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Inhibition of Nox-4 activity by plumbagin, a plant-derived bioactive naphthoquinone

Yaxian Ding, Zi-Jiang Chen, Shiguo Liu, Danian Che, Michael Vetter and Chung-Ho Chang

Abstract

NAD(P)H oxidase contributes to the pathogenesis of cancer and cardiovascular diseases such as hypertension, atherosclerosis, restenosis, cardiac hypertrophy and heart failure. Plumbagin, a plant-derived naphthoquinone, has been shown to exert anticarcinogenic and anti-atherosclerosis effects in animals. However, the molecular mechanisms underlying these effects remain unknown. It is possible that the beneficial effect of plumbagin is due to the inhibition of NAD(P)H oxidase. Human embryonic kidney 293 (HEK293) and brain tumour LN229 cells express mainly Nox-4, a renal NAD(P)H oxidase. We have examined the effect of plumbagin on Nox-4 activity in HEK293 and LN229 cells using lucigenin-dependent chemiluminescence assay. Plumbagin inhibited the activity of Nox-4 in a time- and dose-dependent manner in HEK293 and LN229 cells. Production of superoxide in HEK293 cells was inhibited by diphenyleneiodonium (DPI), a NAD(P)H oxidase inhibitor. The superoxide production in HEK293 cells was NADPH- and NADH-dependent indicating that the superoxide was generated by a NAD(P)H oxidase in HEK293 cells, but not by the redox-cycling of lucigenin. Furthermore, plumbagin inhibited the superoxide production in Nox-4 transfected COS-7 cells. These results indicated that plumbagin directly interacted with Nox-4 and inhibited its activity.

Department of Medicine, Case Western Reserve University and University Hospital of Cleveland, Cleveland, Ohio 44106, USA

Yaxian Ding, Zi-Jiang Chen, Shiguo Liu, Danian Che, Michael Vetter, Chung-Ho Chang

Department of Medicine, Reproductive Research Center, Shandong Provincial Hospital, Shandong University, Jinan, P. R. China

Zi-Jiang Chen

Correspondence: C.-H. Chang, Department of Medicine, Division of Hypertension, School of Medicine W165, Cleveland, Ohio 44106, USA.
E-mail: cxc13@po.cwru.edu

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Introduction

Reactive oxygen species (ROS) have been implicated in various age-related diseases such as cancer, diabetes, hypertension, atherosclerosis, Alzheimer's disease and Parkinson's disease (for review, see Halliwell & Gutteridge 1990; Sagar et al 1992; Ames et al 1993; Alexander 1995; Griendling et al 2000; Zalba et al 2001). A major source of superoxide, one of the ROS, is produced by leakage of the electron transport chain in mitochondria. Besides the electron transport chain, enzymes such as NAD(P)H oxidase and xanthine oxidase generate excessive superoxide.

Superoxide generated by NAD(P)H oxidase contributes to the pathogenesis of cancer and several cardiovascular diseases including hypertension, atherosclerosis, restenosis, cardiac hypertrophy and heart failure (for review, see Griendling et al 2000; Lambeth et al 2000; Sorescu et al 2001; Bokoch & Knaus 2003; Cai et al 2003; Lassegue & Clempus 2003). There are several NAD(P)H oxidase isoforms. Nox-1 is expressed in colon epithelial cells and smooth muscle cells, and may stimulate cell proliferation. Nox-2 is predominantly expressed in professional phagocytes such as neutrophils and plays a crucial role in host defense against microbial infection. Nox-3 is found mainly in embryonic kidney and Nox-5 in spleen and testis. Nox-4 is most abundant in the kidney and is expressed in vascular cells and tissues.

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a compound derived from the roots of *Plumbago zeylanica*. Plumbagin has been shown to exert anticarcinogenic or cytotoxic effects on animals and cancer cells (Parimala & Sachdanandam 1993; Naresh et al 1996; Kini et al 1997; Kuo et al 1997; Sugie et al 1998; Hazra et al 2002) and anti-atherosclerosis effects on hyperlipidaemic rabbits (Sharma et al 1991). However, the mechanisms by which plumbagin exerts these effects remain unknown. Since ROS are involved in the initiation and/or progression of cancer and atherosclerosis, we have examined whether plumbagin affected NADPH oxidase activity in

human kidney embryonic 293 (HEK293) and brain tumour LN229 cells that expressed Nox-4, but not other Nox isoforms (Cheng et al 2001; Shiose et al 2001). We examined the effect of plumbagin on the activity of Nox-4 in transfected COS-7 cells.

Material and Methods

Materials

RPMI medium was obtained from Gibco (Gaithersburg, MD). Protein assay reagent was obtained from Bio-Rad (Hercules, CA). Mouse Nox-4 plasmid was purchased from ATCC (Rockville, MD). GenePORTER transfection reagent was purchased from Gene Therapy System (San Diego, CA). Plumbagin and other common chemicals were purchased from Sigma (St Louis, MO).

Expression of Nox-4 plasmids in COS-7 cells

Mouse Nox-4 plasmid was subcloned into an expression vector pcDNA3.1 via Apa1 and Not1 sites. Nox-4 plasmids (12 μg) were transfected into 60–80% confluent COS-7 cells by the GenePORTER transfection reagent according to manufacture's instructions (Chen et al 2000, 2001). Cells transfected with pcDNA3.1 plasmid were used as the control. One day later, control and transfected cells were used for NAD(P)H oxidase assay.

NAD(P)H oxidase assay

HEK293, LN229 and COS-7 cells were cultured until confluence in 6-cm diameter dishes with EMEM or RPMI (for COS-7 cells) medium containing 5% bovine fetal serum and 10% horse serum. The cells were treated with or without 5, 10 or 20 μM plumbagin or 1 μM diphenyleneiodonium (DPI) for 10 min at 37°C, and washed twice with ice-cold phosphate-buffered saline. The cells were then scraped and suspended in 500 μL homogenization buffer containing 20 mM K_2HPO_4, 1 mM EGTA, 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupetin and 1 mM phenylmethylsulfonyl fluoride. The suspension was homogenized with 50 strokes in a Dounce homogenizer on ice. The activity of NAD(P)H oxidase was then determined by lucigenin-dependent chemiluminescence (Vetter et al 2003). Briefly, 900 μL of an assay buffer containing 50 mM $NaHPO_4$, 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH or NADH was added to 100 μL of the cell homogenate. Chemiluminescent photoemission was determined in relative light units (RLU) with the use of a Lumat LB 9501 Luminometer (Li et al 1998; Beswick et al 2001; Vetter et al 2003).

Statistical analysis

All error bars represent the standard deviation from the mean of three experimental replicates. A two-way analysis of variance was utilized to determine the significance of differences between treatment concentration–response curves. A P value less than 0.05 was considered statistically significant.

Results

Effects of plumbagin on Nox-4 activity in HEK293 and LN229 cells

We examined the effect of plumbagin on NAD(P)H oxidase activity in HEK293 cells that expressed mainly Nox-4 (Shiose et al 2001). HEK293 cells were incubated with 10 μM plumbagin for 10 min or a time course. After incubation, superoxide production was measured by a lucigenin-based chemiluminescence assay (Li et al 1998; Beswick et al 2001; Vetter et al 2003). Plumbagin greatly inhibited Nox-4 activity in the presence of NADPH or NADH in a time-dependent manner (Figure 1). Figure 2

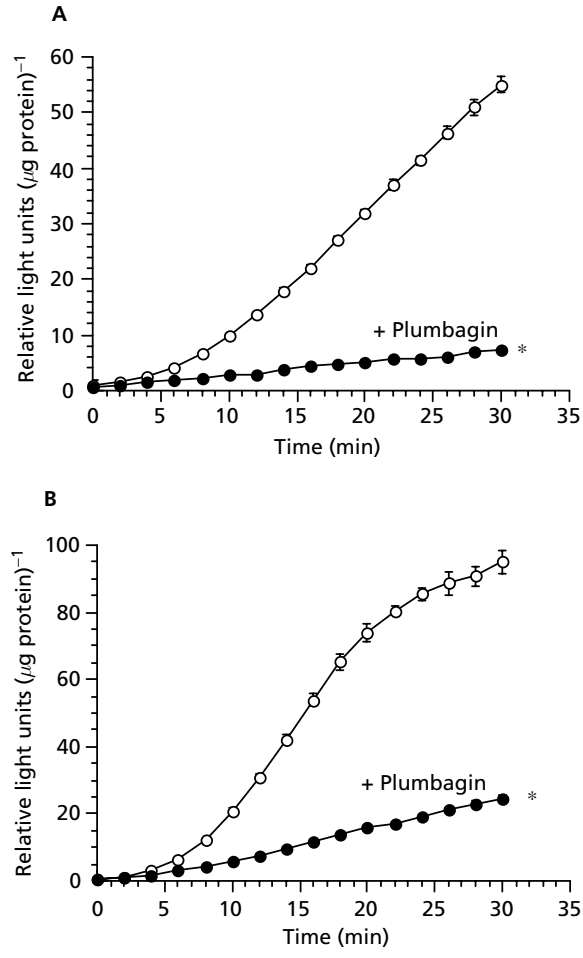
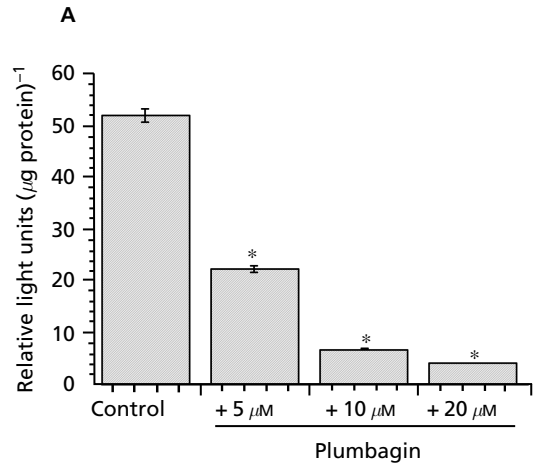


Figure 1 Effects of plumbagin on Nox-4 activity in HEK293 cells. HEK293 cells were exposed to 10 μM plumbagin at 37°C for 10 min. The production of superoxide was measured by lucigenin-dependent chemiluminescence in the presence of NADPH (A) or NADH (B). Data shown are representative of experiments performed in triplicate. The error bar represents the deviation from the mean of the replicates. *$P < 0.0001$ plumbagin compared with control.



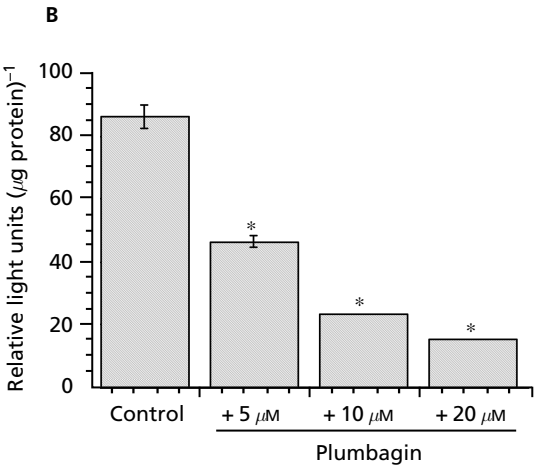
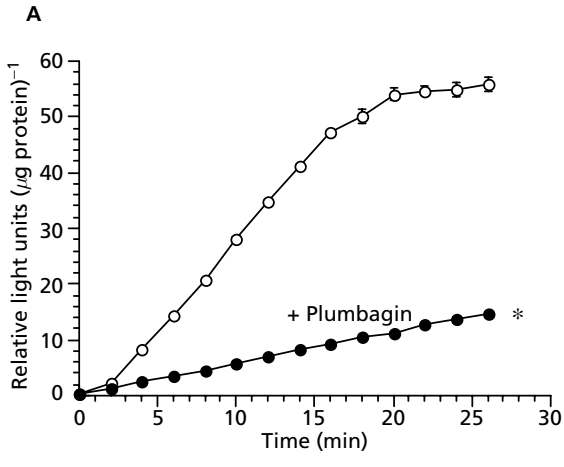


Figure 2 Concentration-dependent effects of plumbagin on Nox-4 activity in HEK293 cells. HEK293 cells were incubated with various concentrations of plumbagin for 10 min. The production of superoxide was measured by lucigenin-dependent chemiluminescence in the presence of NADPH (A) or NADH (B). Data shown are representative of experiments performed in triplicate. The error bar represents the deviation from the mean of the replicates. $*P < 0.0001$ plumbagin compared with control.



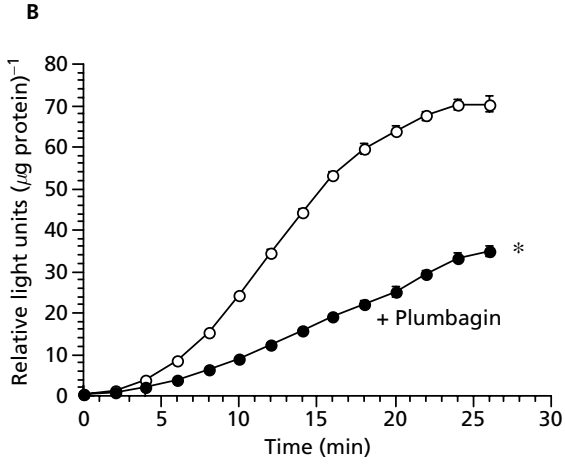


Figure 3 Effects of plumbagin on Nox-4 activity in LN229 cells. LN229 cells were exposed to 10 μM plumbagin at 37°C for 10 min. The production of superoxide was measured by lucigenin-dependent chemiluminescence in the presence of NADPH (A) or NADH (B). Data shown are representative of experiments performed in triplicate. The error bar represents the deviation from the mean of the replicates. $*P < 0.001$ plumbagin compared with control.

shows the dose-dependent inhibition of Nox-4 activity by plumbagin with an IC50 (the concentration for the half-maximal inhibition) around 2 μM. In addition to HEK293 cells, plumbagin inhibited Nox-4 activity in LN229 cells that expressed only Nox-4 (Cheng et al 2001) (Figure 3).

Effects of DPI, NADH and NADPH on Nox-4 activity in HEK293 cells

To confirm that the superoxide generated in HEK293 cells was due to Nox-4, we measured the effect of DPI, a NAD(P)H oxidase inhibitor, on superoxide production in HEK293 cells. Figure 4A shows that addition of DPI inhibited the NADPH-dependent generation of superoxide in HEK293 cells. Interestingly, although higher concentrations can inhibit NADPH-dependent chemiluminescence, 1 μM DPI had little effect on the NADH-dependent superoxide production (Figure 4B). This indicated that the sensitivity of NADPH- and NADH-dependent superoxide production to DPI was very different in HEK293 cells.

The superoxide generation was not observed in the absence of NADPH or NADH (Figure 4), indicating that superoxide in HEK293 cells was produced by a NAD(P)H oxidase and was not due to the redox-cycling of lucigenin.

Effects of plumbagin on Nox-4 activity in transfected COS-7 cells

To determine whether plumbagin directly interacted with Nox-4, we transfected mouse Nox-4 plasmids into COS-7 cells that expressed little NADPH oxidase activity. COS-7 cells transfected with the vector (pcDNA3.1) were used as the control. One day after transfection, the effect of plumbagin on Nox-4 activity was measured. Figure 5 shows

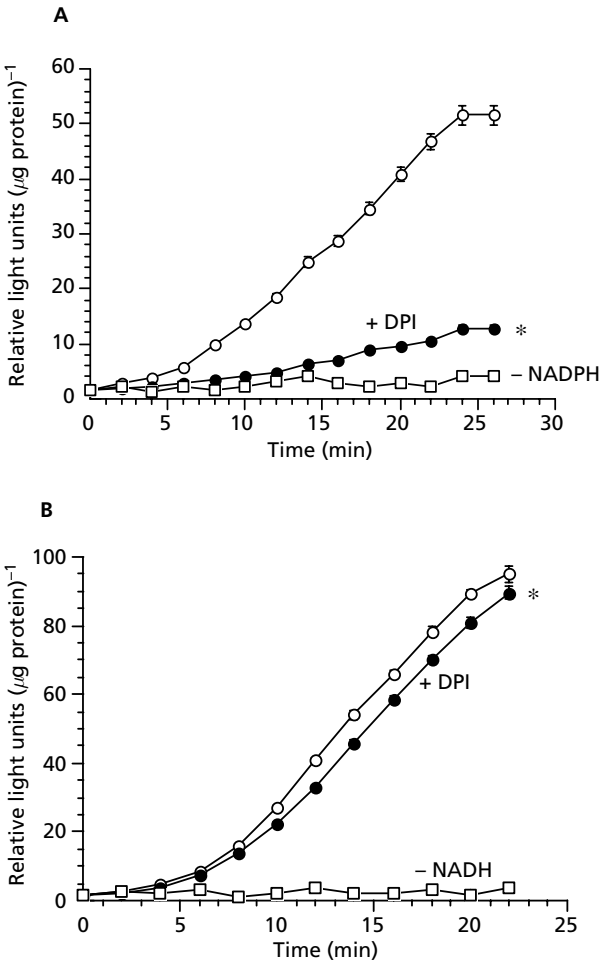


Figure 4 Inhibition of Nox-4 activity by DPI in HEK293 cells. HEK293 cells were exposed to 1 μM DPI at 37°C for 10 min. The NADPH- (A) and NADH-dependent (B) superoxide production was measured by lucigenin-dependent chemiluminescence. Superoxide production in HEK293 cells was measured in the absence of NADPH (A) or NADH (B). The error bar represents the deviation from the mean of the three replicates. A, $*P < 0.0001$ DPI compared with control; B, $*P < 0.05$ DPI compared with control.

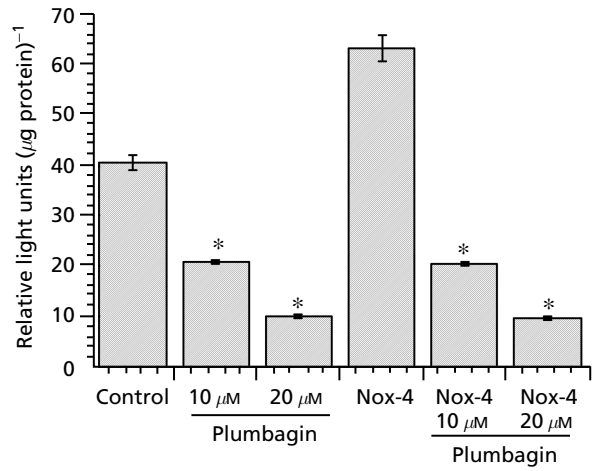


Figure 5 Effect of plumbagin on Nox-4 activity in transfected COS-7 cells. COS-7 cells were transfected with Nox-4 plasmids or a control vector (pcDNA3.1) by employing the GenePORTER transfection reagent. One day after transfection, both cells were incubated with 10 or 20 μM plumbagin at 37°C for 10 min. The production of superoxide was measured by lucigenin-dependent chemiluminescence in transfected and in control cells. The error bar represents the deviation from the mean of the three replicates. $*P < 0.0001$, plumbagin compared with control and plumbagin compared with Nox-4 transfected.

that expression of Nox-4 increased the superoxide production, and that addition of plumbagin inhibited Nox-4 activity.

Discussion

NAD(P)H oxidase is associated with several cardiovascular diseases such as hypertension, atherosclerosis, heart failure and cardiac hypertrophy (for review, see Griendling et al 2000; Lambeth et al 2000; Sorescu et al 2001; Bokoch & Knaus 2003; Cai et al 2003; Lassegue & Clempus 2003). Besides the cardiovascular system, Nox-4, Nox-5 and Nox-1 are expressed in many tumours and transformed cell lines (Cheng et al 2001). Nox-1 and Nox-4 have been shown to regulate cell growth and transformation (Suh et al 1999; Geiszt et al 2000). Therefore, inhibition of NAD(P)H oxidase may provide an opportunity for intervention in a broad range of diseases. In this study, we have investigated the effect of plumbagin on the NAD(P)H oxidase activity in HEK293 and LN229 cells. The results indicated that plumbagin was a potent inhibitor for Nox-4 activity in both cell types.

Using a lucigenin-based chemiluminescent assay, we showed that plumbagin decreased superoxide production in a time- and concentration-dependent manner in HEK293 and LN229 cells. We were able to confirm that the increased luminescence was caused by superoxide because a NAD(P)H oxidase inhibitor DPI decreased the amount of luminescence. Furthermore, our data showed that the production of superoxide depended on the presence of NADPH and NADH, indicating that the superoxide was produced by a NAD(P)H oxidase, but not by redox-cycling of lucigenin.

HEK293 cells have been shown to express Nox-4, but not Nox-1 nor Nox-2 (Shiose et al 2001). Since Nox-3 is expressed only in fetal tissues and Nox-5 is expressed in fetal tissues and adult spleen and testis, Nox-4, the renal NAD(P)H oxidase, is the primary Nox isoform in HEK293 cells. Therefore, the reduction of oxygen radical production by plumbagin in HEK293 cells was likely due to a direct inhibition of Nox-4. Furthermore, plumbagin inhibited the NAD(P)H oxidase activity in LN229 cells that have been shown to express only Nox-4 (Cheng et al 2001). This conclusion was supported by the inhibition of the activity of transfected Nox-4 in COS-7 cells by plumbagin.

Activation of Nox-1 and Nox-2 requires the participation of several other proteins such as $p22^{phox}$, $p47^{phox}$,

$p67^{phox}$, $p41^{nox}$ and $p51^{nox}$ (Banfi et al 2003; Takeya et al 2003; Geiszt et al 2003). However, expression of $p47^{phox}$, $p67^{phox}$, $p41^{nox}$ and $p51^{nox}$ in HEK293 does not affect superoxide production (Banfi et al 2003; Takeya et al 2003; Geiszt et al 2003), indicating that the regulation of Nox-4 is different from Nox-1 and Nox-2. On the other hand, it has been shown that expression of Nox-4 alone produced measurable amounts of superoxide and induced cellular senescence in NIH-3T3 cells (Suh et al 1999; Shiose et al 2001). These results suggested that Nox-4 might have been constitutively active, or that proteins regulating Nox-4 were different from Nox-1 and Nox-2. Since plumbagin inhibited the activity of Nox-4 expressed in COS-7 cells, plumbagin might have directly interacted with Nox-4 and inhibited its activity. However, we can not completely rule out the possibility that plumbagin might have inhibited Nox-4 activity by indirectly interacting with a widely expressed protein in COS-7 cells.

DPI irreversibly inactivates many flavoproteins and is routinely used as a NADPH oxidase inhibitor (Sundaresan et al 1995, 1996; Chakraborty & Massey 2002). For instance, besides NADPH oxidases, DPI inhibits NO synthase, xanthine oxidase, sulfite reductases, and cytochrome P450 (Stuehr et al 1991; Tew 1993; O'Donnell et al 1994; Coves et al 1999). On the other hand, it has not been reported that plumbagin exerts any effects on these enzymes. Therefore, plumbagin may be an alternative choice as a specific NADPH oxidase inhibitor. It is noteworthy that there was a difference in the inhibitory effect of DPI and plumbagin on Nox-4 activity. Although DPI at high concentrations inhibited NADPH- and NADH-dependent superoxide production, DPI at 1 μM had little effect on NADH-dependent superoxide production. However, plumbagin at all tested concentrations significantly inhibited NADPH- and NADH-dependent superoxide production with a similar potency in HEK293 cells, indicating that plumbagin, unlike DPI, had little preference for NADPH or NADH.

The mechanism by which plumbagin inhibited Nox-4 remains unknown. DPI is known to inhibit flavoproteins such as NADPH oxidase by interacting with the protein-bound flavin, subsequently resulting in phenyl adducts of the flavin (Chakraborty & Massey 2002). This covalent modification of the flavin inactivates its redox cycle and the activity of flavoproteins (Chakraborty & Massey 2002). Since the structure of plumbagin differs from that of DPI, it is unknown whether plumbagin inhibited Nox-4 through a similar mechanism. The inhibition of Nox-4 activity by plumbagin was not due to cytotoxic effects because cells were incubated with plumbagin for 10 min and cells remained viable after a 1-h incubation (our unpublished results).

NADPH oxidase is involved in the initiation and progression of atherosclerosis. Plumbagin has been shown to exert anti-atherosclerosis effect in hyperlipidaemic rabbits (Sharma et al 1991). Since vascular smooth muscle cells express Nox-1 and Nox-4, and the expression of Nox-4 is more abundant than Nox-1 (Lassegue & Clempus 2003), it was likely that the anti-atherosclerosis effect of plumbagin was due to the inhibition of Nox-4. Besides atherosclerosis, plumbagin has been shown to exert anticancer effects (Parimala & Sachdanandam 1993; Naresh et al 1996; Kini et al 1997; Kuo et al 1997; Sugie et al 1998; Hazra et al 2002). Many cancer cells have been shown to overproduce reactive oxygen species (Szatrowski & Nathan 1991) and express various Nox isoforms (Cheng et al 2001). For instance, Nox-4 was expressed in ovarian teratocarcinoma and clear cell carcinoma, colon carcinoma and glioblastomas (Cheng et al 2001). It was possible that the anticancer effects of plumbagin were partly due to the inhibition of Nox-4. Further experiments are required to verify this hypothesis.

Conclusions

Plumbagin was a potent inhibitor of Nox-4 in HEK293, LN229, and Nox-4 transfected COS-7 cells. Therefore, plumbagin may be useful for elucidating the roles of Nox-4 in-vivo and may have therapeutic applications in the treatment of cancer, hypertension, and atherosclerosis.

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