.[11,12] Among the ROS, H_2O_2 plays an important role in oxidative stress-mediated diseases and leads to premature senescence phenotype at the cellular level.[13−15] Hence, several antioxidants including *N*-acetyl-L-cysteine have been evaluated as therapeutic targets in aging-related diseases including DM.[9,16] Therefore, we hypothesized that beneficial bioactive natural compounds having an antioxidant effect may be able to control pathogen

Received: April 22, 2013
Revised: June 18, 2013
Accepted: June 27, 2013
Published: June 27, 2013

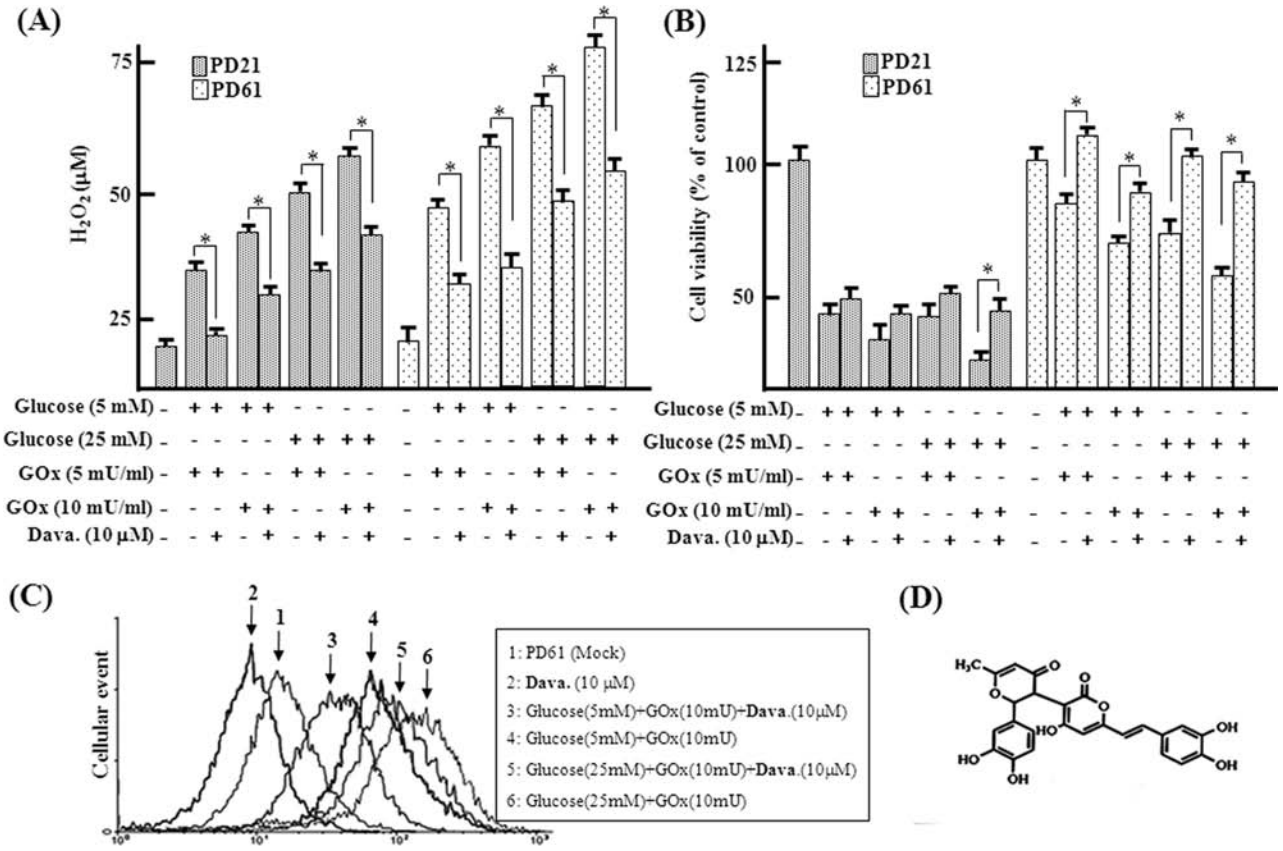

Figure 1. Davallialactone affects cellular toxicity via removal of ROS formation under glucose oxidase induced stress in young (PD 21) and old (PD 61) HDF cells. (A) Amounts of H_2O_2 in the absence or presence of davallialactone (10 μM) before exposure to glucose oxidase (5 and 10 mU/mL) with D-glucose in young (PD 21) and old (PD 61) HDF cells were determined in the culture medium at 24 h as described iunder Materials and Methods. (B) Cell viability was determined using a MTT assay at 24 h in young (PD 21) and old (PD 61) HDF cells. (C) ROS formation was determined by flow cytometry after DCFH-DA treatment at 24 h in old (PD 61) HDF cells. Each value is reported as the mean and standard error of the mean (SEM) of three independent experiments. Panel D shows the chemical structure of davallialactone.

mechanisms of disease complications such as DM and aging-associated inflammation.

In this study we examined whether the antioxidant effects of davallialactone from mushroom contribute to defense mechanisms in both antioxidant and anti-inflammatory responses via reduction of free radical implication against glucose oxidative stress.

MATERIALS AND METHODS

Human Diploid Fibroblast (HDF) Culture. Primary HDF cells were isolated from the foreskins of newborns using the procedure reported elsewhere.[15,17] For induced glucose oxidative stress and free state of glucose in HDF cells, 80% confluence cells were supplemented with 0.5% FBS RPMI 1640 medium before exposure to various glucose oxidase concentrations with D-glucose in the absence or presence of davallialactone for 72 h.

Materials. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), glucose oxidase, D-glucose, X-gal, *O*-nitrophenyl-β-D-galactopyranoside (ONPG), and actin were purchased from Sigma (St. Louis, MO, USA). Antibodies against p- extracellular signal-regulated kinase 1/2 (p-ERK1/2), p-stress-activated protein kinase/c-Jun NH2-terminal kinase (p-SAPK/JNK), and cyclooxygenase-2 (Cox-2) were supplied by Cell Signaling (Beverly, MA, USA). Intracellular adhesion molecule-1 (ICAM-1), interleukin-1 beta (IL-1β), heme oxygenase-1 (HO-1), and c-Jun were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and copper−zinc superoxide dismutases (Cu/ZnSOD), manganese superoxide dismutase (MnSOD), and c-fos were purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA). Davallialactone was extracted from the mushroom *I. xeranticus* (Berk) as previously described.[18]

Measurement of ROS Formation and H_2O_2 Production. The level of ROS formation was assessed using an oxidation-sensitive fluorescent probe DCFH-DA. After a complete reaction, the cells were incubated with 5 μM DCFH-DA for 30 min in the dark at 37 °C, harvested, and followed by FACScan flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

The quantity of H_2O_2 produced by glucose oxidase in culture medium at indicated times was determined using the Biovision hydrogen peroxide assay kit (Biovision Research Products, Milpitas, CA, USA). The absorbance was measured at 570 nm with an enzyme-linked immunosorbant assay (ELISA) reader (Bio-TeK, Winooski, VT, USA).

Cell Viability. Cell viability was determined by the reduction of MTT to formazan. After a complete reaction at 24 h, MTT (1 mg/mL of PBS) was added to each cell of 96-well plates followed by incubation at 37 °C for 3 h; dimethyl sulfoxide (DMSO; 100 μL) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm with an ELISA reader (Bio-TeK).

SA β-gal Staining and Enzyme Assay following Stress-Induced Premature Senescence (SIPS) by Glucose Oxidase. For SIPS, middle-stage HDF cells were exposed to 7 mU/mL glucose oxidase with high-glucose state for 24 h in the absence or presence of davallialactone. After 24 h, the cells were seeded in 100 mm culture dishes (SPL Life Science, Korea) at a 1:4 ratio and subcultured at the 11th day. The control cultures followed the same schedule of medium changes without the glucose oxidase treatment.

The SIPS schedule for senescent status was confirmed by in situ staining for SA β-gal using previously reported methods.[11,15] For the

dx.doi.org/10.1021/jf401691y | J. Agric. Food Chem. 2013, 61, 7089−7095

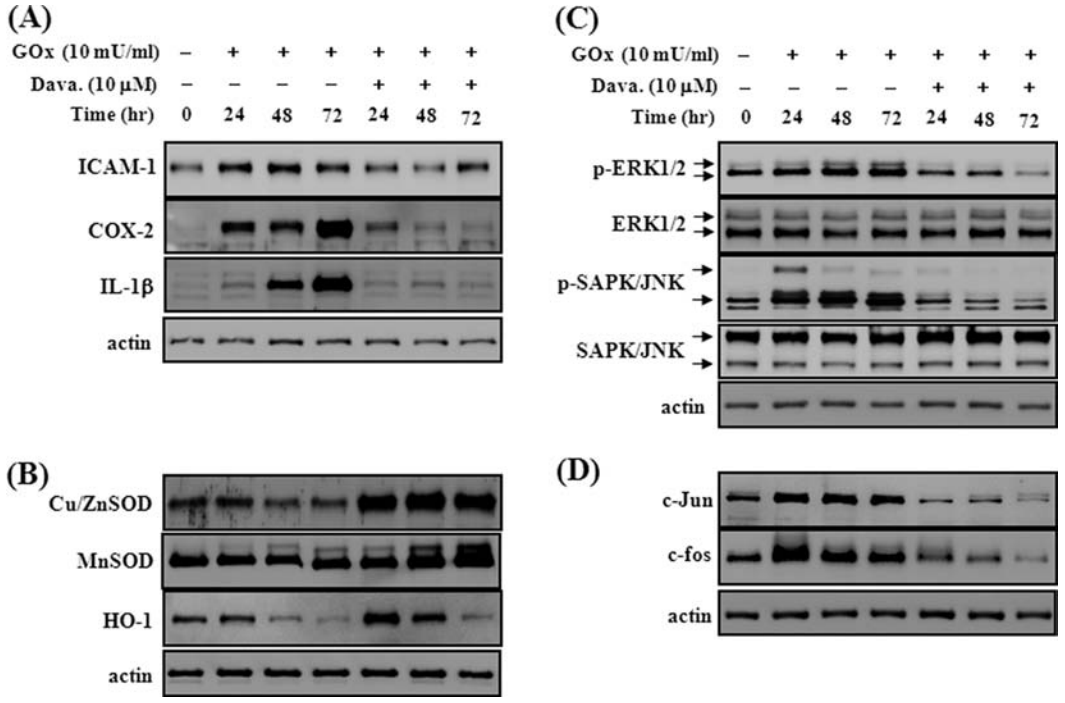

Figure 2. Effect of davallialactone on expression of age-related inflammatory, antioxidant, and MAPKs signaling by glucose oxidase with high-glucose state. Davallialactone (10 μM) was exposed to glucose oxidase (10 mU/mL) with high-glucose state at indicated times in old (PD 61) HDF cells. (A) The expression of inflammatory molecule, ICAM-1, Cox-2, and IL-1β, protein levels in total cell lysate was determined by Western blot analysis. (B) Antioxidant molecule, CuZnSOD, MnSOD, and HO-1, protein levels were determined by Western blot analysis. (C) p-ERK1/2 and p-SAPK/JNK signalings were determined by Western blot analysis. (D) c-Jun and c-fos signalings were determined by Western blot analysis. For loading control, the blots were reprobed with actin antibody. All data are representative of three separate experiments.

ONPG enzyme assay, equal numbers of cells were harvested in a microcentrifuge tube following the SIPS schedule. The activity of SA β-gal was determined using ONPG enzyme according to previously reported methods.[15] The absorbance was measured at 420 nm with an ELISA reader (Bio-TeK).

Western Blot Analysis. Total proteins were extracted from the HDF cells using a lysis buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tri-HCl (pH 8.0), 1%-NP 40, 1 mM aprotinin, 0.1 mM leupeptin, and 1 mM pepstatin and quantified using the Bradford dye-binding procedure (Bio-Rad, Hercules, CA, USA). The protein loading was electrophoresed in 8−15% SDS−polyacrylamide gel under denaturizing conditions and transferred to a Hybond-P membrane (Amersham, Arlington, IL, USA). The membranes were incubated with appropriate primary antibody overnight at 4 °C. After incubation with horseradish peroxidase−IgG-conjugated secondary antibody for 1 h at room temperature, the signals were visualized with chemiluminescent detection using an LAS-4000 CCD imaging system (Fujifilm Corp., Tokyo, Japan).

Statistical Analysis. At least three independent experiments were carried out. The data were analyzed using an ANOVA and Duncan's test. Null hypotheses of no difference were rejected if p values were <0.05.

RESULTS

Davallialactone Reduces ROS Formation and Cellular Toxicity under Glucose Oxidative Stress. Davallialactone was isolated from the mushroom *I. xeranticus* (Berk), and its chemical structure is shown in Figure 1D; it has anti-inflammatory properties through removal of ROS formation in low concentration (5−10 μM) and without cellular cytotoxicity in HDPCs.[3,18] Therefore, in this study, we have decided the optimal concentration is 10 μM. The antioxidant characteristics of davallilactone were evaluated for suppression of glucose oxidative stress by glucose oxidase with high-glucose state in young and old HDF cells. In this study, we used RPMI 1640 culture media for the state of free glucose and added D-glucose for states of low (5 mM) and high glucose (50 mM). The addition of glucose oxidase (5 and 10 mU/mL) with low and high glucose concentrations continuously increases H_2O_2 production for 24 h in young (under PD 21) and old (over PD 61) HDF cells. The state of high glucose significantly increased production of H_2O_2 more than the state of low glucose regardless of glucose oxidase concentration (Figure 1A). However, the production of H_2O_2 was suppressed by davallialactone (10 μM) in young and old HDF cells (Figure 1A).

The influence of glucose oxidase with glucose on cell viability was determined by MTT assay in young and old HDF cells. Treatment with each glucose oxidase concentration (5 and 10 mU/mL) with glucose (5 and 25 mM) results in 50% fewer dead cells compared with mock at 24 h in young cells. On the contrary, cellular toxicity of old cells was reduced against glucose oxidase treatment compared with young cells even though more H_2O_2 was produced (Figure 1B). Particularly, the presence of davallialactone did not affect cell viability in young cells. However, davallialactone clearly affects the protection of cellular toxicity under the same conditions in old cells (Figure 1B).

Furthermore, different concentrations of glucose with glucose oxidase treatment ensure increased ROS formation compared to glucose free old cells (Figure 1C). However, davallialactone removed ROS formation in both glucose-free and glucose against glucose oxidase treatment (Figure 1C).

Effect of Davallialactone on Expression of Aging Related Insult Molecules and Stress Signaling under Glucose Oxidase. To determine whether glucose oxidative

dx.doi.org/10.1021/jf401691y | J. Agric. Food Chem. 2013, 61, 7089−7095

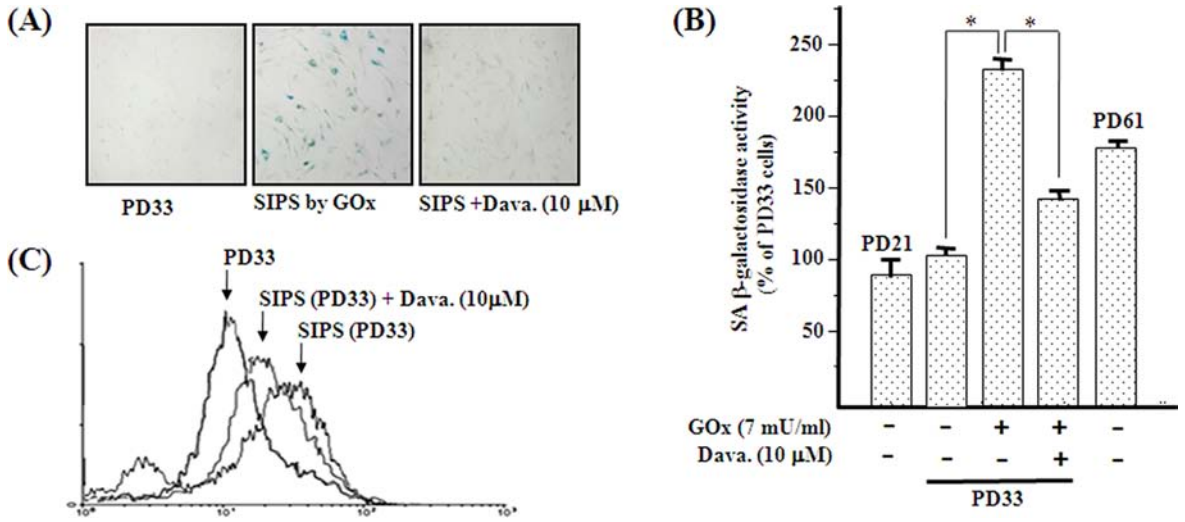

Figure 3. Antiaging effect of davallialactone on SIPS by glucose oxidase with high-glucose state. The middle-stage (PD 33) cells were pretreated with davallialactone (10 μM) before glucose oxidase (7 mU/mL) exposure with high glucose and continuously exposed and treated with davallialactone at 3 days to undergo SIPS for 11 days. (A) The microscope sketches of the cells were determined by SA β-gal staining. (B) Equal numbers of cells were harvested and analyzed by an ONPG enzyme assay at 11 days. (C) The level of ROS formation was determined by flow cytometry after DCFH-DA treatment at 11 days.

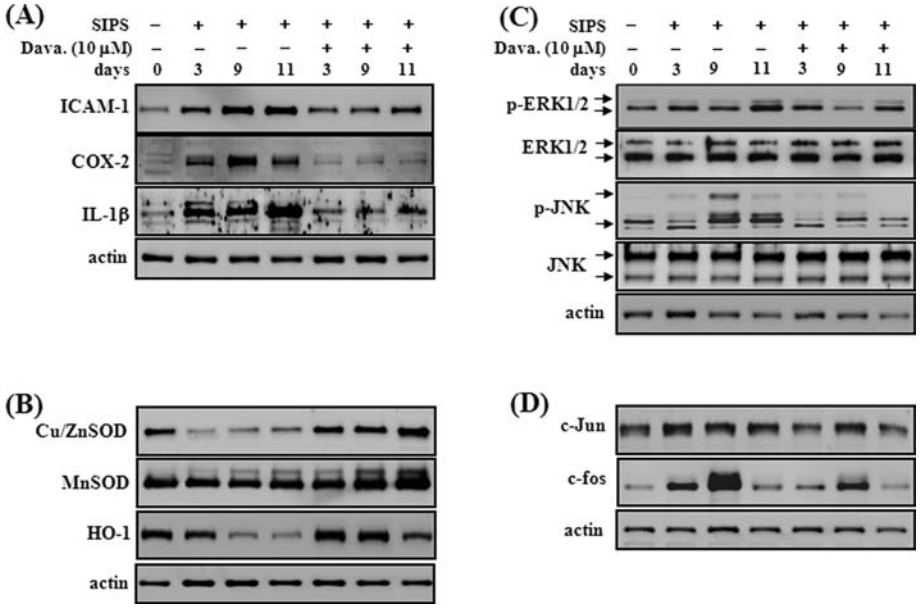

Figure 4. Davallialactone affects reduction of aging insult molecules on SIPS by glucose oxidase. During the induced premature senescence by glucose oxidative stress for 11 days, inflammatory molecules (A), antioxidant molecules (B), MAPKs (C), and AP-1(D) were determined by Western blot.

stress by glucose oxidase (10 mU/mL) with high-glucose (25 mM) state was responsible for inflammation, antioxidant molecules, and stress signaling, the old cells were treated with glucose oxidase for 72 h in high-glucose state, and protein expression of several related markers was assayed by Western blot analysis. The glucose oxidase treatment at the indicated day in HDF old cells revealed gradual aggravation of inflammatory molecules (ICAM-1, Cox-2, and IL-1β; Figure 2A), antioxidant molecules (Cu/ZnSOD, MnSOD, and HO-1; Figure 2B), MAPKs (mitogen-activated protein kinase, p-ERK1/2, p-SAPK/JNK; Figure 2C), and activator protein-1 (AP-1; c-Jun and c-fos; Figure 2D) signal activation. However, the presence of davallialactone in the same condition reduced inflammatory molecules (Figure 2A), induced antioxidant molecules (Figure 2B), and down-regulated MAPKs (p-ERK1/2, p-SAPK/JNK; Figure 2C) and AP-1 (c-Jun and c-fos; Figure 2D) signal activation.

Antiaging Effect of Davallialactone on SIPS by Glucose Oxidase with High-Glucose State. The antiaging effect of davallilacton under glucose oxidase was analyzed through expressional changes of age-related biomarker. The middle-stage HDF cells were induced to undergo SIPS by glucose oxidase with high glucose for 11 days in the absence or presence of davallialactone. To characterize aging, SA β-gal activity and ROS formation were checked after SIPS. The SIPS-HDF cells were seen to significantly increase by the density of SA β-gal stain and enzyme activity compared with PD 21, PD 33, and PD 61 (Figure 3A,B). ROS formation of SIPS-HDF

dx.doi.org/10.1021/jf401691y | J. Agric. Food Chem. 2013, 61, 7089−7095

cells increased more compared with non-SIPS PD 33 cells (Figure 3C). However, the presence of davallialactone suppressed SA β-gal activity and ROS formation (Figure 3A–C).

Davallialactone Affects Reduction of Aging Insult Molecules on SIPS-HDF Cells. In a subsequent experiment on the role of davallialactone in age-related inflammation, antioxidant dysfunction stress signaling was examined by Western blot analysis in SIPS-HDF cells. After inducing SIPS-HDF cells gradually increased expression of inflammatory molecules (ICAM-1, Cox-2, and IL-1β) and dysfunction of antioxidant expression (Cu/ZnSOD, MnSOD, and HO-1) at indicated days (Figure 4A,B). Furthermore, SIPS-HDF cells up-regulated activation of stress signaling such as p-ERK1/2, p-SAPK/JNK (Figure 4C), and c-Jun and c-fos (Figure 4D). However, davallialactone inhibited these inflammatory molecules (Figure 4A) and recovered antioxidant molecules (Figure 4B). Indeed, davallialactone down-regulates MAPKs and AP-1 signaling (Figure 4C,D).

DISCUSSION

Hyperglycemia enhances free radical production and causes oxidative damage. The oxidative injury increases weakened defense and reduces endogenous antioxidants in diabetes.[4] Glucose oxidase yields H_2O_2 via a catalyzed reaction with glucose substrate metabolites. A recent study reported that glucose oxidase induces insulin resistance and plays a central role in the pathogenesis of insulin resistance via glucose oxidative stress.[10] H_2O_2 is an index of oxidative stress to mediators of cell damage in hyperglycemia.[19] This study described the production of H_2O_2 by glucose oxidase with high glucose for glucose oxidative stress state in HDF cells.

In this study, glucose oxidase with high glucose gradually increased the production of H_2O_2 and cellular toxicity in young and old HDF cells. Interestingly, despite less production of H_2O_2 than in old cells, the young cells exacerbated cellular toxicity, so we examined this further with experiments in old cells. The high-glucose state with glucose oxidase in old HDF cells appeared to increase ROS formation and expression of inflammatory molecules, ICAM-1, Cox-2, and IL-1β. This condition causes the reduction of antioxidant molecules, Cu/ZnSOD, MnSOD, and HO-1. Despite a higher accumulation of ROS, the aging cells resist the apoptotic response against oxidative stress via survival mechanisms.[20,21] Oxidative stress in diabetes plays a role in complications leading to increased free radical production and compromised free radical scavenger systems that exacerbate the aging process and inflammatory response.[6,22] Indeed, oxidative stress and inflammation are physiopathological mechanisms related to diabetes and aging.[23] Thus, this phenomenon illustrates that the steady state of glucose oxidative stress leads to diabetes-mediated oxidative stress in HDF cells. However, davallialactone significantly protected cellular toxicity, production of H_2O_2, and ROS formation. Furthermore, davallialactone reduced expression of inflammatory molecules and recovered antioxidant molecules. In a previous study, davallialactone had antioxidant characteristics, anti-inflammatory effect, and protection against cardiotoxicity by stopping ROS formation.[3,24] Several antioxidants are suggested therapeutic strategies in the treatment of complications caused by diabetes.[4,16,25] Thus, the antioxidant effect of davallialactone might be enhanced protection of complication events via induction of a defense enzyme expression against glucose oxidative stress in old HDF cells.

Aging-related hyperglycemia is associated with increased oxidative stress via MAPKs.[26] Moreover, the transcription factor AP-1 is activated by the intracellular redox state in high glucose level.[27] In this study, ERK1/2 and SAPK/JNK among MAPKs were up-regulated by glucose oxidase with high-glucose state, whereas p38 was not detected (data not shown). c-Jun and c-fos among AP-1 were activated in the same condition. However, davallialactone showed marked down-regulation of MAPKs and AP-1 signaling. More evidence suggested that hyperglycemia induced oxidative damage including death pathways implicated in cell apoptosis and necrosis.[28] Several antioxidants contribute to attenuate oxidative damage by down-regulating apoptosis signaling such as MAPKs and AP-1 pathway.[3,29] Therefore, these results verify the involved pathway on the underlying mechanism of glucose oxidative stress, and davallialactone improves the pathogenesis of disease complications via regulating signaling pathways.

Oxidative stress increases the aging process and dysfunction of organs, which lead to chronic inflammation.[6,20] Chronic ailments including diabetes are considered to be an age-associated disease.[30] We hypothesized that glucose oxidative stress also may lead to stress-induced premature senescence at the cellular level. Another thing we focused on is that davallialactone diminished the aging process and aging-related cellular events such as ROS formation and altered molecular expression. In this study, when exposed to glucose oxidase with high glucose, the middle-stage HDF cells exhibited aging phenomena via increased SA β-gal activity and ROS formation. In addition, the SIPS-HDF cells developed gradually increased expression of inflammatory molecules and diminished expression of antioxidant molecules. However, davallialactone robustly attenuated SA β-gal activity and ROS formation and its inflammatory molecules expression. Furthermore, davallialactone recovered expression of antioxidant molecules. Among the many ROS, H_2O_2 plays a major role leading to premature senescence.[13] SIPS cells display replicative senescence-like typical cells as determined by SA β-gal activity, ROS formation, and altered expression of age-related molecules such as expression of ICAM-1.[14,15] Indeed, inflammatory response is related to age-associated disorders including type-2 diabetes.[23] Therefore, these results suggest that glucose oxidative stress can lead to aging process and age-related disease complications, which cause production of H_2O_2 from glucose autoxidation state. On the other hand, davallialactone restores a breakdown of aging processing and inflammatory response via recovered free radical scavenger systems against glucose oxidative stress. Thus, natural compound studies including davallialactone might lead to therapeutic approaches for limiting damage and disease complications from glucose oxidative stress.

In senescence, ERK1/2 signal mediates the role in oxidative stress induced senescence and leads to chronic inflammation by unregulated ROS.[15,31] In addition, accumulation of ROS in cellular senescence appears to activate MAPKS including SAPK/JNK.[32−34] In this study, SIPS-HDF cells appeared to up-regulate ERK1/2, SAPK/JNK, and AP-1 signaling. As a result, glucose oxidative stress is considered to be an age-associated disease complication in both young and aging cells. However, davallialactone resulted in overwhelming down-regulation of its signaling.

New therapeutic drugs from natural compounds have potential property of bioactivity for complicated diseases such as aging and DM. Furthermore, natural bioactive compounds such as flavonoid extract possess antidiabetic properties such as

antioxidant and anti-inflammatory activities.[35] Consequently, davallilactone might have beneficial effects for the breakdown of glucose oxidative stress. Therefore, therapeutic stratagems of natural compounds like davallialactone offer major contributions to the development of treatments of complicated diseases and aging-linked disease complications because of its beneficial antioxidant and anti-inflammatory effects.

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Author Contributions

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Funding

This study was supported by a grant from the Korea Forest Service (S121010L090120), Korea.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

DM, diabetes mellitus; HDF, human diploid fibroblast; SIPS, stress-induced premature senescenc; MAPKs, mitogen-activated protein kinases; SA β-gal, senescence-associated β-galactosidase; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; p-ERK1/2, extracellular signal-regulated kinase 1/2; p-SAPK/JNK, p-stress-activated protein kinase/c-Jun NH_2-terminal kinase; Cox-2, cyclooxyganase-2; ICAM-1, intracellular adhesion molecule-1; IL-1β, interleukin-1 beta; HO-1, heme oxygenase-1; Cu/ZnSOD, copper−zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; DMSO, dimethyl sulfoxide; ONPG, *O*-nitrophenyl-β-D-galactopyranoside; AP-1, activator protein-1; ELISA, enzyme-linked immunosorbant assay

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