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Biochemical and Biophysical Research Communications 330 (2005) 46–52
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Ganoderma lucidum suppresses angiogenesis through the inhibition of secretion of VEGF and TGF-β1 from prostate cancer cells

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Received 17 February 2005

Abstract

Ganoderma lucidum (*G. lucidum*) is a popular medicinal mushroom that has been used as a home remedy for the general promotion of health and longevity in East Asia. The dried powder of *G. lucidum*, which was recommended as a cancer chemotherapy agent in traditional Chinese medicine, is currently popularly used worldwide in the form of dietary supplements. We have previously demonstrated that *G. lucidum* induces apoptosis, inhibits cell proliferation, and suppresses cell migration of highly invasive human prostate cancer cells PC-3. However, the molecular mechanism(s) responsible for the inhibitory effects of *G. lucidum* on the prostate cancer cells has not been fully elucidated. In the present study, we examined the effect of *G. lucidum* on angiogenesis related to prostate cancer. We found that *G. lucidum* inhibits the early event in angiogenesis, capillary morphogenesis of the human aortic endothelial cells. These effects are caused by the inhibition of constitutively active AP-1 in prostate cancer cells, resulting in the down-regulation of secretion of VEGF and TGF-β1 from PC-3 cells. Thus, *G. lucidum* modulates the phosphorylation of Erk1/2 and Akt kinases in PC-3 cells, which in turn inhibits the activity of AP-1. In summary, our results suggest that *G. lucidum* inhibits prostate cancer-dependent angiogenesis by modulating MAPK and Akt signaling and could have potential therapeutic use for the treatment of prostate cancer.

Keywords: *Ganoderma lucidum*; Prostate cancer; PC-3; Angiogenesis; Erk1/2; Akt; NF-κB; AP-1; VEGF; TGF-β1

Although early diagnosis and therapeutic and surgical interventions have increased the survival of prostate cancer patients, prostate cancer remains the second leading cause of cancer death among men [1]. One of the potential therapeutic strategies for the treatment of cancer is the inhibition of angiogenesis. Angiogenesis, the formation of blood vessels by capillaries sprouting from pre-existing vessels, is important for normal physiological processes such as embryogenesis, growth, and wound healing, as well as pathological processes such as tumor growth and metastasis [2]. Angiogenesis is a complex multistep process involving close orchestration of endothelial cells, extracellular matrix, and angiogenic factors. Prostate cancer has been shown to produce angiogenic factors such as vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1) [3]. Expression of VEGF and TGF-β1 is controlled by two distinct families of transcriptions factors AP-1 and NF-κB [4,5]. AP-1 and NF-κB play a crucial role in a variety of biological responses, such as cell proliferation, cell survival, cell transformation, immune response, and cancer therapy resistance [6,7]. AP-1 and NF-κB are also constitutively active in a variety of cancers including prostate cancer, and their activity was linked to the negative prognosis of cancer progression [8–10].

The dried powder of a medicinal mushroom *Ganoderma lucidum*, which has been recommended in

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doi:10.1016/j.bbrc.2005.02.116

traditional Chinese medicine during the last two thousand years, is currently popularly used as a dietary supplement in the form of spores, fruiting body or mushroom extract [11]. The nature of the biologically active components of *G. lucidum*, especially their antitumor activity, has been reported in a number of previously published studies. For example, some of the triterpenes isolated from *G. lucidum* demonstrated cytotoxicity against mouse cancer cells in vitro [12] and inhibited the growth and cancer metastases in mice [13,14]. Polysaccharides from *G. lucidum* inhibited the growth of tumors in mice [15,16] and induced expression of a variety of inflammatory cytokines [17,18]. Mechanistically, the antitumor effects of *G. lucidum* have been implicated in the inhibition of post-translational modification of Ras oncoprotein [19], the induction of cell cycle arrest by down-regulating cyclin D1, and the induction of apoptosis by up-regulating a proapoptotic Bax protein in breast cancer cells [20]. *G. lucidum* inhibited the growth of hepatoma cells by inhibiting protein kinase C (PKC) and activating JNK and p38 MAPKs [21]. We have previously demonstrated that *G. lucidum* suppresses the motility of highly invasive breast and prostate cancer cells by inhibiting the transcription factors NF-κB and AP-1, resulting in the down-regulation of expression of uPA and its receptor uPAR [22]. More recently, we have shown that *G. lucidum* inhibits proliferation, induces apoptosis in prostate cancer cells [23], and suppresses cell invasion and anchorage-independent growth of breast cancer cells [24].

In the present study, we examined the effect of *G. lucidum* on angiogenesis induced by cancer cells. We found that *G. lucidum* inhibits the first step in angiogenesis, tube formation of the human aortic endothelial cells (HAECs). Thus, *G. lucidum* can affect endothelial cells directly or indirectly by the inhibition of suppression of secretion of VEGF and TGF-β1 from prostate cancer cells. In summary, our data provide the molecular mechanism by which *G. lucidum* can inhibit angiogenesis related to prostate cancers.

Materials and methods

Materials. G. lucidum (ReishiMax) was purchased from Pharmanex (Provo, UT). According to the manufacturer, this sample contains powdered extract (20:1) with spores and is standardized to 13.5% polysaccharides and 6% triterpenes [25]. Stock solution was prepared by dissolving ReishiMax in sterile water at a concentration of 50 mg/ml and stored at 4 °C.

Cell culture. The human prostate cancer cell line PC-3 was obtained from ATCC (Manassas, VA). PC-3 cells were maintained in F-12 medium containing penicillin (50 U/ml), streptomycin (50 U/ml), and 10% fetal bovine serum (FBS). Human aortic endothelial cells (HAECs) were obtained from Clonetics (Walkersville, MD) and maintained in CSC medium supplemented with CSC growth factors (Clonetics), as well as 10% FBS and 1% antibiotic–antimycotic solution.

In vitro endothelial cell morphogenesis assay (capillary morphogenesis). HAEC differentiation into "capillary-like" structures was observed using a two-dimensional Matrigel-based assay as we previously described [26]. Initially, 200 μl of ice-cold growth factor reduced Matrigel (Becton–Dickinson Labware, Bedford, MA), an extracellular matrix preparation derived from the Engelbreth–Holm–Swarm tumor, was placed into each well of a 24-well tissue culture treated plate. HAECs were harvested, resuspended in serum-free EBM, and plated at 3.5×10^4 cells/well coated with Matrigel. HAECs were further incubated with conditioned media from PC-3 cells, which were prepared by the incubation of PC-3 cells in the presence of *G. lucidum* (0–1.0 mg/ml) for 24 h. Endothelial HAECs differentiated into capillary-like structures within 16 h of incubation at 37 °C in the presence of 5% CO_2. These structures were examined microscopically (40×) using an inverted Olympus CK40 microscope. In order to facilitate analysis of the structures, non-adherent cells incorporated in excess medium were removed from each well prior quantitative analysis. Photomicrographs were taken to assess the extent of capillary-like structural formation. Quantification of the capillary-like structures was performed counting the number of nodes/field, where a node is defined as an intersection of at least three cells. Each sample was assayed in triplicate and reproduced in at least two additional experiments.

DNA-binding assay. PC-3 cells were treated for 24 h with *G. lucidum*, nuclear extracts were prepared, and DNA-binding assay with ^{32}P-labeled AP-1 was performed as previously described [22]. Oligonucleotide probe containing consensus sequence for AP-1 was purchased from Promega (Madison, WI).

DNA transfection and AP-1 reporter gene assay. PC-3 cells were transiently transfected with AP-1-CAT reporter constructs and β-galactosidase expression vector pCH110, as previously described [22]. Twenty-four hours after transfection, cells were treated with indicated amounts of *G. lucidum* for an additional 24 h at 37 °C. CAT assay was performed as described [22]. Data points represent the average ± SD of 3–6 independent transfection experiments.

ELISA analysis. PC-3 cells were treated with *G. lucidum* (0–0.5 mg/ml) for 24 h, cell media were collected, and secretion of VEGF and TGF-β1 was determined using a respective Quantikine human immunoassay kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Western blot analysis. PC-3 cells were treated with *G. lucidum* (0–1.0 mg/ml) for 24 h and whole cell extracts were prepared as previously described [23]. Equal amounts of proteins (20 μg/lane) were separated on NuPAGE 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane (Millipore, Bedford, MA). The protein expression was detected with the corresponding primary antibodies: anti-Erk1/2, anti-phospho-Erk1/2, anti-Akt, anti-phospho-Akt (Thr^{308}), and anti-phospho-Akt (Ser^{473}) antibody (Cell Signaling, Beverly, MA), respectively. Protein expression was visualized using the ECL Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, UK).

Results

Ganoderma lucidum inhibits capillary morphogenesis in vitro

Capillary morphogenesis (tube formation) of human endothelial cells is an important step in angiogenesis associated with the growth and progression of cancer. Because previous reports have suggested that *G. lucidum* possesses anticancer activities, we evaluated the potency of *G. lucidum* for the inhibition of capillary morphogenesis of human aortic endothelial cells (HAECs). As seen

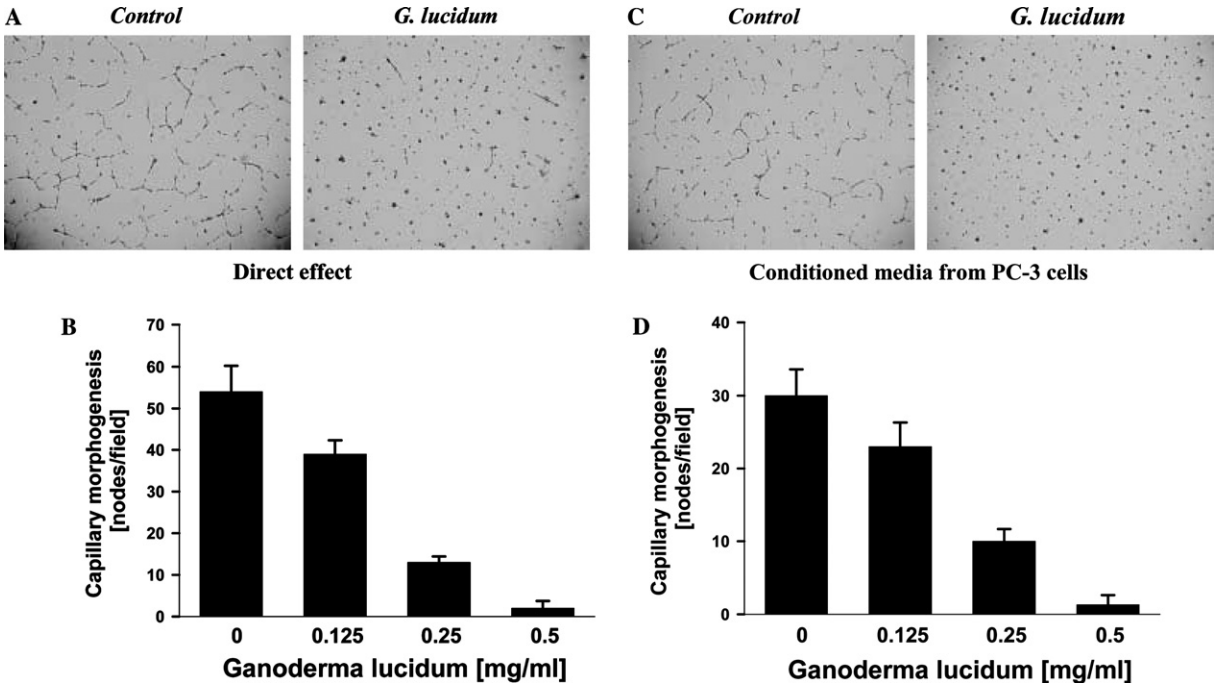

Fig. 1. *Ganoderma lucidum* inhibits capillary morphogenesis of aortic endothelial cells. (A) Human aortic endothelial cells were seeded onto Matrigel, and the cells were incubated in the absence or presence of *G. lucidum* (0.5 mg/ml) as described in Materials and methods. (B) Capillary morphogenesis was performed as described in (A) in the presence of *G. lucidum* (0–0.5 mg/ml). Capillary network formation was quantified as described in Materials and methods. (C) PC-3 cells were incubated for 24 h in the absence or presence of *G. lucidum* (0.5 mg/ml), cell media were collected (conditioned media) and used in capillary morphogenesis assay with human aortic endothelial cells as described in (A). (D) Capillary morphogenesis was performed with conditioned media (PC-3 cells treated with *G. lucidum* (0–0.5 mg/ml) for 24 h) as described in (C), and capillary network formation was quantified as described in Materials and methods.

in Fig. 1A, capillary morphogenesis of HAECs was markedly suppressed by *G. lucidum*, and the inhibitory effect of *G. lucidum* on tube formation of HAECs was dose dependent (Fig. 1B).

We have recently demonstrated that *G. lucidum* also inhibits growth and invasive behavior of prostate cancer cells PC-3 [23]. In order to evaluate if the inhibition of capillary morphogenesis of HAE cells is mediated through prostate cancer cells, conditioned media from PC-3 cells treated with *G. lucidum* were used in the capillary morphogenesis assay. As seen in Fig. 1C, cell media from PC-3 cells treated with *G. lucidum* markedly decreased capillary morphogenesis of HAE cells. Furthermore, increased concentration of *G. lucidum* (0–0.5 mg/ml) suppressed capillary morphogenesis in a dose-dependent manner (Fig. 1D). Therefore, *G. lucidum* inhibits capillary morphogenesis of endothelial cells directly as well as indirectly through the suppression of factors secreted from prostate cancer cells.

Ganoderma lucidum suppresses secretion of VEGF and TGF-β1 from prostate cancer cells

Angiogenic factors VEGF and TGF-β1 were previously implicated in angiogenesis related to prostate cancer. As we have shown above, conditioned media from prostate cancer cells PC-3 treated with *G. lucidum* markedly inhibited capillary morphogenesis of endothelial cells, suggesting that *G. lucidum* may inhibit secretion of factors inducing angiogenesis from PC-3 cells. In order to evaluate the effect of *G. lucidum* on secretion of VEGF, PC-3 cells were treated for 24 h with *G. lucidum* (0–0.5 mg/ml) and the levels of VEGF in cell media were assessed. As seen in Fig. 2A, VEGF secretion from PC-3 cells is noticeably inhibited by *G. lucidum* in a dose dependent manner. We also evaluated the effect of *G. lucidum* on the secretion of another angiogenic factor, TGF-β1. The levels of TGF-β1 in media from PC-3 cells treated with *G. lucidum* (0–0.5 mg/ml) were also markedly decreased in a dose–response manner (Fig. 2B). Thus, inhibition of capillary morphogenesis of HAE cells by *G. lucidum* is caused by the inhibition of secretion of proangiogenic factors VEGF and TGF-β1 from prostate cancer cells.

Suppression of VEGF and TGF-β1 is mediated through AP-1

Promoter regions of VEGF and TGF-β1 contain binding sites for transcription factor AP-1 [27,28]. We have also previously demonstrated that unpurified sample of mushroom *G. lucidum* inhibited AP-1 in prostate

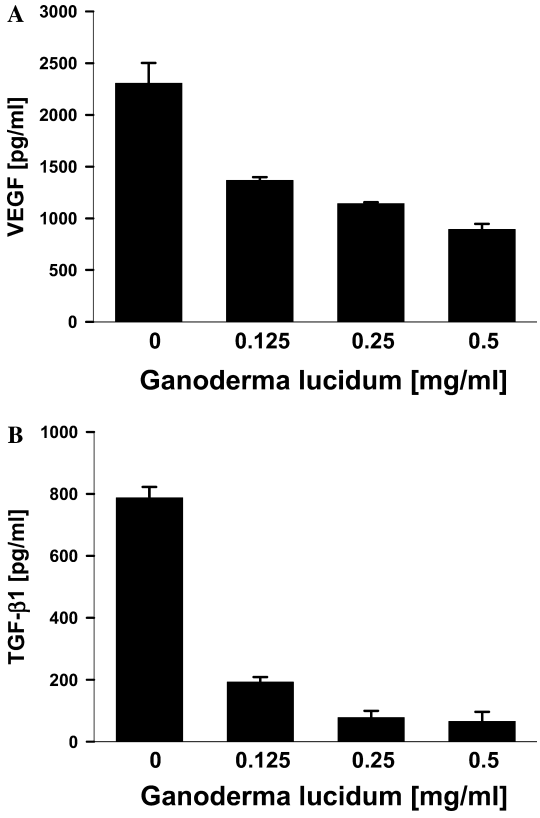

Fig. 2. Effect of *G. lucidum* on secretion of VEGF and TGF-β1 from prostate cancer cells. (A) PC-3 cells were treated with *G. lucidum* (0–0.5 mg/ml) for 24 h, media were collected, and secretion of VEGF was determined as described in Materials and methods. Each bar represents the mean ± SD (pg/ml of secreted VEGF)) of minimum three experiments. (B) PC-3 cells were treated with *G. lucidum* (0–0.5 mg/ml) for 24 h, media were collected, and secretion of TGF-β1 was determined as described in Materials and methods. Each bar represents the mean ± SD (pg/ml of secreted TGF-β1) of minimum three experiments.

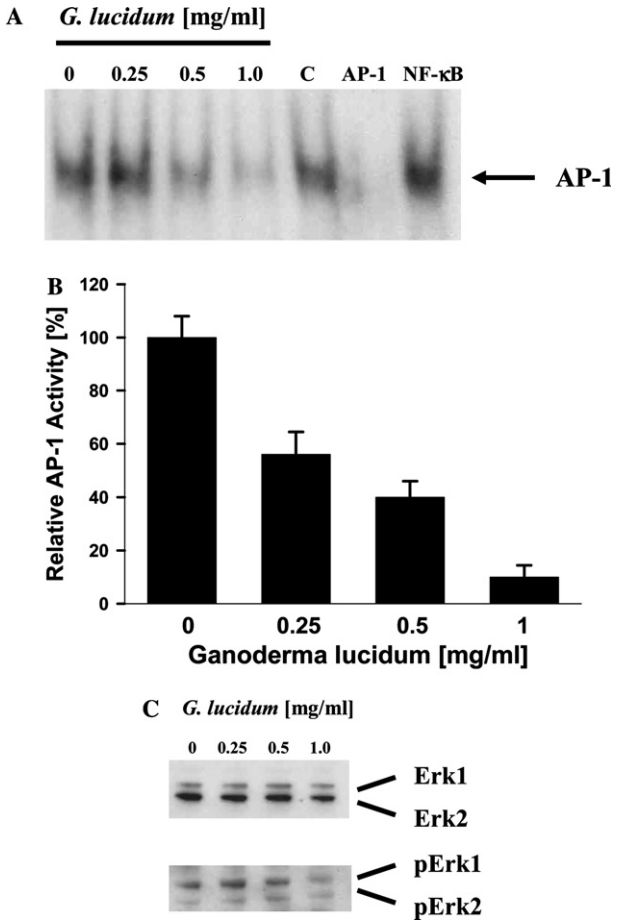

Fig. 3. *Ganoderma lucidum* inhibits AP-1 and Erk1/2 in PC-3 cells. (A) DNA-binding assay with ^{32}P-labeled AP-1 was performed with nuclear extracts prepared from PC-3 cells treated for 24 h with *G. lucidum* (0–1.0 mg/ml), as described in Materials and methods. The specificity of AP-1 binding was confirmed with cold AP-1 and NF-κB probes. (B) AP-1 activity was assessed in PC-3 cells transfected with AP-1-CAT reporter construct and β-galactosidase expression vector, and treated for 24 h with *G. lucidum* (0–1.0 mg/ml), as described in Materials and methods. The results are expressed as the percentage of relative AP-1 activity. Each bar represents the mean ± SD of three experiments. (C) Phosphorylation of Erk1/2 determined in PC-3 cells treated with *G. lucidum* (0–1.0 mg/ml) for 24 h. Whole cell extracts were prepared, and subjected to Western blot analysis with anti-Erk1/2, anti-phospho-Erk1/2 antibodies as described in Materials and methods. The results are representative of three separate experiments.

cancer cells PC-3 [22]. However, in the present study we used an extract of *G. lucidum* with characterized and standardized amount of polysaccharides and triterpenes. To examine whether *G. lucidum* inhibits DNA-binding activity of AP-1, nuclear extracts from PC-3 cells treated with *G. lucidum* (0–1.0 mg/ml) were prepared and gel shift analysis was performed with ^{32}P-labeled AP-1 probe. As seen in Fig. 3A, *G. lucidum* inhibits DNA-binding activity of AP-1 in a dose–response manner. Next we investigated if *G. lucidum* also inhibits transactivation activity in a reporter gene assay. PC-3 cells were transiently transfected with AP-1-CAT reporter gene construct and the cells were treated with *G. lucidum* (0–1.0 mg/ml). As expected, *G. lucidum* inhibited activation of AP-1 in a dose dependent manner (Fig. 3B).

Activity of AP-1 is controlled, through the induction of expression of AP-1 subunit c-fos, by mitogen-activated protein kinase (MAPK) Erk1/2 [29]. Therefore, we hypothesized that *G. lucidum* modulates the activity of Erk1/2 through the phosphorylation of Erk1/2. Whole cell extracts were prepared from PC-3 cells treated with *G. lucidum* (0–1.0 mg/ml) for 24 h and subjected to Western blot analysis with phospho-Erk1/2 antibody. As seen in Fig. 3C, *G. lucidum* also inhibited phosphorylation of Erk1/2. Therefore, inactivation of Erk1/2 by *G. lucidum* resulted in the inhibition of transactivation as well as DNA-binding activity of AP-1 in prostate cancer cells.

Ganoderma lucidum suppresses AP-1 activity through Akt kinase

Modulation of AP-1 activity by NF-κB was recently demonstrated in pancreatic tumor cells [4].

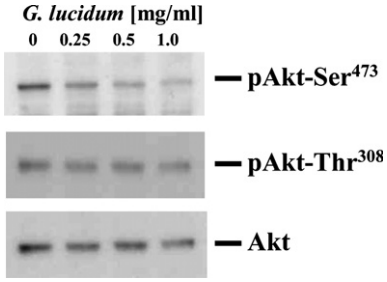

Fig. 4. *Ganoderma lucidum* inhibits phosphorylation of Akt in PC-3 cells. PC-3 cells were treated with *G. lucidum* (0–1.0 mg/ml) for 24 h. Whole cell extracts were prepared, and subjected to Western blot analysis with anti-pAkt-Ser^{473}, anti-pAkt-Thr^{308}, and anti-Akt antibodies as described in Materials and methods. The results are representative of three separate experiments.

Furthermore, we have also shown that *G. lucidum* markedly suppressed NF-κB activity in prostate cancer cells PC-3 [23]. Therefore, we hypothesized that the suppression of AP-1 could be the result of the inhibition of the activity of Akt kinase, which is up-regulated in PC-3 cells [30] and controls the activation of NF-κB [31]. In order to evaluate the effect of *G. lucidum* on Akt, we determined the phosphorylation of Akt at Thr^{308} and Ser^{473}, which is required for the activity of Akt [32]. PC-3 cells were treated with *G. lucidum* (0–1.0 mg/ml) for 24 h, whole cell extracts were prepared and subjected to Western blot analysis with phospho-Akt-Ser^{473}, phospho-Akt-Thr^{308}, and Akt antibody, respectively. As seen in Fig. 4, *G. lucidum* markedly inhibited Akt phosphorylation at Ser^{473} in a dose–response manner. The Akt phosphorylation at Thr^{308} as well as the expression of Akt were not affected by *G. lucidum* (Fig. 4). Thus, *G. lucidum* suppresses Akt kinase activity, followed by the inhibition of NF-κB, resulting in the inhibition of AP-1 in PC-3 cells.

Discussion

Popular oriental medicinal mushroom *G. lucidum* has been used in traditional Chinese medicine to promote general health and to treat a variety of diseases [11]. We have previously demonstrated that spores or fruiting body of *G. lucidum* inhibited invasive behavior of prostate cancer cells by the suppression of uPA signaling [22]. More recently, we have shown that extract from *G. lucidum*, containing standardized amount of biologically active polysaccharides and triterpenes, suppressed cell growth and induced apoptosis of prostate cancer cells [23].

In the present study, we evaluated the effect of extract from *G. lucidum* on angiogenesis related to prostate cancer. Here we show that *G. lucidum* suppresses capillary morphogenesis of aortic endothelial cells. Our data suggest that this effect is mediated through the inhibition of secretion of angiogenic factors VEGF and TGF-β1 from prostate cancer cells PC-3. Mechanistically, *G. lucidum* inhibits activities of kinases Erk1/2 and Akt leading to the inhibition of AP-1, which results in the down-regulation of expression of VEGF and TGF-β1.

Increased levels of epidermal growth factor receptor (EGFR) signaling were detected in advanced stages of prostate cancer and implicated in the autonomous growth, proliferation, and metastasis of prostate cancers [33,34]. EGFR signaling activates Ras-Raf pathway leading to the activation of MAPK/Erk1/2 [35]. Furthermore, constitutive activation of Erk1/2 has been demonstrated in more aggressive androgen-independent prostate cancers [36], suggesting Erk1/2 as a converging point for membrane receptor- as well as non-receptor-mediated mitogenic signaling. Therefore, Erk1/2 can be a suitable target for the prevention and intervention of growth and metastasis of prostate cancer [37]. Finally, natural flavonoid silibinin isolated from milk thistle inhibited Erk1/2 in prostate cancer cells [38], and as we demonstrate here, *G. lucidum* inhibits activity of Erk1/2 by the inhibition of phosphorylation of Erk1/2 in androgen-independent prostate cancer cells PC-3.

Another EGFR-driven pathway involves phosphatidylinositol 3-kinase (PI3K) and serine threonine kinase Akt [39]. As recently demonstrated, Akt expression was markedly up-regulated in PC-3 cells [30] and phosphorylation of Akt at Ser^{473} has been associated with a poor clinical outcome of cancer patients [40]. Therefore, phospho-Akt (Ser^{473}) was suggested as a potential biomarker that can distinguish between tumors with a high potential for recurrence [41]. Inhibition of phosphorylation of Akt at Ser^{473} has been recently demonstrated in prostate cancer cells with natural compounds such as indole-3-carbinol, quercetin, genistein as well as fermentation product of soy extract GCP (genistein combined polysaccharide) [42–45]. Here we show that *G. lucidum* specifically inhibits phosphorylation of Akt at Ser^{473} in prostate cancer cells PC-3, which is in agreement with our recent data demonstrating inhibition of Akt activity by *G. lucidum* in breast cancer cells [46].

In the present study, we also demonstrate that *G. lucidum* suppresses the activity of AP-1. Therefore, inhibition of Erk1/2 by *G. lucidum* results in the direct suppression of AP-1 activity. Alternatively, AP-1 can also be controlled indirectly through the modulation of NF-κB activity [4], and we have recently demonstrated that *G. lucidum* inhibits transactivation of NF-κB in PC-3 cells [23]. As we demonstrated above, *G. lucidum* suppresses the activity of Akt, which in turn inhibits NF-κB through IκB kinase (IKK) [47,48], and finally results in the inhibition of AP-1. Alternatively, Akt can modulate the activity of AP-1 through the c-Jun N-terminal kinase (JNK) [49]. Collectively, inhibition of Erk1/2 as well as Akt by *G. lucidum* results in the suppression of AP-1 activity in prostate cancer cells (Fig. 5). Previous studies demonstrated the inhibition of VEGF as well as

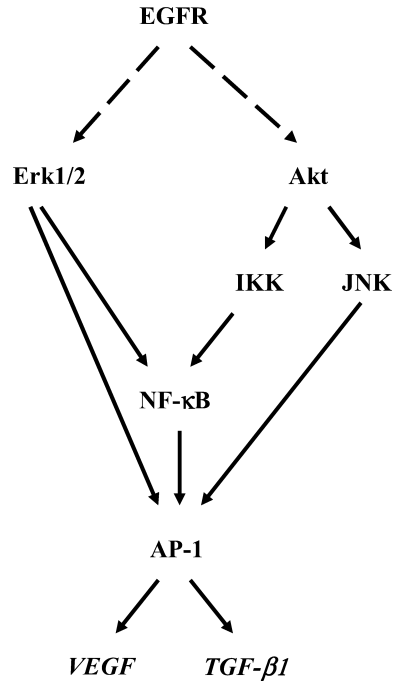

Fig. 5. Schematic representation of *G. lucidum*-mediated inhibition of VEGF and TGF-β1 secretion through Erk1/2 and Akt pathways.

TGF-β1 through the suppression of NF-κB, respectively [50,51]. Since NF-κB binding sites are not in the promoter regions of VEGF or TGF-β1 [27,28], the suppression of secretion of VEGF and TGF-β1 by *G. lucidum* is caused by the inhibition of different signaling pathways resulting in the final inhibition of DNA-binding and activation of AP-1, followed by the down-regulation of expression of VEGF and TGF-β1 (Fig. 5).

In summary, here we show a possible mechanism by which extract from mushroom *G. lucidum* inhibits angiogenesis related to prostate cancer. The biological effects of *G. lucidum* on PC-3 cells are mediated by the inhibition of phosphorylation of Erk1/2 and Akt kinases finally resulting in the inhibition of AP-1-dependent expression of VEGF and TGF-β1, and inhibition of capillary morphogenesis of endothelial cells. Further studies are in progress to identify biologically active compounds responsible for the anti-angiogenic activity of *G. lucidum*.

Acknowledgments

We thank Tatiana Valachovicova for technical assistance. This work was supported by a grant from Showalter Foundation to D.S.

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