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Plumbagin activates ERK1/2 and Akt via superoxide, Src and PI3-kinase in 3T3-L1 Cells

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ABSTRACT

Plumbagin, derived from the plant *Plumbago zeylanica*, has been shown to chronically activate ERK1/2 and inhibit Akt activity in cancer cells. However, the acute effects of plumbagin on ERK1/2 and Akt activities remain unknown. In this study, we examined the effects of plumbagin on ERK1/2 and Akt activities in 3T3-L1 cells. Exposure of 3T3-L1 cells to plumbagin generated superoxide and activated both ERK1/2 and Akt. The plumbagin-stimulated ERK1/2 and Akt activities were sensitive to an antioxidant NAC, superoxide dismutase mimetic MnTBAP, superoxide scavenger Tiron and NAD(P)H oxidase inhibitor DPI. Plumbagin-stimulated ERK1/2 activity was attenuated by the MEK1/2 inhibitor PD98059 and Ras inhibitor manumycin A, whereas plumbagin-stimulated Akt activity was blocked by the PI3K inhibitor LY294002. Both plumbagin-stimulated ERK1/2 and Akt activities were attenuated by PP2, a Src inhibitor. Interestingly, inhibition of phosphatidylinositol 3-kinase (PI3-kinase), but not Akt, activity leaded to attenuation of plumbagin-stimulated ERK1/2 activity. These results suggest that plumbagin activates NAD(P)H oxidase, Src, and PI3K, and that the activated PI3K or PDK1 subsequently stimulate Akt and Ras–Raf–MEK1/2–ERK1/2 in 3T3-L1 cells.

1. Introduction

Reactive oxygen species including superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radicals have been suggested to play a major role in the pathogenesis of a number of diseases such as cancer, diabetes, hypertension, atherosclerosis, and Alzheimer's disease. Insulin resistance and beta cell dysfunction are key features of the type 2 diabetes (for a recent review, see Frey et al., 2008; Klaunig and Kamendulis, 2004; Bedard and Krause, 2007; Madamanchi et al., 2005; Simmons, 2006). There is considerable evidence showing that visceral adiposity is closely linked to insulin resistance (Moller and Kaufman, 2005). 3T3-L1 cells are a widely used model system for adipocytes. In 3T3-L1 adipocytes, reactive oxygen species induced by hyperglycemia (Lin et al., 2005), hyperinsulinemia (Ge et al., 2008), dexamethasone (Houstis et al., 2006), tumor-necrosis factor-α (Houstis et al., 2006), or advanced glycation end products (Unoki et al., 2007) have been associated with the development of insulin resistance. The downstream pathways of reactive oxygen species responsible for the insulin resistance are multiple and involve JNK, p53, phosphatidylinositol 3-kinase (PI3-kinase), p38 MAPK, NF-kB, and some PKC isoforms (for review, see Simmons, 2006; Evans et al., 2003; Rudich et al., 2007).

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a compound derived from the roots of *Plumbago zeylanica*, has been shown to exert anti-carcinogenic, anti-microbial and anti-atherosclerosis effects (Naresh et al., 1996; Sugie et al., 1998; Sharma et al., 1991; Mossa et al., 2004; Ding et al., 2005; Sandur et al., 2006; Hsu et al., 2006). The underlying mechanisms by which plumbagin exerts these effects remain unclear. Plumbagin is known to produce reactive oxygen species such as superoxide anion and hydrogen peroxide (Kawiak et al., 2007; Inbaraj and Chignell, 2004; Srinivas et al., 2004; Powolny and Singh, 2008; Wang et al., 2008). Plumbagin appears to generate reactive oxygen species through multiple mechanisms. Depending on cell types, plumbagin could produce reactive oxygen species via the redox cycling (Inbaraj and Chignell, 2004), the leakage of the mitochondrial respiratory chain (Kuo et al., 2006), or the depletion of intracellular glutathione levels (Powolny and Singh, 2008; Wang et al., 2008). The generation of reactive oxygen species by plumbagin may account for its cyto-toxic or apoptotic effects.

The Ras/Raf/MEK/ERK and PI3 kinase/PDK1/Akt pathways are known to regulate cell proliferation, differentiation and survival. Plumbagin has been shown to chronically stimulate ERK1/2 in human melanoma cells (Wang et al., 2008) but inhibit Akt activity in breast cancer cells (Kuo et al., 2006). Unexpectedly, the effect of plumbagin on ERK1/2 activation was reactive oxygen species-independent in

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human melanoma cells (Wang et al., 2008). However, the acute effects of plumbagin on ERK1/2 and Akt activity have never been reported. Obesity is known to be associated with cellular oxidative stress (Rudich et al., 2007) that modulates cellular functions by the acute protein phosphorylation/dephosphorylation and long-term gene regulation. The oxidative stress-mediated signaling pathways in adipose tissues are not fully elucidated. In this report, we studied the acute signaling pathways initiated by reactive oxygen species generated by plumbagin in 3T3-L1 preadipocytes.

2. Materials and methods

2.1. Antibodies and chemicals

Antibodies against phospho-ERK1/2 and ERK1/2 antibodies were purchased from Biosource International (Camarillo, CA). Antibodies against PKCδ, phosphor-Src, phospho-Akt, and Akt antibodies were purchased from Cell Signaling (Beverly, MA). Antibodies against Src antibodies were obtained from Upstate Biochemical Inc. (Lake Placid, NY). Immobilon-P membrane was purchased from Millipore (Bedford, MA). 3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d] pyrimidin-4-ami ne (PP2) and Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP) were purchased from Calbiochem (San Diego, CA). Dihydroethidium (DHE) was obtained from Invitrogen (San Diego, CA, USA). The BCA protein assay kit and the enhanced chemiluminescence (ECL) detection system kit were purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). The Akt inhibitor (Akt inhibitor VIII trifluoroacetate salt hydrate), 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), dibenziodolium chloride (DPI), N-Acetyl-L-cysteine (NAC), 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt, pyrocatechol-3,5-disulfonic acid disodium salt (Tiron) and other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Cell culture

3T3-L1 cells were maintained at 37 °C in high glucose Dulbecco's modified Eagle's medium (DMEM) with 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% calf serum in a 5% CO_2 environment. Near confluent 3T3-L1 cells were incubated with serum-free media for 18 h to arrest and synchronize the cell growth. After this time period, the cells were treated with the various concentrations of plumbagin for 10 min or 20 μM plumbagin for the indicated periods. In some experiments, cells were pretreated with or without the antioxidant N-acetyl-cysteine (NAC), superoxide scavenger Tiron, superoxide dismutase mimetic MnTBAP, NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI), specific Src family kinase inhibitor PP2, MEK kinase inhibitor PD98059, or PI3K inhibitor LY294002 before the addition of plumbagin. Cells were processed for the measurement of cellular reactive oxygen species and expression of ERK1/2, Akt, and Src at the end of incubation.

2.3. Measurement of reactive oxygen species

Level of intracellular superoxide anion (O_2^-) was assessed spectrofluorimetrically by the oxidation of a specific probe: dihydroethidium (DHE). 3T3-L1 cells were seeded in a 6 wells plate and were serum-starved for 18 h. The cells were pretreated with or without 2 mM NAC or 30 μM MnTBAP, and then exposed to 20 μM plumbagin for specified time intervals. After stimulation, cells were washed with serum-free DMEM and loaded with 2 μM DHE. After incubation for 30 min at 37 °C in the dark, the cells were washed two times with phosphate-buffered saline. The red fluorescing at 610 nm which reflects reactive oxygen species generation in cells was detected by the fluorescence microscopy (Olympus). Scale bars were generated and inserted by Olympus DP controller software.

2.4. Immunoblotting

3T3-L1 cells were starved for 18 h in DMEM, and then stimulated with 20 μM plumbagin for the indicated times at 37 °C. The cells were washed with ice-cold PBS and lysed in RIPA buffer (20 mM Tris–HCl (pH 8.0), 10% glycerol, 150 mM NaCl, 1% Nonidet P-40 (NP-40) and 0.42% NaF) containing protease inhibitors (5 μg/mL aprotinin, 5 μg/mL leupetin, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 20 mM NaF). Cell lysates were centrifuged at 12,000 rpm for 15 min to remove insoluble materials. For immunoblotting, equal amounts of cell lysates from the control and plumbagin-treated cells were subjected to 8% SDS-PAGE and then transferred to the polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% nonfat powdered milk in Phosphate buffered saline (PBS) containing 0.1% Tween-20. The membranes were incubated with primary antibodies specific to total or phospho-specific ERK1/2, Akt, or Src at 37 °C for 1–2 h. After three times of 10 min washing with PBS plus 0.1% Tween-20 (PBST), the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h. Proteins were visualized with the enhanced chemiluminescence blotting detection system (Chen et al., 2004; Vetter et al., 2003).

2.5. Statistical analyses

The experimental results are expressed as the mean values ± S.E.M. and are accompanied by the number of observations. Data were assessed by the Student's *t*-test method. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Plumbagin induces reactive oxygen species generation in 3T3-L1 cells

To investigate whether plumbagin produces reactive oxygen species in 3T3-L1 cells, we measured the intracellular generation of reactive oxygen species by dihydroethidium (DHE) method which detects superoxide and by (2′,7-dichlorofluorescin diacetate) DCFDA method which senses H_2O_2. 3T3-L1 cells were treated with plumbagin for the indicated time. As shown in Fig. 1, the time-dependent reactive oxygen species generation induced by plumbagin was detected by DHE, but not DCFDA (data not shown), method in 3T3-L1 cells. These results suggest that plumbagin generates superoxide anions. To confirm the observation that plumbagin generates superoxide, we examined whether antioxidants affect plumbagin-stimulated reactive oxygen species production in 3T3-L1 cells. Fig. 2 shows that addition of NAC (a reactive oxygen species scavenger) or MnTBAP (a superoxide dismutase mimetic) inhibited the generation of reactive oxygen species by plumbagin.

3.2. Plumbagin induces ERK1/2 phosphorylation in 3T3-L1 cells

Superoxide generated by NAD(P)H oxidase and H_2O_2 have been shown to activate ERK1/2 (Milligan et al., 1998; Mehdi et al., 2007; Anilkumar et al., 2008). Since plumbagin generates superoxide, we examined whether plumbagin stimulates ERK1/2 activation in 3T3-L1 cells. Cultured cells were treated with 20 μM plumbagin for the indicated time periods. As shown in Fig. 3A, plumbagin induced ERK1/2 phosphorylation with a maximal effect around 10 min and the plumbagin-stimulated ERK1/2 phosphorylation returned to the control level at around 1 h. Plumbagin stimulated ERK1/2 activity in a dose-dependent manner with a maximal effect at around 20 μM (Fig. 3B). In contrast, pretreatment of the cells with an MEK inhibitor