수의학 박사 학위 논문

JAK/STAT 인산화 및 VEGFR-2/Akt/mTOR 신호전달 억제를 통한 독사조신의 난소암 억제 기전 규명

Antineoplastic Effects of Doxazosin on Human Ovarian Cancer by Inhibiting JAK/STAT Phosphorylation and VEGFR-2/Akt/mTOR Signaling Pathways

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ABSTRACT

Antineoplastic Effects of Doxazosin on Human Ovarian Cancer by Inhibiting JAK/STAT Phosphorylation and VEGFR-2/Akt/mTOR Signaling Pathways

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Doxazosin, a commonly prescribed treatment for patients with benign prostatic hyperplasia, serves as an α1-blocker of the adrenergic receptors. In this study, we calculated its effect on the ovarian carcinoma cell system and animal model. Doxazosin induces dose-dependent growth and time-dependent suppression and is additively activated through IFN-α or IFN-γ stimulation. At the same time, they both enhanced G_1 phase arrest, as well as the activity of caspase-3 and PARP, and the reduction of cyclin D1 and CDK4 protein levels.

Doxazosin growth suppression was abolished either by the Janus family of tyrosine kinase (JAK) or the signal transducer and activator of transcription (STAT) inhibitor treatment. The activity of JAK/STAT was dependent on the level of doxazosin, suggesting a requirement of doxazosin for the activation of JAK/STAT. Furthermore, doxazosin plus IFN-α or doxazosin plus IFN-γ additively suppressed the activation of the JAK/STAT signals through phosphorylation of JAK and STAT, thus affecting the activation of subsequent downstream signaling components PI3K, mTOR, 70S6K and PKCδ. And *in vivo* study demonstrated that doxazosin significantly suppressed tumor growth in an ovarian xenograft model, inducing apoptotic cell death by up-regulating the expression of p53, whereas c-Myc expression was markedly reduced. Therefore, our data findings indicate that doxazosin can modulate the apoptotic effects of IFN-α and IFN-γ through the JAK/STAT signaling pathways. Collectively, we indicate that this action may be a potent chemotherapeutic property against ovarian carcinoma. Doxazosin also inhibited vascular endothelial growth factor (VEGF)-induced HUVEC migration as well as capillary-like structure tube formation *in vitro*. It also suppresses the expression of HIF-1α and VEGF in ovarian carcinoma cells. Doxazosin inhibited phosphorylation of vascular endothelial growth factor receptor-2 (VEGFR-2) and

transcription of VEGFR-2. Doxazosin inhibited PI3K, Akt, PDK1 and mTOR phosphorylation but had no effect on ERK1/2 phosphorylation by VEGF treatment. Our results provide evidence for the cellular function in endothelial cell system that is relevant to angiogenesis through the inhibition of the Akt/mTOR phosphorylation by interacting with VEGFR-2. Furthermore, doxazosin prohibited VEGFR-2 phosphorylation and suppressed tumor vascularization in a xenograft model of human ovarian cancer. We found the biological function of doxazosin to be a potent anti-ovarian cancer agent by inhibition of JAK/STAT phosphorylation and anti-angiogenic agent by suppression of VEGFR-2 signaling pathway. Therefore, doxazosin combination therapies may be a more useful approach for more advanced ovarian cancers and recurrent patients

Keywords: doxazosin, ovarian cancer, IFN, JAK, STAT, VEGFR-2, Akt, mTOR,

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CONTENTS

ABBREVIATIONS

4E-BP1: eukaryotic translation initiation factor 4E-binding protein 1

CDK4: cyclin-dependent kinase 4

Doxazosin: 1-(4-amino-6, 7-dimethoxy-2-quinazolinyl)-4-(1,4-benzodioxan-2-ylcarbonyl)

ERK1/2: extracellular signal-regulated kinase 1/2

HIF-1α: hypoxia inducible factor 1 α

HUVECs: human umbilical vein endothelial cells

IFN: interferon

IκBα: B-cells inhibitor-alpha

JAK: Janus family of tyrosine kinase

mTOR: the mammalian target of rapamycin

P70S6K: ribosomal protein S6 kinase

PARP: poly (ADP-ribose) polymerase

PDK1: 3-phosphoinositide-dependent protein kinase 1

PI3K: phosphatidylinositol-3-kinase

PKCδ: protein kinase C δ

STAT: signal transducer and activator of transcription

TGF-β1: transforming growth factor-β1

Tyk2: tyrosine kinase 2

VEGF: vascular endothelial growth factor

VEGFR-2: vascular endothelial growth factor receptor-2

LITERATURE REVIEW

Ovarian cancer chemotherapy

Ovarian cancer is the second most lethal gynecological cancer in Korea, after uterine cervix cancer (Table 1). In 2008, the ovarian cancer incidence rate in Korea was similar to that in women worldwide and lower than that in Western countries (Figure 1). The crude ovarian cancer rate in Korea was 8.0 in 2011 (Figure 2). The incidence has increased steadily in Korean women because Korean women showed rapid changes in nutritional, reproductive, and anthropometric factors (Park *et al.,* 2010). In 2014, there will be an estimated 21,980 new ovarian cancer cases in the USA, with 14,270 deaths (Informed from NCI's PDQ®, 2014).

Most patients with ovarian cancer are diagnosed at a late stage due to the lack of effective screening methods and specific symptoms associated with early stage disease (Buys *et al.,* 2011; Dasari *et al.,* 2014). Ovarian cancer-associated mortality rates have increased in Korea (Park *et al.,* 2010). Ovarian epithelial cancer, the most common type of ovarian cancer (observed in approximately 90% of cases) is thought to originate from epithelial cells covering the ovaries. Although ovarian cancers are all epithelial in origin, they display four

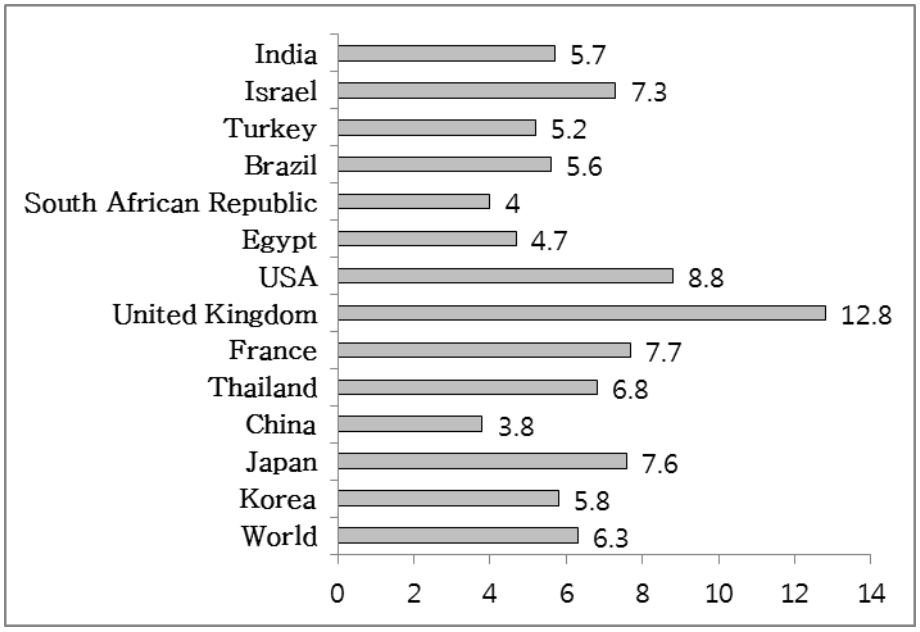
distinct histologies—serous, mucous, clear cell, and endometrioid—that correlate with distinct gene expression patterns and with distinct sensitivity to therapies. At early stages (stages 1–2), current therapies include surgery, chemotherapy, and/or radiation therapy. At later stages, debulking surgery is combined with platinum– or taxane–based chemotherapy (intraperitoneal or external) and, potentially, radiation therapy. However, 80% of patients will relapse after first–line platinum– or taxane–based chemotherapy, and conventional therapeutic protocols have so far given disappointing results. There is a need for new therapeutic drugs, and agents that target neo–angiogenesis are an interesting prospect. Ovarian cancer comprises a variety of tumors that involve the ovarian tissues and arise from Müllerian tissues, including the distal fallopian tube and endometrium. Histologic and clinical features, as well as associated finding such as mutations, expression profiles, pathway activation, and genome variation, are summarized in Table 2 (Bookman *et al.,* 2014).

Table 1. Major cancer sites by sex, Korean, 2011
(Modified from 'annual report of cancer statistics in Korea in 2011, NCC', 2013).

(Unit: cases, %, rate per 100,000)

Rank	Site	Cases	%	CR	ASR
	All cancers	**107,866**	**100.0**	**431.0**	**316.7**
1	**Thyroid**	**33,562**	**31.1**	**134.1**	**113.8**
2	**Breast**	**15,942**	**14.8**	**63.7**	**50.0**
3	**Colon and rectum**	**10,955**	**10.2**	**43.8**	**27.6**
4	**Stomach**	**10,293**	**9.5**	**41.1**	**26.9**
5	**Lung**	**6,586**	**6.1**	**26.3**	**15.5**
6	**Liver**	**4,274**	**4.0**	**17.1**	**10.5**
7	**Cervix uteri**	**3,728**	**3.5**	**14.9**	**11.7**
8	**Gallbladder etc**	**2,514**	**2.3**	**10.0**	**5.6**
9	**Pancreas**	**2,273**	**2.1**	**9.1**	**5.2**
10	**Ovary**	**2,010**	**1.9**	**8.0**	**6.2**

* Age adjusted to the Korea standard population



(Incidence rate per 100,000, age-standardized, Segi standard population)

Figure 1. Global incidence rates for ovarian cancer in year 2008 (Park *et al.*, 2010).

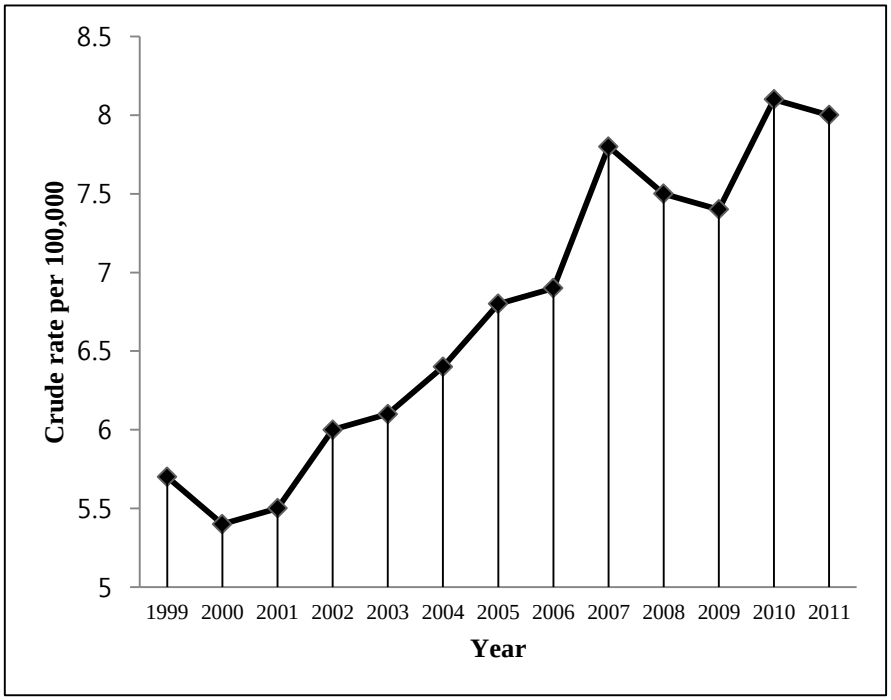


Figure 2. Korean crude rates for ovarian cancer in year 2011 (From data of 'annual report of cancer statistics NCC', 2013).

Table 2. Disease types and characteristics of ovarian cancer (Bookman *et al.,* 2014).

Destriptors	HGSC	CCC	EC	MC	LGSC
Percentage of cases					
FIGO I-II	39% *	33%	22%	5%	1%
FIGO III-IV	86% *	2%	7%	2%	3%
Genetic risk factors	BRCA1/2	HNPCC	HNPCC	None known	None known
Enviromental risk factors	↓ Risk with OC and tubal ligation; ↑ Risk with HRT	None Known	↓ Risk with OC and tubal ligation; ↑ Risk with HRT	None known	None known
Precursor lesions	Serous tubal intraepithelial carcinoma	Endometriosis	Endometriosis	Unknown	Serous borderline
Clinical presentation	ascites, GI symptoms	Adnexal mass	Adnexal mass	Adnexal mass	Gi symptoms
Pattern of spread	Peritoneal, nodal	Peritoneal, nodal, hematogenous	Peritoneal, nodal, hematogenous	Peritoneal +/- Pseudomyxoma peritoneli	Peritoneal, nodal
Chemotherapy response	Sensitive, then resistant	Resistant	Sensutive	Resistant	Resistant
Molecular genetics	p53, BRCA1, BRCA2, HRD with genomic instability	PI3K, ARID1A, MSI	PTEN, β-catenin, ARID1A, MSI	KRAS, HER2	BRAF, KRAS, NRAS
Knowledge gaps	Overcoming drug resistance, targeting tumor stem cells	Risk modification effective chemotherapy	Risk modification	Effective chemotherapy	Effective chemotherapy
Prevention	OC, tubal ligation, RRBSO	None known	OC, tubal ligation, RRBSO, avoid HRT	None known	None known
Screening	None known	None known	None known	None known	None known
Potential targets	PARPi, angiogenesis	Angiogenesis	Hormone receptors, mTOR	HER2/neu	BRAF, MEK

CCC:clear cell carcinoma; EC:endometrioid carcinoma; GI:gastrointenstinal; HGCS:high-grade serous carcinoma; HRD:homologous recombination deficiency; HRT:hormone replacement therapy (postmenopausal); LGSC: low-grade serous carcinoma; MC:mucinous carcinoma; MSI:microsattelite instability; OC:oral contraceptive; RRBSO:risk-reducing bilateral salpingo-oophorectomy; * Includes mixed histology tumors with high-grade features.

As described by the American Cancer Society, chemotherapy is the use of drugs to treat cancer. The standard approach is to combine anti-cancer drugs, such as cisplatin, carboplatin and a taxanes (paclitaxel and docetaxel). Ovarian tumors are attenuated or even eliminated by chemotherapy, but some cancer cells may eventually regrow. Chemotherapeutic drugs kill cancer cells but also damage some normal cells (Informed from NCI's PDQ®, 2014). For example, cisplatin has been linked to crosslinking with the purine bases in DNA. This interferes with DNA repair mechanisms, causing DNA damage and subsequently inducing apoptosis in cancer cells. However, there are numerous undesirable side effects such as severe kidney problems, allergic reactions, decreased immunity, gastrointestinal disorders, hemorrhage, and hearing loss, especially in younger patients. Despite its severe side effects and the development of resistance, cisplatin is used as the major treatment of ovarian cancer (Dasari *et al.,* 2014). Taxane is a well-known mitotic inhibitor that binds to microtubules, thereby inhibiting their reorganization. Taxanes such as paclitaxel (Taxol) and docetaxel (Taxotere) are first-line treatments for breast, ovarian, lung, and other metastatic cancers, but they cause multidrug resistance and side effects (Dasari *et al.,* 2014; Fauzee *et al.,* 2011). A recent targeted therapy in ovarian cancer is the humanized monoclonal antibody

bevacizumab (Avastin), which blocks angiogenic signaling. Bevacizumab has been shown to decrease or delay the growth of ovarian cancer. However, there have been problems with perforations of the bowel wall during treatment, which can be fatal to patients (Keating *et al.,* 2014). Traditional approaches to anti-cancer drug development are being increasingly challenged by the numerous potential targets and candidate drugs. Thus, it is sometimes useful to study multiple markers and cancer-related molecular cascades to reduce the side effects (Bookman *et al.,* 2014).

Interferons and JAK/STAT pathway

Interferons (IFNs) are a glycoprotein known as a part of the cytokine superfamily, and they regulate the response of the immune system, such as the infection against viruses, parasites, bacteria, and even the response for cancer cells by mediating the action of various types of genes (Chawla-Sarkar *et al.*, 2003; Cheon *et al.*, 2014; Morikawa *et al.*, 1987; Stark *et al.,* 1998; Takaoka *et al.,* 2003). IFN-α is a key indicator for viral infections through Toll-like receptors (TLRs), where macrophages and natural killer (NK) cells are induced, which enhances lymphocytes antigen presentation (Cheon *et al.*, 2014; Pirhonen *et al.,* 2007). IFN-γ is a secreted protein that serves as a regulator of macrophage activation, where it appertains to the type II class of the interferon family. It is also activated by specific antigen presentation and can induce apoptosis. In addition, IFN-γ can induce the anti-tumor effect, anti-viral, and immunoregulatory of the type I interferons, such as IFN-α, IFN-β, IFN-ω, IFN-κ and IFN-ε (Burke *et al.*, 1999; Schroder *et al.,* 2004). In the biological function, IFNs are multifunctional factors that signal through the JAK/STAT pathway in the activation of STAT1/2 (Figure 3) (Pestka *et al.,* 2004; Platanias *et al.,* 2005).

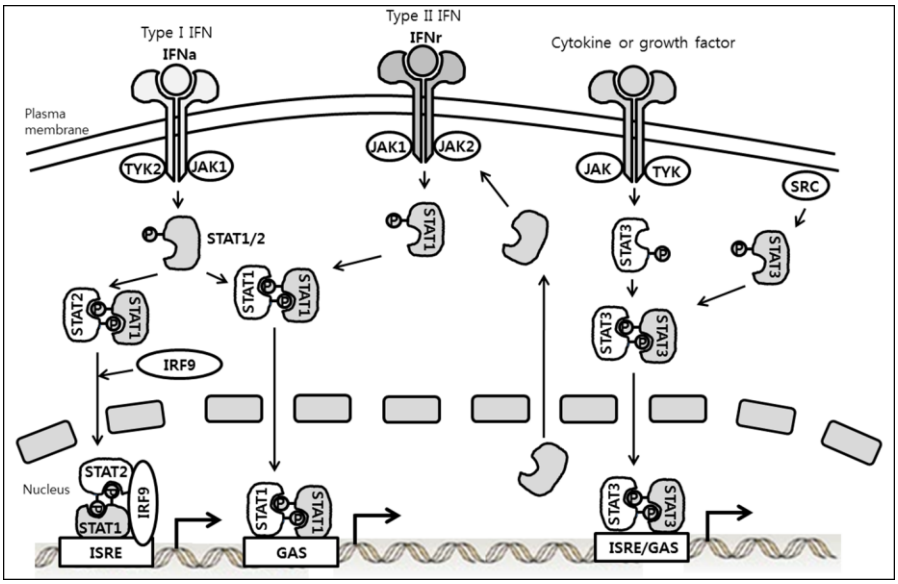


Figure 3. Interferon receptors and activation of classical JAK-STAT pathways (Modified from Platanias *et al.,* 2005).

The JAK/STAT pathways are involved in the regulation of various cellular events including cell proliferation, differentiation, hematopoiesis, development, and apoptosis. They are activated by the binding of cell-surface receptors, such as interferons, growth factors, and interleukins (Aaronson *et al.,* 2002; O' Shea *et al.,* 2004). Interrupted or non-regulated JAK/STAT function can result in various proliferative disorders and immune disorders. Instance, STAT3 is continuously up-regulated in various tumors while JAK activation induces cell proliferation, cell migration, and apoptotic cell death (Darnell *et al.,* 2005). Interestingly, STAT3 up-regulates various proteins involved in cell cycle progression, such as c-Myc, and cyclin D1, and induces anti-apoptotic proteins, such as Bcl-2 and Bcl-xL (Barre *et al.,* 2003). Thus, STATs target proteins are important mediators for the regulation of cell cycle progression from the G_1 to S phase.

VEGFR-2/AKT/mTOR pathway

Angiogenesis is initiated by growth factors such as VEGF and bFGF(basic fibroblast growth factor) and is a potential treatment for vascular injuries (Yoshiji *et al.,* 1996; Gavin *et al.*, 2004; Kraus *et al.,* 2004). During cancer progression, endothelial cell activity plays an essential role in modulating various vascular physiological and pathological functions. VEGFR-2 plays a pivotal role in the activation of downstream components that are responsible for proliferation, including endothelial cell invasion, migration, differentiation and embryonic angiogenesis (Breier *et al.,* 2000; Ferrara *et al.,* 2000; Meyer *et al.,* 2003) in contrast, VEGFR-1 has no role in endothelial cell proliferation (Meyer *et al.,* 2004). VEGFR-2 exerts its function through the PI3K/Akt/mTOR signaling pathway in endothelial cells, the activation status of VEGFR-2 was studied by determining phosphorylation of VEGFR-2 itself together with phosphorylation of downstream targets of mTOR (Figure 4)(Trinh *et al.,* 2009). In addition, Trinh observed the correlation between the activated status of the VEGFR-2 and downstream markers of the AKT/mTOR signal pathway, pS6 and p4E-BP1 protein, using human ovarian cancer samples (Table 3) and suggested that anti-VEGF treatments are suppressors of acting as a surrogate AKT/mTOR signalling inhibitors on ovarian tumor cells (Trinh *et al.*, 2009).

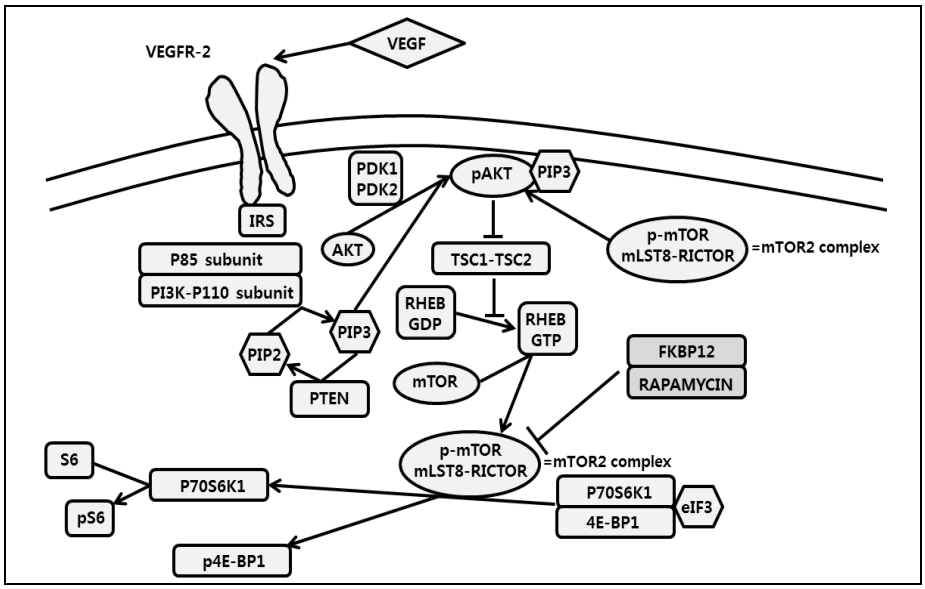


Figure 4. VEGFR-2 activation VEGFR-2 exerts its function through the PI3K/Akt/mTOR signaling pathway in endothelial cells (Modified from Trinh *et al.,* 2009).

Table 3. Immunostaining and marker correlative study summary of clinical data (Trinh *et al.*, 2009).

(N=89 patients)

	N	pVEGFR-2 (Tyr996)	pVEGFR-2 (Tyr951)	pS6	p4E-BP1
FIGO I	**19**	**270**	**275**	**20**	**100**
FIGO II	**4**	**285**	**100**	**45**	**125**
FIGO III	**56**	**210**	**100**	**20**	**90**
FIGO IV	**10**	**300**	**180**	**150**	**160**
		NS	**NS**	**NS**	**NS**
Grade 1	**20**	**300**	**200**	**30**	**90**
Grade 2	**27**	**180**	**80**	**15**	**80**
Grade 3	**42**	**300**	**100**	**20**	**160**
		P=0.06	**NS**	**NS**	**P=0.085**
Serous papllliary	**47**	**270**	**180**	**20**	**80**
Mucinous	**4**	**195**	**250**	**20**	**95**
Endometrioid	**23**	**210**	**80**	**25**	**160**
Clear cell	**10**	**195**	**50**	**40**	**95**
		NS	**NS**	**NS**	**NS**
Total	**89**	**240(0-300)**	**160(0-300)**	**20(0-300)**	**100(0-300)**

Median H-scores are reported. P-values (Kruskal -Wallis tests) are reported in case of significance ($P<0.05$) or in case of trend ($P<0.10$).

Doxazosin

Doxazosin, is a quinazoline compound that is a selective inhibitor of the α1-adrenergic receptors. The chemical name of doxazosin mesylate is 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(1,4-benzodioxan-2-ylcarbonyl) piperazine methanesulfonate (Kirby *et al.,* 1997). They have function to block α-receptors in relaxing blood vessels and reducing the speed and force of the heart contractions (Akduman *et al.,* 2001). It is used for treatment of patients with benign prostatic hyperplasia, a noncancerous enlargement of the prostate gland, because α1-blockers relax the smooth muscle tissue surrounding the prostate, easing urine to flow and decreasing bladder outlet obstruction (Benning *et al.,* 2002; Tahmatzopoulos *et al.,* 2004). Some previous studies also reported that α1-adrenoceptor antagonists stimulate apoptotic cell death via TGF-β1 signaling and the activation of clear factor of kappa light polypeptide gene enhancer in IκBα in prostate tumor cells (Benning *et al.,* 2002; Partin *et al.,* 2003). Doxazosin also stimulates apoptotic cell death in response to abnormal cell-matrix interactions (Keledjian *et al.,* 2003). Garrison et al. reported that specific caspase-8 inhibitors could block doxazosin-induced apoptotic cell death in benign prostate cells (Garrison *et al.,* 2006).

Objective

In previous studies, doxazosin relaxed the smooth muscles surrounding the prostate and stimulated apoptotic cell death in prostate hyperplasia (Keledjian *et al.,* 2003; Garrison *et al.,* 2006). However, the biological roles of these doxazosin-regulated processes, as well as the molecular mechanism behind the anti-angiogenic effects of doxazosin, remain poorly understood. This study evaluated the effects of doxazosin on ovarian cancer as an anti-tumorigenesis and anti-angiogenic agent by *in vitro* and *in vivo* studies.

CHAPTER I

DOXAZOSIN SUPPRESSES JAK/STATs PHOSPHORYLATION THROUGH IFN-α/γ INDUCED APOPTOSIS

Abstract

Doxazosin, a commonly prescribed treatment for patients with benign prostatic hyperplasia, serves as an $\alpha 1$-blocker of the adrenergic receptors. In this study, we calculated its effect on the ovarian carcinoma cell system. Doxazosin induces dose-dependent growth suppression and is additively activated through IFN-α or IFN-γ stimulation. At the same time, they both enhanced G_1 phase arrest, as well as the activity of caspase-3, and the reduction of cyclin D1 and CDK4 protein levels. Doxazosin growth suppression was abolished either by the JAK or the STAT inhibitor treatment. The activity of JAK/STAT was dependent on the level of doxazosin, suggesting a requirement of doxazosin for the activation of JAK/STAT. Furthermore, doxazosin plus IFN-α or doxazosin plus IFN-γ additively suppressed the activation of the JAK/STAT signals through phosphorylation of JAK and STAT, thus affecting the activation of subsequent downstream signaling components PI3K, mTOR, 70S6K, and PKCδ. *In vivo* study demonstrated that doxazosin significantly suppressed tumor growth in an ovarian xenograft animal model, inducing apoptotic cell death by up-regulating the expression of p53, whereas c-Myc expression was markedly reduced. Therefore, our data findings indicate that doxazosin can modulate the

apoptotic effects of IFN−α and IFN−γ through the JAK/STAT signaling pathways. Collectively, we indicate that this action may be a potent chemotherapeutic property against ovarian carcinoma.

Introduction

Doxazosin, 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(1,4-benzodioxan-2-ylcarbonyl) piperazine methanesulfonate, quinazoline compound, is known as an α1-blocker of adrenergic receptors (Kirby *et al.,* 1997). Generally, α1-blockers have similar abilities to α-blockers during biological functions, where they function to block α-receptors in the blood and heart physiological system, such as relaxing blood vessels and reducing the speed and force of the heart contractions (Akduman *et al.*, 2001).

Interferons (IFNs) are a glycoprotein known as being part of the cytokine superfamily, and are known to regulate the response of the immune system, such as during invasions from viruses, parasites, bacteria, and cancer cells. IFNs directly control the immune system response and decrease the growth of carcinoma cells by mediating the action of various types of genes (Stark *et al.,* 1998; Chawla-Sarkar *et al.,* 2003). In addition, IFNs regulate the phosphorylation of STAT by interacting with their specific receptor. IFN-α is directly developed from leukocytes and are mostly involved in both anti-viral and immunoregulatory activities at target cells. IFN-α is also a key indicator for viral infections through Toll-like receptors (TLRs), where macrophages and natural killer (NK)

cells are induced, which enhances antigen presentation to lymphocytes (Morikawa *et al.,* 1987; Takaoka *et al.,* 2003; Pirhonen *et al.,* 2007). IFN-γ is a secreted protein that serves as a regulator of macrophage activation, where it appertains to the type II class of the interferon family. It is also activated by specific antigen presentation and can induce apoptosis. In addition, IFN-γ can induce the anti-tumor effect, anti-viral, and immunoregulatory of the type I interferons, such as IFN-α, IFN-β, IFN-ω, IFN-κ and IFN-ε (Rinderknecht *et al.,* 1984; Dufour *et al.,* 2004; Schroder *et al.,* 2004). Previous studies have demonstrated that IFN-γ can either direct anti-proliferative activity or induce apoptotic cell death in some ovarian carcinoma cell types, as well as in primary carcinoma cells and mouse tumor model systems (Chawla-Sarkar *et al.,* 2003; Burke *et al.,* 1999; Kim *et al.,* 2002; Wall *et al.,* 2003).

The JAK/STAT pathways are involved in the regulation of the immune system for various cellular events including cell proliferation, differentiation, hematopoiesis, development, and apoptosis. They are activated by the binding of cell-surface receptors, such as the bindings by interferons, growth factors, and interleukins (Aaronson *et al.,* 2002; O' Shea *et al.,* 2004). Interrupted or non-regulated JAK/STAT functionality can result in various cancers and immune disorders. For instance, STAT3 is continuously up-regulated in various tumors,

including major carcinomas and some hematologic cancers, while JAK activation induces cell proliferation, cell migration, and apoptotic cell death (Darnell *et al.,* 2005).

Doxazosin is also prescribed to patients with benign prostatic hyperplasia, a non-cancerous enlargement of the prostate gland, because α1-blockers alleviate the smooth muscles surrounding the prostate, easing urine flow and decreasing bladder outlet obstruction (Benning *et al.,* 2002; Tahmatzopoulos *et al.,* 2004). However, the side effects of α1-blockers include fainting, headache, orthostatic hypotension, heart palpitations, dry mouth, and nasal congestion. These drugs can also cause erectile dysfunction, although not as frequently as some other blood pressure medications. In some studies, α1-adrenoceptor antagonists have induced apoptotic cell death through the TGF-β1 signaling IκBα activation in prostate carcinoma cells (Benning *et al.,* 2002; Partin *et al.,* 2003). Doxazosin has also been validated to induce apoptotic cell death, in which the antagonistic effector is triggered by abnormal cell-matrix interactions (Keledjian *et al.,* 2003). In a recent study, doxazosin-mediated apoptosis could be blocked by specific caspase-8 inhibitors in benign prostate cells (BPH). Caspase-8 activation is initiated by its interaction with FADD and their subsequent recruitment by doxazosin (Garrison *et al.,* 2006). Howevere, the details of the molecular

mechanism of these doxazosin-mediating processes are still poorly understood.

This study was designed to explore the major roles of the critical components in the cellular signaling pathways, such as JAK/PI3K/STAT phosphorylation, accompanied by the patterns of expression of apoptosis-related proteins by doxazosin-induced apoptosis along with the underlying molecular mechanisms of ovarian carcinoma cells.

Materials and Methods

Cell lines, drug reagents and antibodies

Human ovarian cancer cell lines (OVCAR-3, 2774 and SKOV-3) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Human IFN-α and IFN-γ were obtained from Sigma (St. Louis, MO). The chemical doxazosin was also purchased from Sigma (St. Louis, MO). The JAK1/2 kinase inhibitor INCB18424 (Ruxolitinib) and STAT1 inhibitor (NSC118218) were obtained from Selleck Chemicals (Houston, TX), and stock solutions were prepared in DMSO. NSC74859 (S31-201), a specific STAT3 inhibitor, was purchased from Calbiochem Chemicals (La Jolla, CA). The following primary antibodies were used in this study: anti-JAK1, anti-phospho-JAK1, anti-JAK2, anti-phospho-JAK2, anti-STAT1, anti-phospho-STAT1, anti-STAT3, anti-phospho-STAT3, anti-caspase-3, and anti-cMyc, anti-Bcl-2, anti-Bax, anti-p53, anti-survivin, and anti-COX-2 (Cell Signaling, Beverly, MA), anti-PARP, anti-XIAP (BD Biosciences, San Jose, CA), anti-cyclin D1, anti-CDK4, anti-Akt, anti-phospho-Akt, anti-TYK2, anti-phospho-TYK2, anti-PI3K, anti-phospho-PI3K, anti-mTOR, anti-phospho-mTOR, anti-PKCδ, anti-phospho-PKC, anti-STAT2, anti-phospho-STAT2, anti-p70S6K, and anti-phospho-p70S6K (Santa Cruz Biotechnology,

Santa Cruz, CA), anti-p16, anti-p21, and anti-p27 (Oncogene, San Diego, CA).

Cytotoxicity assay

Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-^{2}H-tetrazolium bromide (MTT) assays. Briefly, cells were seeded at 5.5×10^{3} cells per well in 96-well plates. Then, the cells were treated with doxazosin, IFN-α or IFN-γ and 20 μl of MTT solution (Sigma, 5 mg/ml) were added to each well. The plate was incubated for an additional 4 h at 37℃. The MTT reaction was terminated with DMSO.

Cell cycle analysis

Ovarian carcinoma cells were plated onto chamber slides at a density of 4.5×10^{4} cells per well and were then treated. A cell cycle distribution was performed through fluorescence-activated cell sorting (FACS). Cells were harvested by trypsin treatment and rinsed twice with phosphate buffered saline (PBS). After centrifugation, cells were incubated with FITC-labeled Annexin V and propidium iodide (PI) for 15 min according to the manufacturer's protocols (BD PharMingen, Mississauga, ON).

Western blotting

Cells and tissues were collected, rinsed twice with cold PBS, and lysed using RIPA lysis buffer with protease inhibitor cocktail (Sigma) at 4℃ for 50 min. Cell lysates containing equal amounts of protein were subsequently separated with 8~12% SDS-PAGE, where they were then transferred onto Hybond-ECL nitrocellulose membrane (GE Healthcare, UK). After blocking, the membranes were incubated with the appropriate primary antibodies at 4℃ overnight. The membranes were washed thrice with TBST buffer and incubated in either goat anti-rabbit or anti-mouse secondary antibodies.

Xenograft mouse model

Specific pathogen-free BALB/c - nu/nu mice (5~6 weeks old) were purchased from Orientbio (Sungnam, Korea). All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Research Institute of the National Cancer Center. To establish ovarian tumors in mice, 1.0×10^6 SKOV-3 cells were injected subcutaneously with Matrigel (BD Bioscience, MA) into the left mid-dorsal region of each nude mouse. Tumors were allowed to grow for 10 days. Tumor sizes were measured by caliper measurements 3 times every week. On day 10, oral treatment of 3 mg/kg of doxazosin was done and repeated 5 times per week for 30 days. Mice were sacrificed

one day after the final treatment, and tumors were then fixed 10% neutral bufferd formalin (NBF) for paraffin blocks and stored at −80℃ for further study.

Statistical analysis

Data are expressed as the mean±SD of error for the mean of the triplicate experiments. Statistical analyses were performed using Student's t−test for comparisons between the two groups. The index for statistical significance was $P<0.05$. The values with 95% confidence ($P<0.05$) are depicted with an asterisk (*) on each graph.

Results

Doxazosin inhibits cell proliferation in a dose-dependent manner, and additively enhances apoptotic cell death by IFN-α and IFN-γ treatment

To study the biological function of doxazosin during IFN-α or IFN-γ treatment in ovarian carcinoma cells, we elected to use a treatment of doxasozin which is known as an α1-blocker of adrenergic receptors. The cell viability of SKOV-3 cells treated with various concentrations (0, 10, 20, 30, and 40 μM) of the doxazosin drug were decreased in a dose-dependent manner, where the doses were required to suppress the growth of the cells by 40%, which exceeded 20 μM on SKOV-3 ovarian carcinoma cell line (Figure 5). Similar results of the pro-apoptotic effect were shown by other ovarian carcinoma cells, such as 2774 (Figure 6) and OVCAR-3 (data not shown). We then examined the apoptotic activity of doxazosin through IFN-α and IFN-γ treatment, respectively. As presented in Figure 7, the effect of each doxazosin, IFN-α, and IFN-γ on SKOV-3 cells, decreased the number of cells, which is indicative of cell viability. IFN-α treatment significantly reduced the proliferation in the cells. The cell viability of IFN-α-treated SKOV-3 cells was inhibited to approximately 60% when compared to the untreated SKOV-3 cells. In contrast,

doxazosin and IFN-γ weakly inhibited the SKOV-3 cells. We also calculated the potential additive or synergistic effects of doxazosin in combination with IFN-α or IFN-γ in ovarian tumors. As shown in Figure 8, co-treatment with doxazosin and IFN-γ or IFN-α had an additive effect, indicating that doxazosin plus either, IFN-α or IFN-γ additively inhibits cell growth in a time-dependent manner. The cytotoxicity of the doxazosin/IFN-α or IFN-γ combination on carcinoma cells were evaluated using fluorescence activated cell sorting (FACS) with the FITC-labeled Annexin V. As presented in Figure 9, pre-treatment with IFN-α or IFN-γ dramatically promoted doxazosin-induced apoptotic cell death, showing that IFN-α or IFN-γ enhances doxazosin-mediated cell growth inhibition. Caspases can be activated through intrinsic or extrinsic signaling pathways. Caspase-3 is a critical apoptotic molecule, as it is responsible for the proteolytic cleavage of various regulatory proteins, such as PARP. To confirm the increased activity of the cleaved caspase-3, the immunoblotting of major caspase-3 and cleaved caspase-3 involved in the apoptotic pathways is presented in Figure 10. The immunoblotting results that were clearly observed dramatically enhance the activity of caspase-3 with doxazosin alone, doxazosin plus IFN-α, or doxazosin plus IFN-γ treatment. Subsequently, the cleavage of PARP induced by

doxazosin alone was also additively induced by the combination with each IFN-α and IFN-γ treatment (Figure 11).

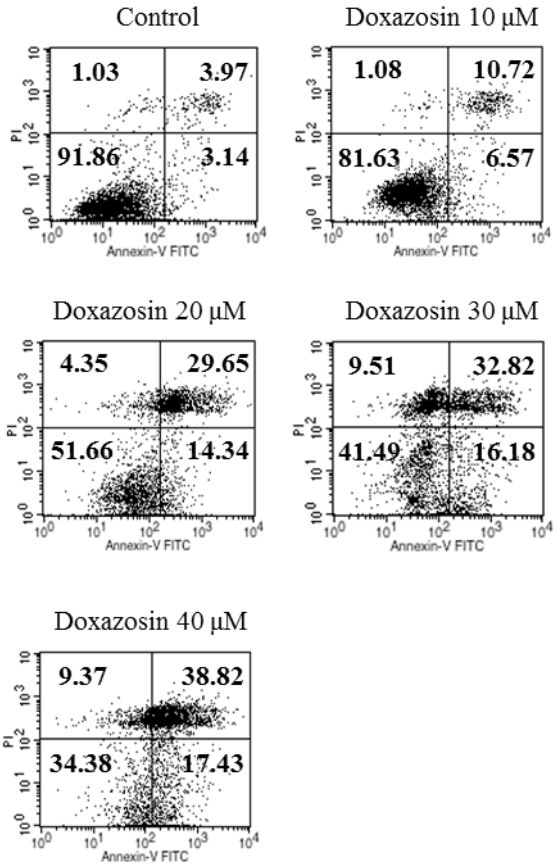


Figure 5. Effects of doxazosin on cell proliferation in SKOV-3 ovarian carcinoma cells.

Exponentially-growing cells were treated with increased concentrations of doxazosin (0~40 μM). Doxazosin drug were decreased in a dose-dependent manner, where the doses were required to suppress the growth of the cells by 40% in SKOV-3 ovarian cancer cells.

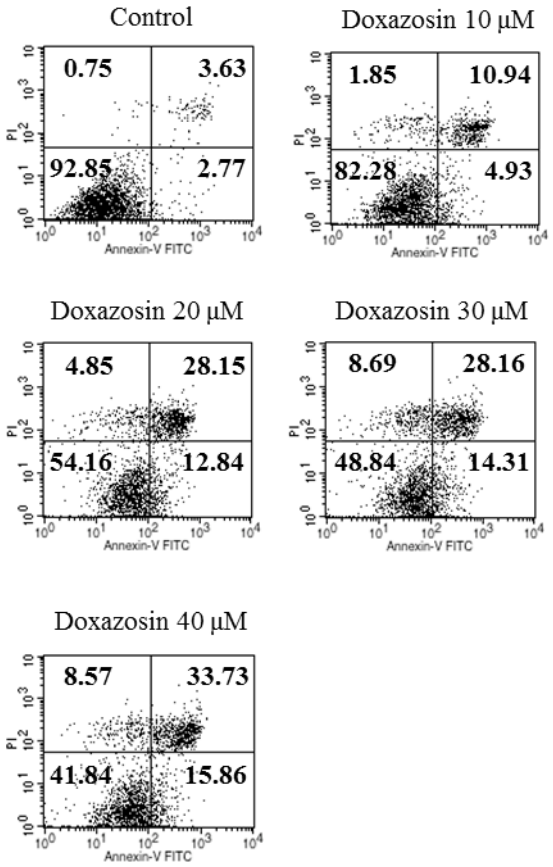


Figure 6. Effects of doxazosin on cell proliferation in 2774 ovarian carcinoma cells.

Exponentially-growing cells were treated with increased concentrations of doxazosin (0~40 μM). Doxazosin drug were decreased in a dose-dependent manner, where the doses were required to suppress the growth of the cells in 2774 ovarian cancer cells.

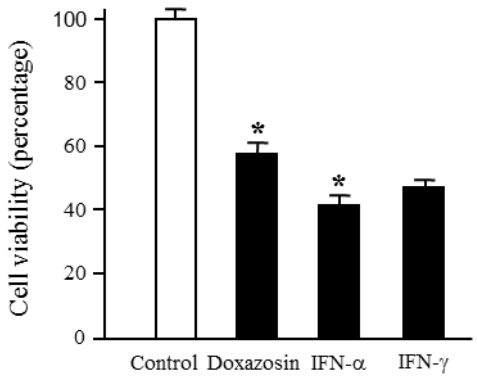


Figure 7. Effects of doxazosin, IFN−α and IFN−γ stimulation on ovarian carcinoma cell viability.

SKOV−3 cells were treated with doxazosin, IFN−α or IFN−γ and the cell viability was measured using a MTT colorimetric assay. Significant differences of 95% confidence (P<0.05) are depicted with an asterisk (*) for each graph.

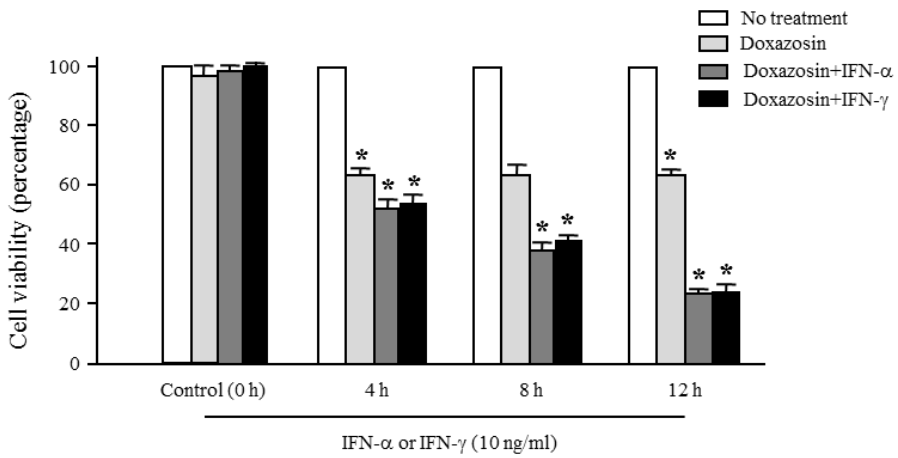


Figure 8. Additive effects of doxazosin by IFN-α or IFN-γ stimulation on ovarian carcinoma cell viability.

IFN-α and IFN-γ-stimulated SKOV-3 cells were treated for the indicated times with doxazosin. Significant differences of 95% confidence (P<0.05) are depicted with an asterisk (*) for each graph.

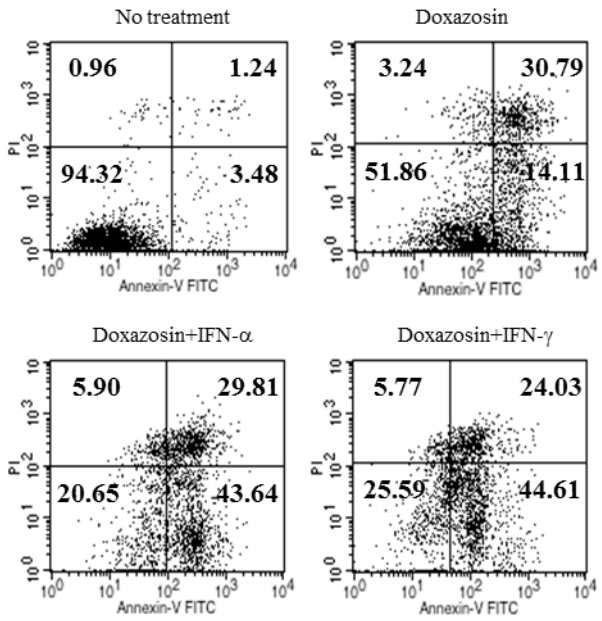


Figure 9. Additive effects of doxazosin by IFN-α or IFN-γ stimulation on ovarian cell proliferation.

Cells were treated with control (untreated) or doxazosin alone or in combination (doxazosin plus IFN-α and doxazosin plus IFN-γ) where the data calculated the additive effects of doxazosin in combination using the flow cytometry system.

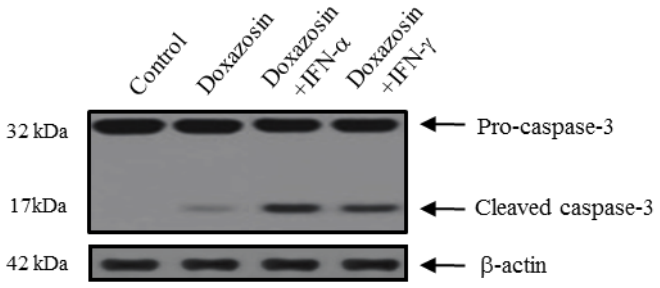


Figure 10. Caspase-3 cleavages activated by doxazosin, doxazosin plus IFN-α, or doxazosin plus IFN-γ treatments.
Soluble protein extracts were introduced by immunoblotting for pro-caspase-3 and cleaved caspase-3. β-actin was used to verify equal loading.

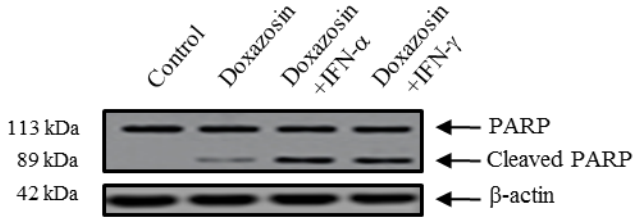


Figure 11. PARP cleavages activated by doxazosin, doxazosin plus IFN-α, or doxazosin plus IFN-γ treatments.
Soluble protein extracts were introduced by immunoblotting for PARP and cleaved PARP. β-actin was used to verify equal loading.

Effect of doxazosin on IFN-α or IFN-γ-stimulated expression of cell cycle modulator proteins

To investigate whether cell cycle arrest is related to the expression of the cell cycle regulatory proteins, as well as changes to the cell cycle distribution, cells were treated at various times with the indicated doxazosin, IFN-α, IFN-γ or combination, followed by FACS, and were then immunoblotted for analysis. As presented in Figure 12 (*upper* panel), the cell cycle profile of the control (untreatment) cells did not change significantly, except that of the S phase section gradually decreasing due to doxazosin, IFN-α or IFN-γ treatment, respectively. In addition, the cell cycle profile of the cells treated with the doxazosin plus IFN-α or doxazosin plus IFN-γ combinations arrested the G1 phase when compared to the doxazosin single-drug treatment Figure 12 (*lower* panel). Interestingly, the greatest pro-apoptotic effect occurred with the combination of 20 μM of doxazosin and 10 ng of IFN-α. These results suggest that the induction of apoptotic cell death is contributed by the doxazosin plus IFN-α-mediated anti-tumor effect. The expression of cyclin D1 and CDK4, which are relevant to the conversion from the G_1 to S phase, was inhibited in a time-dependent manner. In addition, cyclin-dependent kinase inhibitors p21 and p27, which typically cause cells to delay cell cycle progression in the G_1 phase, were induced in

the doxazosin, IFN-α or IFN-γ-treated cells (Figure 13). Consistent with these findings, a decrease in cyclin D1 and CDK4 protein levels in response to the combined treatment was greater than that of doxazosin, IFN-α or IFN-γ-treated alone cells (Figure 14). Taken together, our findings indicate that doxazosin, IFN-α and IFN-γ inhibit cell proliferation by enhancing G_1 phase arrest in ovarian carcinoma cells.

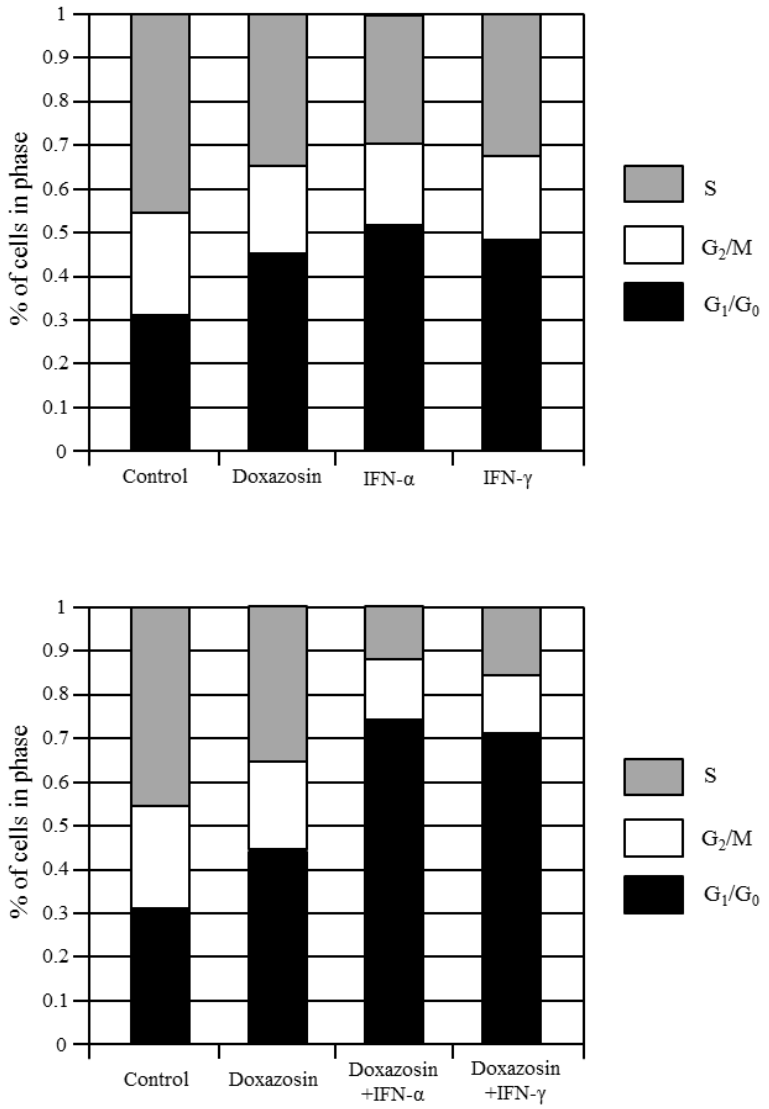


Figure 12. Effects of doxazosin on IFN-α or IFN-γ stimulation on cell cycle distribution.

Cells were pre-treated with IFN-α or IFN-γ then with doxazosin (*lower* panel), collected, and analyzed using flow cytometry. The cell cycle distribution was assessed form the presence of apoptotic cells using PI, FACS.

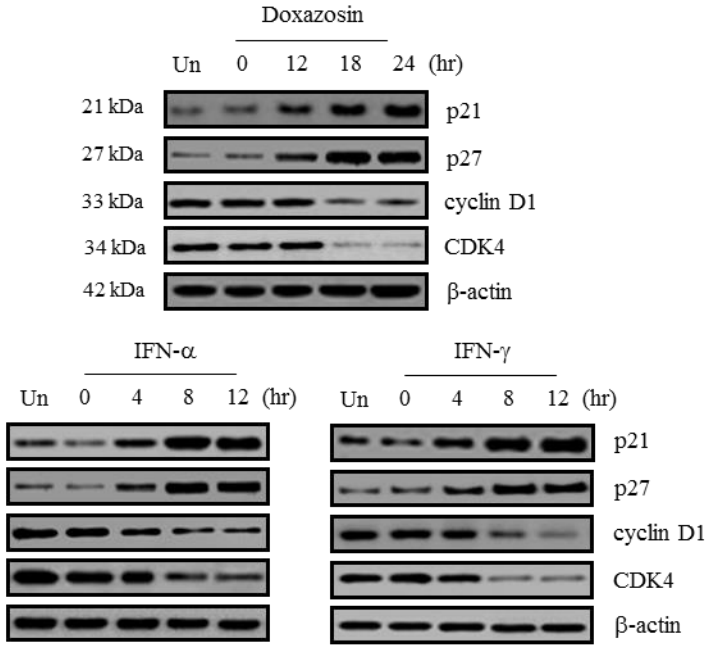


Figure 13. Effects of doxazosin and IFN-α or IFN-γ-stimulated on the expression levels of cell cycle-associated proteins.

Cells were treated to compare with a time-course of shorter times by using the indicated control (untreated cells), doxazosin alone, IFN-α alone, IFN-γ alone. Cell lysates were immunoblotted with the indicated antibodies (p21, p27, cyclin D1, and CDK4). β-actin was used as a loading control.

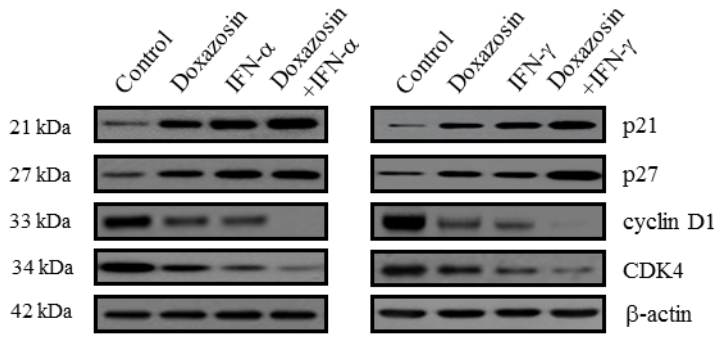


Figure 14. Effects of doxazosin with IFN-α or IFN-γ combination on the expression levels of IFN-α or IFN-γ stimulated cell cycle-associated proteins.

Cell lysates were immunoblotted with the indicated antibodies (p21, p27, cyclin D1, and CDK4). β-actin was used as a loading control.

Doxazosin inhibits phosphorylation of JAK/STAT

Considering the propagation of JAK/STAT activation in malignant cancer and the requirement of the various biological/physiological responses for cell proliferation, understanding how JAK/STAT activation regulates the phosphorylation levels and cellular signal transduction processes is very important. JAK proteins are phosphorylated when cytokines bind to their specific receptors, subsequently activating STATs. STATs are a family of cytoplasmic proteins with Src homology-2 (SH2) domains that act as signal messengers and transcription factors (Dell'Albani *et al.*, 2003). Based on the findings presented, we examined whether doxasoxin suppressed the phosphorylation of JAK and STATs in carcinoma cells. As presented in Figure 11 and Figure 12, phosphorylation of JAK and STAT were dramatically reduced by the doxazosin drug. In addition, JAK and STAT inhibitor treatment exactly recovered doxazosin-reduced phospho rylation of both JAK1/2 and STAT1/3. Consistent with the results observed in carcinoma cells, inhibitors for JAK and STAT also significantly blocked doxazosin-reduced JAK or STAT activity (Figure 15 and Figure 16). Taken together, these results suggest new insight into the anti-tumor effects of this potentially important new chemotherapeutic agent in ovarian carcinoma.

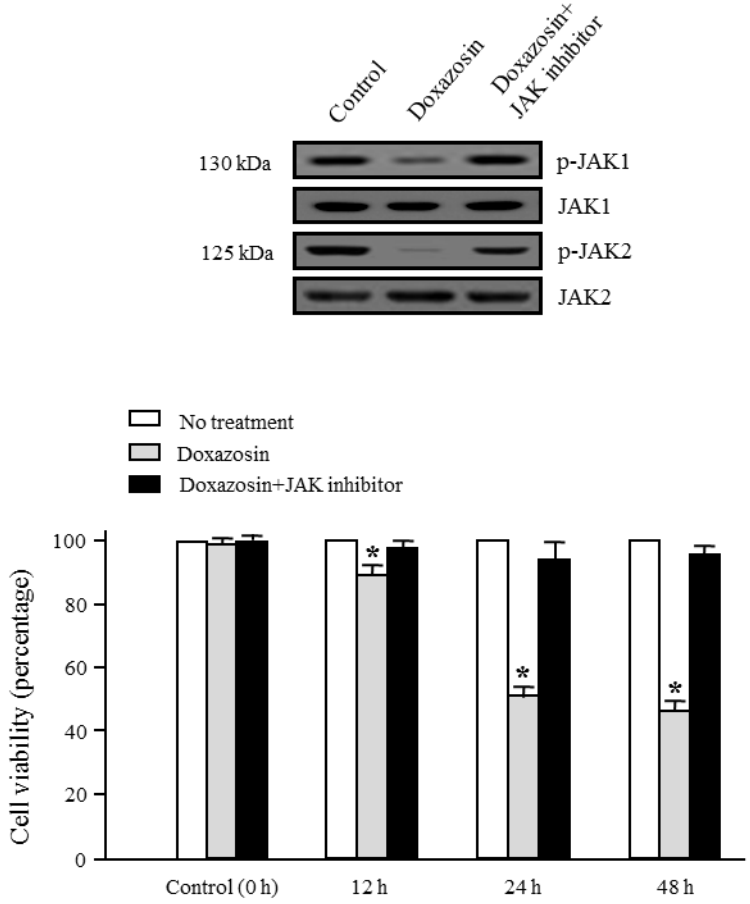


Figure 15. JAK inhibitors block doxazosin-mediated JAK dephosphorylation.

Cells were treated with control (untreated cells), doxazosin, doxazosin plus JAK1/2 inhibitor respectively. Levels of JAK1 (total and p-Y1022/1023), JAK2 (total and p-Y1007/1008) were developed by Western blotting(*upper* panel). Cells were treated for the indicated times with the indicated drug agents (*lower* panel). Experiments were performed in triplicate and error bars are shown as the mean±SD. *P<0.05.

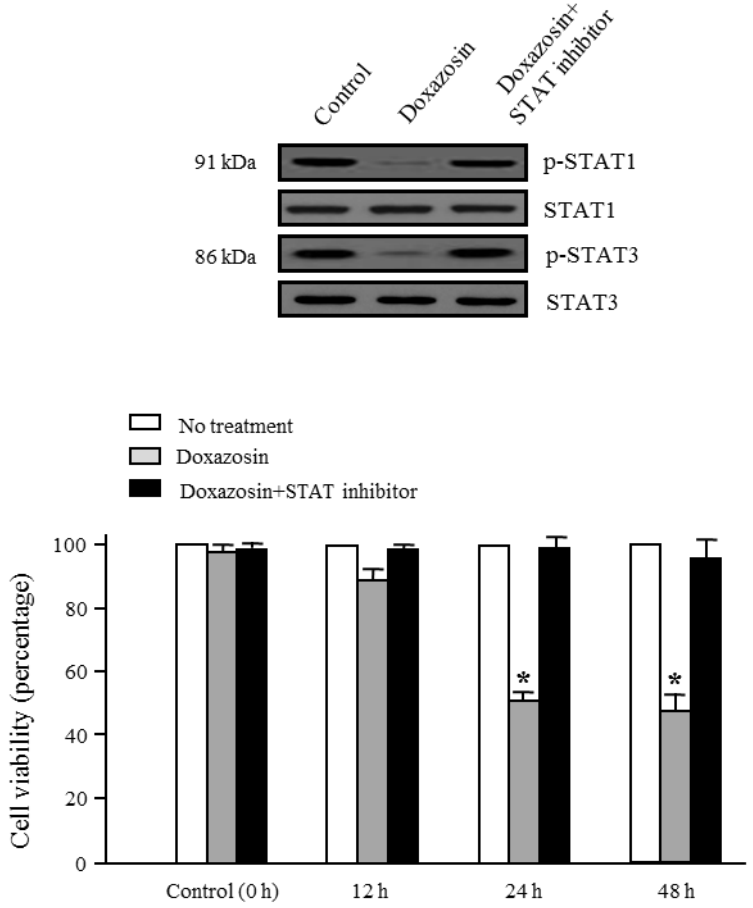


Figure 16. STAT inhibitors block doxazosin-mediated STAT dephosphorylation.

Cells were treated with control (untreated cells), doxazosin, doxazosin plus STAT1/3, respectively. Levels of STAT1 (total and p-Y701) and STAT3 (total and p-Y705) were developed by Western blotting(*upper* panel).. Cells were treated for the indicated times with the indicated drug agents (*lower* panel). Experiments were performed in triplicate and error bars are shown as the mean±SD. *P<0.05.

Decreased phosphorylation of PI3K/Akt/mTOR as well as 70S6K and PKC δ following doxazosin treatment

IFNs are crucial regulators of the JAK/STAT signaling pathway. The IFN-α/γ receptors consist of two subunits of IFNR-1 and IFNR-2. Generally, activation of the JAK kinase family (JAK1 and TYK2) followed by the phosphorylation of the STAT1/2 proteins. In contrast, IFN-γ leads to the phosphorylation of the JAK1/2 tyrosine kinases, resulting in the phosphorylation of STAT1 through the binding of its receptor. As with previous evidence, immunoblotting was employed to evaluate the effect of doxazosin on the JAK/STAT signaling pathway molecules, such as Tyk2, PI3K, Akt, mTOR, 70S6K, and PKC δ phosphorylation. Generally, active PI3K/Akt induces cell proliferation as well as protein synthesis through the activation of the mTOR and 70S6K genes. Cells were treated with 20 μM doxazocin, 20 μM doxazoxin plus 10 ng IFN-α or 20 μM doxazosin plus 10 ng IFN-γ respectively. As presented in Figure 17 and Figure 18, doxazoxin down-regulates phospho-JAK1/2 and STAT1/3, but not the total JAK1/2 and STAT1/3. Consistent with this, doxazosin plus IFN-α or IFN-γ additively inhibited the activation of the JAK/STAT pathway, and thus affected subsequent downstream signaling components, including PI3K, mTOR, 70S6K, and PKC δ activation. Overall the results validated that doxazosin

can potentiate the effects of IFN−α or IFN−γ through JAK/STAT/mTOR signaling pathway−dependent apoptotic cell death, as well as increasing G_1 phase arrest of the cell cycle progression, which includes cell migration and metastasis in ovarian tumorigenesis.

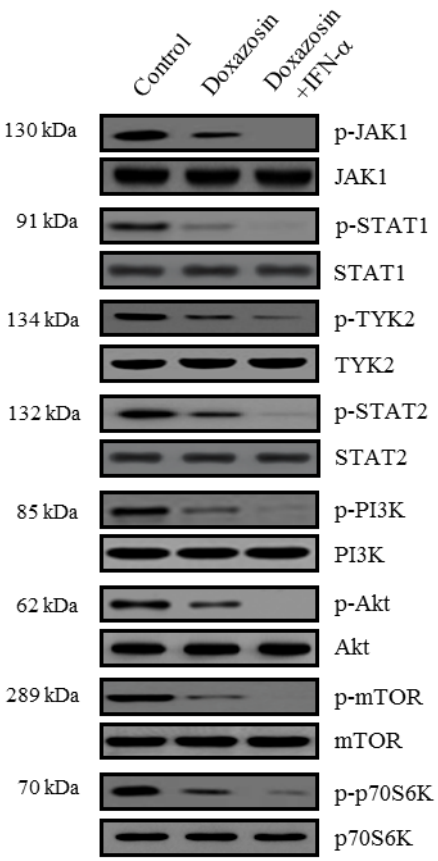


Figure 17. Doxazosin and doxazosin plus IFN-α combination down-regulates the phosphorylation of the major components of the JAK/STAT/mTOR-mediated signaling pathways.

Cells were treated with control (untreated cell), doxazosin alone, doxazosin plus IFN-α or doxazosin plus IFN-α. Western blots for the non-phosphorylated proteins were used as a loading control (JAK1, Tyk2, STAT1, STAT2, PI3K, Akt, mTOR, p70S6K).

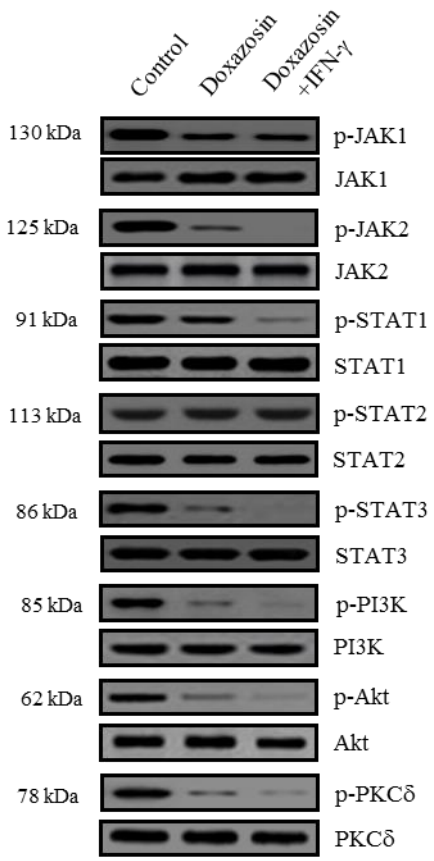


Figure 18. Doxazosin and doxazosin plus IFN-γ combination down-regulates the phosphorylation of the major components of the JAK/STAT/mTOR-mediated signaling pathways.

Cells were treated with control (untreated cell), doxazosin alone, doxazosin plus IFN-γ or doxazosin plus IFN-γ. Western blots for the non-phosphorylated proteins were used as a loading control (JAK1, JAK2, STAT1, STAT2, STAT3, PI3K, Akt, PKC δ).

Doxazosin inhibits tumor growth in nude mice

We investigated the growth-inhibitory effect of doxazosin on ovarian carcinoma cells *in vivo*. For this purpose, Exponentially-growing SKOV-3 ovarian cells were implanted subcutaneously into immune-deficient BALB/c-nu/nu mice. We allowed the tumors to grow until they reached a mean volume of 50 mm^3. Tumor growth was inhibited by doxazosin at the 20th day, which reached a mean volume of 100 mm^3 (Figure 19). There were no differences in body weight loss or liver toxicity (data not shown). The volume of the treated tumors was 50~65% smaller than those of the control mice (Figure 20). Next, we examined the effect of doxazosin on p53 stabilization in tumor tissues collected from the control and doxazosin treated mice. Doxazosin treatment was observed to remarkably increase the level of p53 protein expression, whereas c-Myc expression was markedly reduced (Figure 21). Subsequently, to further clarify the biological mechanism of doxazosin-induced cell cycle arrest. The protein levels of cell progression were examined with tumor tissue lysates. The expression levels of cyclin D1 and CDK4 protein, which is associated with the transition of G_1 to S phase was significantly reduced, whereas the CDK cell cycle inhibitors p16 and p27, which are related with the interruption of cell cycle progression in the G_1 or G_2/M phase, were enchanced (Figure 22, *upper panel*). In

addition, to address whether doxazosin induces apoptotic cell death, we evaluated the activation of caspase-3 for stimulation of apoptosis, as well as the activation of PARP through western bloti analysis. Expression of cell death-related proteins such as caspase-3 and PARP was remarkably increased in doxazosin-treated tumors (Figure 22, *lower panel*). These results indicate that doxazosin is capable of inhibiting tumor growth by inducing apoptotic cell death *in vivo*. We next showed that Bax expression was increased in the doxazosin-treated group, but not in the control group (Figure 23, *upper panel*). Meanwhile, the protein expression of anti-apoptotic/oncogenic/anti-proliferation genes, such as Bcl-2, surviving, COX-2, ICAM-1, XIAP were decreased in the doxazosin treatment group compared to the control group (Figure 23, *lower panel*). Taken together, these results showed that the treatment with doxazosin significantly inhibited tumor growth n the mouse ovarian cancer cell xenograft model.

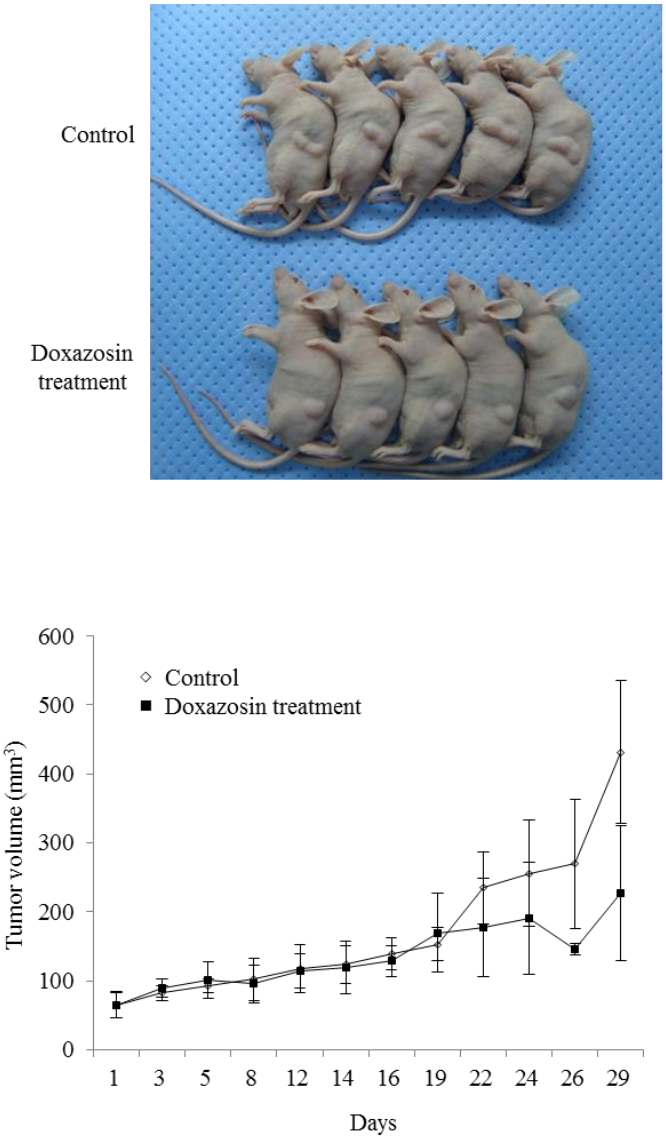


Figure 19. Doxazosin suppresses tumor growth on ovarian carcinoma xenograft mice.

Subcutaneous tumors derived from the SKOV-3 (1.0×10^6) cells were treated with doxazosin as indicated and tumor growth was inhibited by doxazosin at the 20th day (*lower* panel). *$P<0.05$ compared to the control.

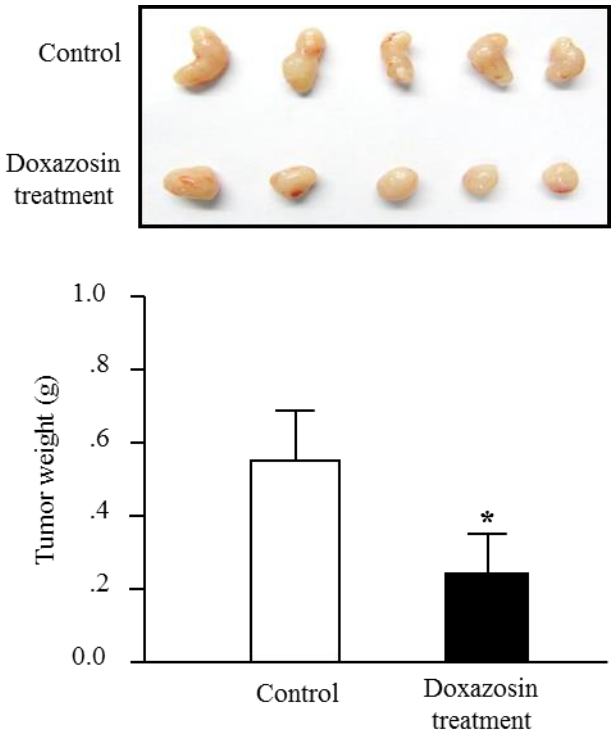


Figure 20. Doxazosin suppresses tumor progression in nude mice.

After sacrifice, image of the xenograft tumor mass treated with or without doxazosin (*upper* panel). Inhibitory effects of doxazosin on xenograft tumor weight indicated that the treatment mice tumors were 50~65% smaller than those of the control mice tumors (*lower* panel). *P<0.05 compared to control.

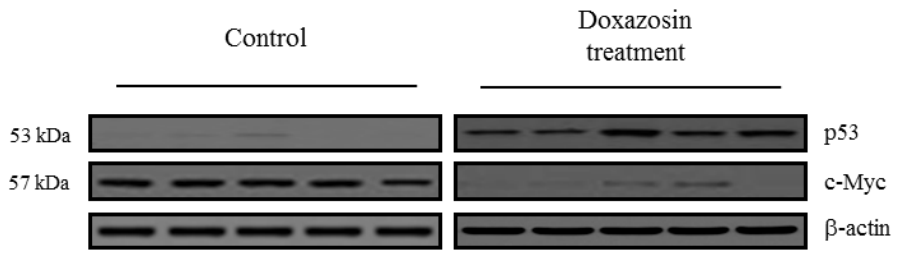


Figure 21. Effect of doxazosin treatment on the expression level of p53 in SKOV-3 carcinoma cell-derived tumor.

The expression level of p53 in SKOV-3 carcinoma cell-derived xenograft tumors excised on the 31st day post-treatment, p53 and c-Myc expressions measured using Western blot analysis.

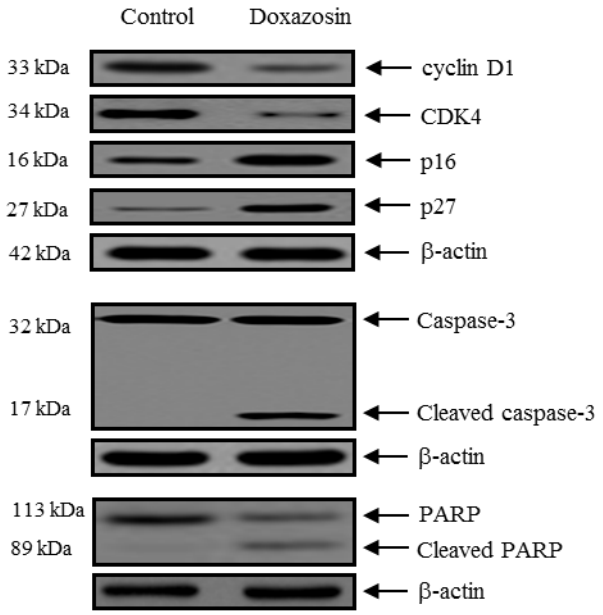


Figure 22. Effect of doxazosin treatment on the expression level of cell cycle and apoptosis in SKOV-3 carcinoma cell-derived tumor.

From xegrafted mice, soluble protein extracts were detected by immunobloting for the indicated proteins (Cyclin D1, CDK1, p16, p27, caspase-3 and PARP). β-actin was used to verify equal loading. Un-cleaved caspase-3 and PARP and their cleaved products are indicated.

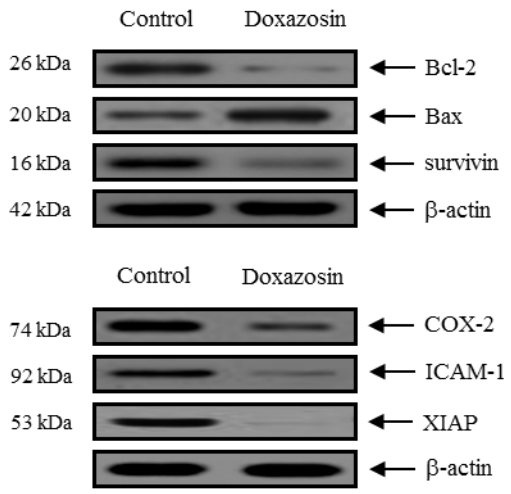


Figure 23. Effect of doxazosin treatment on the expression level of anti-apoptotic/oncogenic/anti-proliferation gene in SKOV-3 carcinoma cell-derived tumor.

From xegrafted mice, soluble protein extracts were detected by immunoblotting for the indicated proteins (Bcl-2, Bax, survivin, COX-2, ICAM-1-3 and XIAP).

Discussion

Our studies were planned to analyze the mechanism of the effects of doxazosin on the JAK/STAT signaling pathway by IFN-α or IFN-γ stimulation in ovarian carcinoma cells. This was due to the fact that the JAK/STAT pathways are a rising interest for targeting cancer-associated diseases, which also include cancer treatment. Interferons are multifunctional cytokines, as major modulators of the innate and adaptive immune system that signal through the JAK/STAT pathway in the activation of STAT1/2 (Pestka *et al.*, 2004). In case of a response to IFN-α which is preceded by IFN-α binding to cell surface receptors, JAK kinases (TYK2 and JAK1) are activated, leading to tyrosine phosphorylation of STAT1 and STAT2. Generally, cytokines and their cognate transmembrane receptors are the essential activators of the JAK/STAT pathway. IFNs regulate both anti-viral and immunomodulatory activities, while mediating intracellular effects, such as the anti-tumor and anti-angiogenic effects. Recently, DAPk1 is a major regulator of IFN-γ-induced apoptotic cell death in human ovarian carcinoma cells (Yoo *et al.,* 2012). In this study, our results strongly indicate a new crucial molecular mechanism for doxazosin, which can be reported as a key regulatory factor that can target the JAK/STAT signaling cascade. As shown in

Figure 9, pre-treatment with IFN-α or IFN-γ significantly increased doxazosin-induced apoptotic cell death, which was observed through IFN-α or IFN-γ up-regulating doxazosin-modulated cell growth inhibition. Furthermore, the cleavage of PARP, which can be activated by doxazosin alone, was also additively promoted via the combinations with IFN-α and IFN-γ treatment (Figure 11).

Activation of STATs can be mediated through IFN-α, IFN-β, or IFN-γ signaling where it is subsequently translocated to the nucleus. IFN-α/β directly binds to their receptors and trigger auto-phosphorylation of the JAK protein families, such as JAK1, JAK2, and TYK2. Previous studies have shown that STAT1 is involved in mechanisms like cell proliferation and apoptotic cell death, so phosphorylation-independent STAT1 biological functions have been postulated (Mui *et al.,* 1999; Chatterjee-Kishore *et al.,* 2000). Meyer et al. (Meyer *et al,.* 2002) have reported that STAT1 can be found in both the cytoplasm and the nucleus without cytokine induction of cells. Also, cytomegaloviral proteins demonstrated a dual biological function for STAT2 in anti-viral responses and IFN-γ signaling (Zimmermann *et al.,* 2005). In addition, Dengue virus NS5 suppresses IFN-α signaling through the blocking of STAT2 phosphorylation (Mazzon *et al.,* 2009). Other recent studies have revealed STAT2 as a key component of the

STAT1-independent mechanism for the protection against DENV infection in mice, which has validated that STAT1 and STAT2 possess the ability to independently limit the severity of DENV pathogenesis (Perry *et al.,* 2011). Interestingly, STAT3 up-regulates various proteins involved in cell cycle progression, such as Fos, c-Myc, and cyclin D1, and enhances anti-apoptotic proteins, such as Bcl-2 and Bcl-xL (Barre *et al.,* 2003). Thus, STAT3 target proteins are important mediators for the regulation of cell cycle progression from the G_1 to S phase.

The effects of doxazosin on the biological roles of the IFN-α or IFN-γ cascade in ovarian tumors have not been fully understood. To explain the biological mechanism of cell proliferation suppression by doxazosin concerning cell cycle changes, we observed both, an inhibition of cell cycle-regulatory proteins such as cyclin D1 and CDK4, and expression of p21 in doxazosin-treated carcinoma cells. As indicated in Figure 12 (*upper* panel), the cell cycle profile of the control (un-treatment) cells were not affected significantly, except at the S phase section which was gradually down-regulated by doxazosin, IFN-α or IFN-γ treatment. Additionally, the cell cycle profile of the cells treated with doxazosin plus IFN-α or doxazosin plus IFN-γ combinations were arrested at the G_1 phase when compared to the doxazosin

single-drug treatment (*lower* panel). Interestingly, the greatest pro-apoptotic effect occurred with the combination of 20 μM of doxazosin and 10 ng/ml of IFN-α. These results indicate that the activation of apoptosis is contributed by the doxazosin plus IFN-α combination-modulated anti-tumor effect. The expression of cyclin D1 and CDK4, which are relevant for the conversion from the G_1 to S phase, was suppressed in a time-dependent manner. The present study also demonstrated that doxazosin significantly down-regulates the phosphorylation of JAK/STAT3 in SKOV-3 cells. JAK and STAT inhibitor treatment completely restored the doxazosin-reduced phosphorylation of each JAK1/2 and STAT1/3. Consistent with the results observed in the carcinoma cells, inhibitors for JAK and STAT also strongly disturbed doxazosin-reduced JAK or STAT activity (Figure 15 and Figure 16). Subsequently, doxazosin plus IFN-α or IFN-γ additively suppressed the activation of the JAK/STAT pathway, and thus affecting the subsequent downstream signaling components, which include PI3K, mTOR, 70S6K, and PKC δ activation.

In conclusion, we have shown that doxazosin can significantly inhibit the JAK/STAT signaling pathway through caspase-dependent apoptosis, as well as cell cycle arrest by IFN-α or IFN-γ stimulation. Growth suppression was associated in the ovarian tumor system with the down-regulation of the JAK/STAT activity that resulted in down-

stream signaling suppression. These findings provide the first exact information on the biological pro-apoptotic mechanisms of doxazosin in ovarian carcinoma cells. Therefore, combination therapies may be a more useful approach for more advanced ovarian cancers and recurrent patients.

CHAPTER II

DOXAZOSIN INHIBITES TUMOR GROWTH AND ANGIOGENESIS BY DECRASING VEGFR-2/Akt/mTOR SIGNALING AND VEGF AND HIF-1α EXPRESSION

Abstract

Doxazosin is an α1-adrenergic receptor blocker that also exerts antitumor effects. However, the underlying mechanisms by which it modulates PI3K/Akt intracellular signaling are poorly understood. In this study, we reveal that doxazosin functions as a novel antiangiogenic agent by inhibiting vascular endothelial growth factor (VEGF)-induced cell migration and proliferation. It also inhibited VEGF-induced capillary-like structure tube formation *in vitro*. Doxazosin inhibited the phosphorylation of VEGF receptor-2 (VEGFR-2) and downstream signaling, including phosphatidylinositol-3'-kinase (PI3K), Akt, 3-phosphoinositide-dependent protein kinase 1 (PDK1), mammalian target of rapamycin (mTOR), and hypoxia-inducible factor 1α (HIF-1α). However, it had no effect on VEGF-induced extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. Furthermore, doxazosin reduced tumor growth and suppressed tumor vascularization in a xenograft human ovarian cancer model. These results provide evidence that doxazosin functions in the endothelial cell system to modulate angiogenesis by inhibiting Akt and mTOR phosphorylation and interacting with VEGFR-2.

Introduction

Angiogenesis is an important but complex process that occurs during endothelial cell development, growth, and movement, as well as wound healing and endothelial cell-mediated degradation of the extracellular matrix. Multistep angiogenesis is vital for cell division and metastasis in most solid tumors. Angiogenesis, the constitution of new blood vessels forming from pre-existing vessels, occurs during embryonic development as well as invasive tumor growth and tumor pathogenesis (Folkman *et al.,* 1992; Risau *et al.,* 1997). Angiogenesis is activated by various signaling molecules and growth factors, including basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF). VEGF is a major modulator of endothelial cell function, such as blood vessel formation during embryonic development, and plays a vital role in the proliferation, migration, and invasion of vascular endothelial cells (Ferrara *et al.,* 2002). Angiogenesis is initiated by growth factors such as VEGF and is a potential treatment for vascular injuries (Yoshiji *et al.,* 1996; Gavin *et al.,* 2004; Kraus *et al.,* 2004). During cancer, endothelial cell activity plays an essential role in modulating various vascular physiological and pathological functions. Although VEGF receptor 1 (VEGFR-1)

and VEGFR-2 are structurally very similar, they have different biological roles. For example, VEGFR-1 negatively regulates embryonic vasculogenesis and stimulates tumor angiogenesis by activating macrophages, whereas VEGFR-2 is predominantly responsible for both tumor angiogenesis and embryonic vasculogenesis (Sawano *et al.*, 1996; Ferrara *et al.,* 2004; Takahashi *et al.,* 2011; Shibuya *et al.,* 2013) VEGFR-2 also plays a pivotal role in the activation of downstream components that are responsible for proliferation, including endothelial cell invasion, migration, differentiation, and embryonic angiogenesis (Breier *et al.,* 2000; Ferrara *et al.,* 2000; Meyer *et al.,* 2003) in contrast, VEGFR-1 has no role in endothelial cell proliferation (Meyer *et al.,* 2004).

Doxazosin functions as an α1-adrenergic receptor blocker (Kirby *et al.,* 1997). It is used to treat patients with benign prostatic hyperplasia, a noncancerous enlargement of the prostate gland, because α1-blockers relax the smooth muscles surrounding the prostate, easing urine flow and decreasing bladder outlet obstruction (Benning *et al.,* 2002; Tahmatzopoulos *et al.,* 2004). Some previous studies also reported that α1-adrenoceptor antagonists stimulate apoptotic cell death via transforming growth factor-β1 (TGF-β1) signaling and the activation of clear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha (IκBα)

in prostate tumor cells (Benning *et al.,* 2002; Partin *et al.,* 2003). Doxazosin also stimulates apoptotic cell death in response to abnormal cell-matrix interactions (Keledjian *et al.*, 2003). Garrison and Kyprianou reported that specific caspase-8 inhibitors could block doxazosin-induced apoptotic cell death in benign prostate cells (Garrison *et al.,* 2006). Nevertheless, the biological roles of these doxazosin-regulated processes, as well as molecular mechanism behind the anti-angiogenic effects of doxazosin, remain poorly understood.

Endothelial cells play a vital role in regulating various vascular biological effects and related diseases, including tumor growth and maintenance. Most studies on angiogenesis have focused on endothelial cell proliferation, migration, and capillary-like tubule formation. In the current study, we investigated the anti-angiogenic effects of doxazosin in tumors using *in vitro* human umbilical vein endothelial cell (HUVEC) and *in vivo* model systems. We also identified the molecular pathways responsible for Akt- and mammalian target of rapamycin (mTOR)-dependent endothelial cell growth during tumorigenesis. Doxazosin inhibited the phosphorylation of signaling modulators downstream of phosphatidylinositol-3' -kinase (PI3K) including Akt, phosphoinositide-dependent protein kinase 1 (PDK1), and mTOR by interacting directly with VEGFR-2. Therefore, this interaction results in potent anti-

angiogenic and antitumor effects. The current findings suggest that doxazosin plays a vital role in regulating cellular angiogenesis.

Materials and methods

Cell lines, drug reagents and antibodies

Human ovarian cancer cell lines (SKOV-3 and OVCAR-3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA), were grown in DMEM (Life Technologies, Gaithersburg, MD) medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 units/ml) at 37℃ in a humidified 5% CO_2 incubator. Primary HUVECs (Clonetics, San Diego, CA) were grown on 0.3% gelatin-coated dishes (Sigma, St. Louis, MO) using EGM-2 Bullekit medium (Clonetics). Rapamycin was purchased from Cell Signaling (Berverly, MA). Doxazosin and other chemicals were purchased from Sigma (St. Louis, MO). The following primary antibodies were used in this study: anti-phospho-VEGFR-2 (Y1175), anti-VEGFR-2, anti-phospho-PI3K, anti-PI3K, anti-phospho-Akt, anti-Akt, anti-HIF-1α, anti-phospho-ERK1/2, anti-ERK1/2, anti-PCNA(Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-PDK1, anti-PDK1, anti-phospho-4E-BP1, anti-4E-BP1, anti-phospho-mTOR, anti-mTOR, anti-phospho-p70S6K, anti-p70S6K, anti-PCNA, anti-Cyclin D1, anti-survivin, anti-cleaved Caspase3(Asp175) (all from Cell Signaling Technology), anti-CD31, anti-phospho-STAT3(Tyr705) (Abcam, Cambridge,

UK), anti-$VEGF_{165}$ (Ab-1; Oncogene, Cambridge, MA) and anti-β-actin (Sigma).

[^{3}H]thymidine incorporation analysis

[^{3}H]thymidine incorporation was assessed as described previously (Meng *et al.* 2006; Xia *et al.*, 2007). Briefly, HUVECs were plated into gelatin-coated plates at a density of 1.6×10^4 cells/well in DMEM containing 10% FBS and 1% penicillin/streptomycin on day 0. After 18 h, the cells were rinsed twice with M199 and then incubated in M199 containing 1% FBS for 6 h. Cells were first incubated with doxazosin, and were then induced with VEGF (10 ng/ml, R&D Systems, Minneapolis, MN) for 24 h in M199 containing 1% FBS. [^{3}H]thymidine (0.5 Ci/ml; Amersham, Arlington, IL) was added 4 h prior to analysis. High-molecular-mass compounds with [^{3}H]-radioactivity were then precipitated using 10% trichloroacetic acid for 1 h at 4℃. Cells were solubilized in 0.2 N NaOH containing 0.1% sodium dodecyl sulfate (SDS), and [^{3}H]thymidine incorporation was calculated using a liquid scintillation counter (Beckman Coulter, Franklin Lakes, NJ).

Cell migration analysis

Twenty-four well Transwell chambers (8.0 μm pore size; Costar, New York, NY) were used to assay migration and

invasion (Meng *et al.*, 2006; Xia *et al.,* 2007). For migration analyses, the lower surface of a filter was coated with 10 g/ml of gelatin overnight. M199 containing 1% FBS and 10 ng/ml VEGF was added to the lower wells. Cells were harvested by trypsinization and washed. Next, 1.3 × 10^5 cells were resuspended in 100 ㎕ of fresh DMEM, added to the upper chamber, and incubated at 37℃ for 24 h. Cells that had migrated to the lower chamber were fixed with methanol, and stained with 10 mg/ml H&E. Cells that remained on the surface of the upper filter were removed by wiping with a cotton swab. Cell migration was then quantified by counting the number of stained cells in five random areas of each membrane.

In vitro tube formation analysis

Growth factor-reduced Matrigel was added to a 24-well plate and polymerized for 30 min at 37℃ (Xia *et al.*, 2007). Untreated, mock-treated, or doxazosin-treated HUVECs (3.3 × 10^5 cells/well) were then added to the surface of the Matrigel. The seeded cells were incubated for 48 h with or without 10 ng/ml of VEGF in M199 containing 1% FBS. Images were then captured at 40× magnification after washing. The length of the formed tubes was measured using an inverted microscope equipped with a digital CCD camera and ImageLab software (MCM Design, Hillerød, Denmark). The control sample

(VEGF-induced control) was defined as 100% tube formation, and the percent increase or decrease in tube formation relative to the control was measured for each sample.

PI3K activity analysis

In vitro kinase assays were performed as described previously (Arbiser *et al.,* 2007; Duronio *et al.,* 1997; Lee *et al.,* 1999). Briefly, cells were seeded at a density of 1.4×10^6 cells/well. After an overnight incubation, the cells were treated with various concentrations of doxazosin for 6 h and then lysed in 1% NP-40 lysis buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM sodium orthovanadate. After the removal of insoluble materials by centrifugation, the supernatants were incubated at 4℃ for 1 h with anti-p85 antibodies, followed by protein A-agarose beads for an additional 1 h at 4℃. The resulting immunoprecipitates were incubated in a kinase reaction buffer mixture containing 200 μg/ml phosphatidylinositol 3-phosphate and 2 μCi of [-^{32}P] ATP for 15 min at 37° C. The reaction products were developed using autoradiography, and the radioactive lipids were quantified using liquid scintillation counting.

Western blotting analysis

Cells were rinsed with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer supplied with protease inhibitors. The concentration of protein samples was then evaluated using a Bradford protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded onto 8-12% SDS-polyacrylamide gel electrophoresis (PAGE) gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). After blocking, the membranes were incubated at room temperature with primary antibodies for 1 h. They were then rinsed three times in wash buffer, followed by incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein bands were developed using an enhanced chemiluminescence (ECL) detection system. Protein lysates were prepared by homogenizing the frozen tumor tissues. Protein quantitation and immunoblotting were then performed as described above with 30 μg protein/sample.

Xenograft mouse model

Specific pathogen-free BALB/c-nu/nu mice (5~6 weeks old) were purchased from Orientbio (Sungnam, Korea). SKOV-3 cells (1.0×10^6) were injected subcutaneously with Matrigel (BD Bioscience, MA) into the left flank region of each nude mouse.

Tumor sizes were measured by caliper measurements 3 times every week. On day 12, per oral treatment of 3 mg/kg of doxazosin was done and repeated 5 times per week for 24 days. Mice were sacrificed one day after the final treatment and tumors were then excised and stored at −80℃ for further study. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Research Institute of the National Cancer Center.

Immunohistochemistry

Tumor samples were collected from mouse xenografts and fixed in 10% neutral-buffered formalin (Sigma-Aldrich). Slides were then stained using H&E (Sigma-Aldrich) according to the manufacturer's instructions. For immunohistochemistry, paraffin-embedded ovarian tumor tissues were serially sectioned into 5 μm slices. The prepared slides were then deparaffinized, rehydrated in xylene and graded alcohols, and then rinsed in PBS. The slides were also incubated in 5% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity. Sections were incubated with a saturating concentration of anti-mouse CD31 (platelet-derived endothelial cell adhesion molecule; PECAM-1) (Abcam) antibody overnight at 4℃, followed by a alkaline phosphatase (AP)-conjugated secondary antibody at room temperature for

1 h. Microvessel density (MVD) was quantified in five randomly selected individual tumor fields (at 40× magnification) per sample, and the number of microvessels was counted under a high-powered microscope (400× magnification).

Statistical analysis

Results were analyzed statistically using Student's *t*-test for comparisons between two groups. Data are presented as the means ± SDs, or SEM for triplicate experiments. Statistical significance was defined as $P<0.05$. Values with 95% confidence ($P<0.05$) are depicted with an asterisk (*) on each graph. Statistical significance was defined as $P<0.01$. Values with 99% confidence ($P<0.01$) are depicted with an asterisk (**) on each graph.

Results

Doxazosin significantly suppressed VEGF-induced cell proliferation, migration, and capillary-like tubule formation in HUVECs

Physiological activity of endothelial cell plays a vital role in regulating various vascular biological functions and diseases, including tumor growth and maintenance. The detailed molecular mechanism on doxazosin effects in endothelial cells during tumor growth has not been yet. Generally, angiogenesis was investigated based on cell proliferation, migration, and capillary-like tubule formation in endothelial cells.

The migration of endothelial cells is essential during angiogenesis. Therefore, we explored whether doxazosin modulated the effects of VEGF on cell migration using a modified Boyden Transwell chamber system in HUVECs. As expected, VEGF promoted the migration of doxazosin-treated cells compared to control cells. Doxazosin inhibited migration in a dose-dependent manner, and the maximal effect was seen at a concentration of 20 μM (Figure 24). No migration was observed after 24 h of treatment with increasing concentrations (0-25 μM) of doxazosin (data not shown). These results suggest that VEGF-stimulated endothelial cell migration and angiogenesis might be inhibited specifically by doxazosin.

The *in vitro* anti-angiogenic activity of doxazosin was analyzed by assessing its effects on the VEGF-stimulated proliferation of endothelial cells using [^{3}H]thymidine incorporation. Doxazosin inhibited VEGF-induced HUVEC DNA synthesis significantly (Figure 25). This anti-proliferative effect was not due to the cytotoxicity of doxazosin in endothelial cells, since doxazosin had no effect on the viability of HUVECs, as assessed using Trypan Blue exclusion (data not shown). These results suggest that doxazosin regulates angiostasis and potently inhibits VEGF-induced pivotal events during angiogenesis, including endothelial cell proliferation and migration *in vitro*.

We next assessed the anti-angiogenic effects of doxazosin on VEGF-induced capillary-like tubule formation using HUVECs grown on Matrigel *in vitro*. As shown in Figure 26, treatment with VEGF increased the formation of extended, strong capillary-like tubular structures compared with control cells. In contrast, doxazosin treatment abrogated VEGF-induced capillary-like tubule formation. Collectively, these results strongly suggest that doxazosin specifically regulates VEGF-induced angiogenesis *in vitro*.

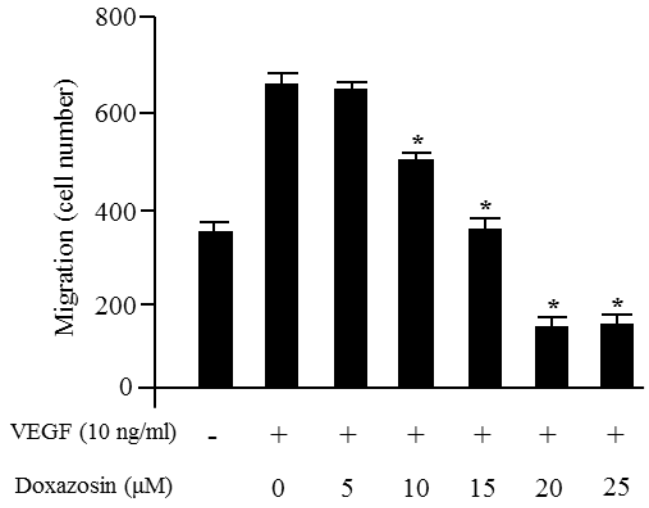


Figure 24. Doxazosin inhibited migration in a dose-dependent manner.

To address whether doxazosin-treatedcontrols the effects of VEGF on endothelial cell migration, they were assessed using the Boyden chamber for the migration assay system. Cells were plated in the top chamber of the Boyden Transwell chamber (pore size; 8 μm). All cells were treated with increasing concentrations of doxazosin, fixed, and then stained with H & E. The numbers of the migrated cells were calculated under a light microscope. Three independent experiments were assayed in triplicate. Data are presented as the mean±SD. *P<0.05 versus control group.

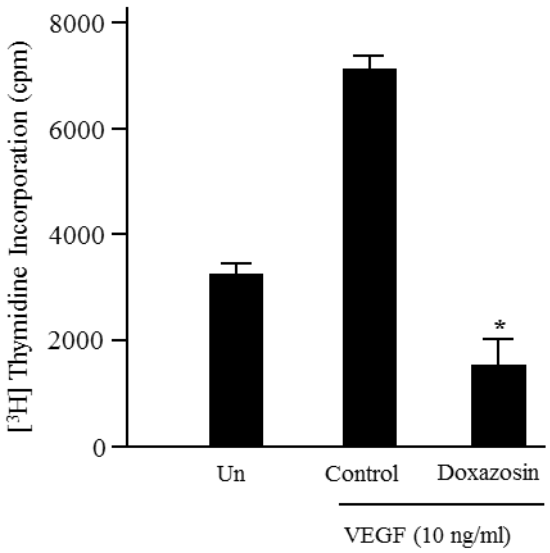


Figure 25. Treatment of doxazosin decreases angiogenic activity in endothelial cell proliferation.

The c.p.m. value of [^{3}H]thymidine was evaluated using a liquid scintillation counter. The data are the mean±SD of three independent experiments. Data are presented as the mean±SD. *P<0.05 versus control group.

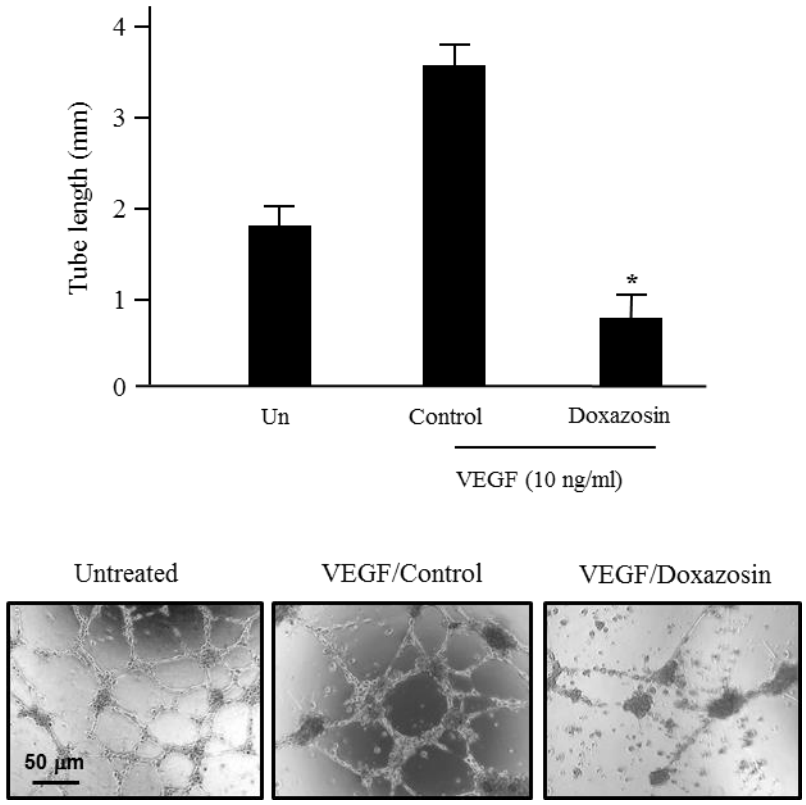


Figure 26. Treatment of doxazosin decreases angiogenic activity in endothelial cell tube formation.

After treatment with doxazosin, they were grown on growth factor-reduced Matrigel. Cells were incubated with or without 10 ng/ml of VEGF. The formation of tubular-like structures was monitored by the inverted microscope. Tube lengths were quantified and expressed as mean±SD. Experiments were repeated three times and a representative result was shown. *P<0.05 compared to control.

Doxazosin suppresses PI3K and Akt phosphorylation in a concentration-dependent manner

The phosphorylation of PI3K/Akt is an essential process for signaling pathways in tumor angiogenesis. Akt plays a vital role as a down-stream messenger of PI3K and is also controlled by several growth factors, such as insulin-like growth factor-1 (IGF-1), and transforming growth factor-β1 (TGF-β1), and epidermal growth factor (EGF). Therefore, we evaluated whether doxazosin decreased PI3K and Akt phosphorylation in SKOV-3 and OVCAR-3 (data not shown) ovarian carcinoma cells. Lysates from SKOV-3 cells treated with various concentrations (0~25 μM) of doxazosin were analyzed by Western blot analysis, which observed that the VEGF-induced phosphorylation of PI3K and Akt that played an important role in VEGF-stimulated angiogenesis. As shown in Figure 27 and Figure 28, the activity of PI3K-treated with various concentrations of doxazosin was inhibited a dose-dependent manner, the maximum effect was observed with 20 μM. Consistent with this result, 20 μM doxazosin also decreased PI3K phosphorylation significantly. Akt is an essential downstream component of PI3K signaling. As expected, doxazosin gradually reduced Akt phosphorylation in a dose-dependent manner (Figure 29). Similarly, doxazosin also lowered Akt phosphorylation in HUVECs (data not shown).

Collectively, these results suggest that PI3K/Akt-dependent signaling cascades play important roles in the effects of doxazosin in endothelial cells. The magnitude of the observed effects was comparable to those exerted by well-known PI3K and mTOR inhibitors such as wortmannin and rapamycin, respectively.

As expected, doxazosin conspicuously decreased the phosphorylation of Akt (Ser473) (Figure 30) and the phospho rylation of eukaryotic translation initiation factor 4E-BP1 (Tyr37/46) (Figure 31), as well as the phosphorylation of PDK1 (Ser241) (Figure 32), one of the best- characterized targets of the mTOR. Subsequently, when doxazosin was co-treated with rapamycin, an inhibitor of mTOR, p-4E-BP1 was inactivated. These results provide evidence to support our hypothesis that doxazosin inhibits PI3K/Akt activity.

We next assessed the effects of doxazosin on downstream mediators of the PI3K/Akt pathways. Doxazosin dramatically diminished the phosphorylation of mTOR at Ser-2448 and p70S6K at Thr-421 (Figure 32). Therefore, we next determined whether doxazosin led to VEGF-induced activation of the Akt/PDK1/mTOR complex. As shown in Figure 33, doxazosin decreased the VEGF-induced phosphorylation of Akt (Ser-473 and Thr-308), PDK1 (Ser-241), and mTOR (Ser-2448), but had no effect on the phosphorylation of

ERK1/2. These results strongly suggest that doxazosin decreases VEGF-dependent Akt, PDK1, and mTOR phospho rylation.

PI3K/Akt signaling not only mediates VEGF-induced cell proliferation and migration, but also the expression of VEGF and HIF-1α (Tahmatzopoulos *et al.,* 2004). Consistent with these findings, doxazosin treatment abrogated VEGF and HIF-1α expression completely. These results suggest that VEGF-induced protein activation is inhibited specifically by doxazosin in HUVECs (Figure 34) and ovarian carcinoma cells (Figure 35). Collectively, these observations suggest that doxazosin inhibits the autocrine effects of VEGF in endothelial cells, exerts direct antiangiogenic effects, and inhibits tumor growth and metastasis.

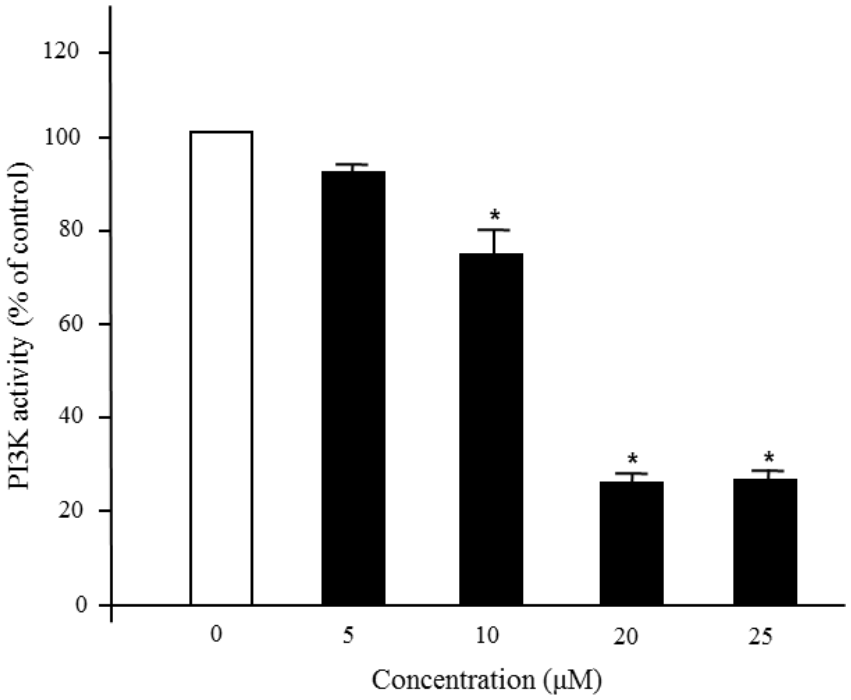


Figure 27. Doxazosin down-regulates PI3K activity (From Park *et al.*, 2014).

The inhibitory effect on PI3K activity in the presence of doxazosin was measured using the *in vitro* PI3 kinase assay system. *P<0.05 compared to control group.

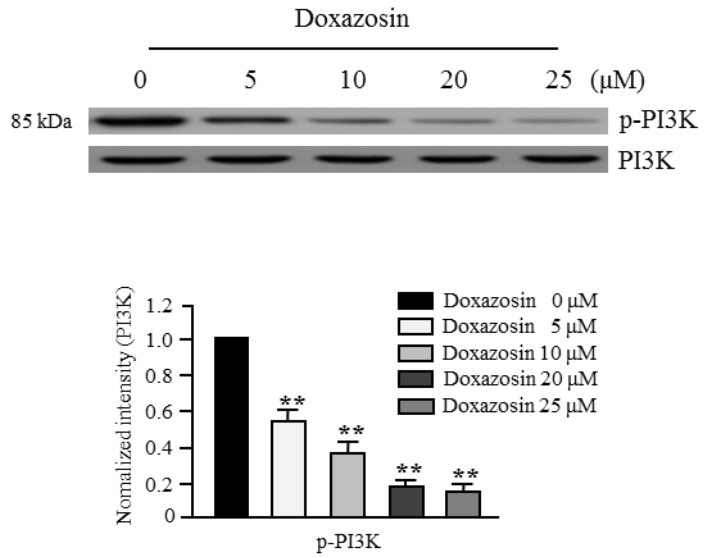


Figure 28. Doxazosin down-regulates PI3K phosphorylation.

Cells were treated with increasing concentrations of doxazosin, harvested, and detected to immunoblotting for the indicated dose proteins. PI3K was used to verify the equal loading of the samples. **P<0.01 compared to control.

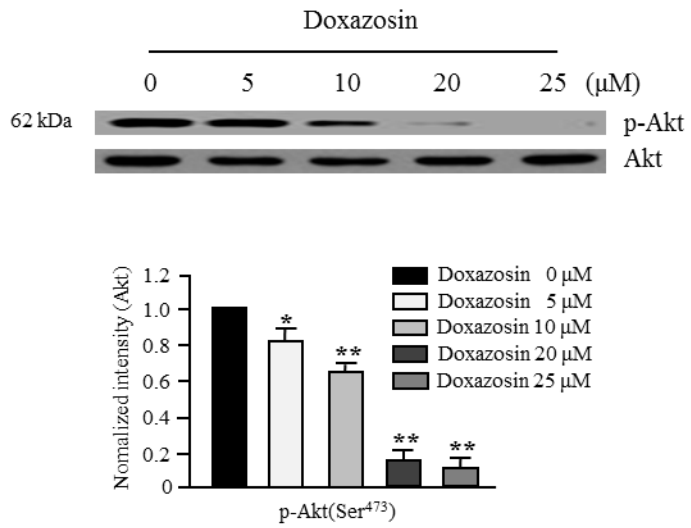


Figure 29. Doxazosin down-regulates Akt phosphorylation on dose dependent manner.

Cells were treated with various concentrations of doxazosin. Phosphorylation of Akt was evaluated using Western blot analysis. *P<0.05 compared to control; **P<0.01 compared to control.

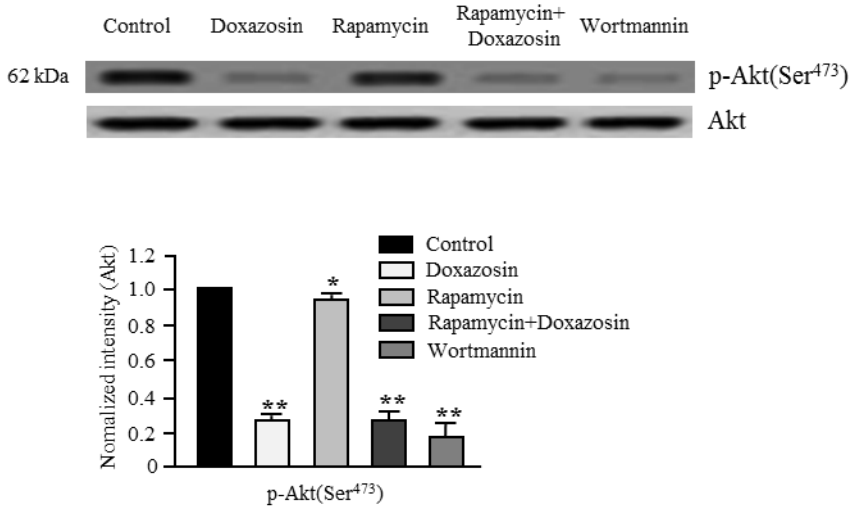


Figure 30. Doxazosin down-regulates Akt phosphorylation.

Cells were treated with doxazosin, rapamycin as an mTOR inhibitor, rapamycin plus doxazosin, and wortmannin as a PI3K inhibitor, respectively. Immunoblotting for un-phosphorylated Akt was used as a loading control. *P<0.05 compared to control; **P<0.01 compared to control.

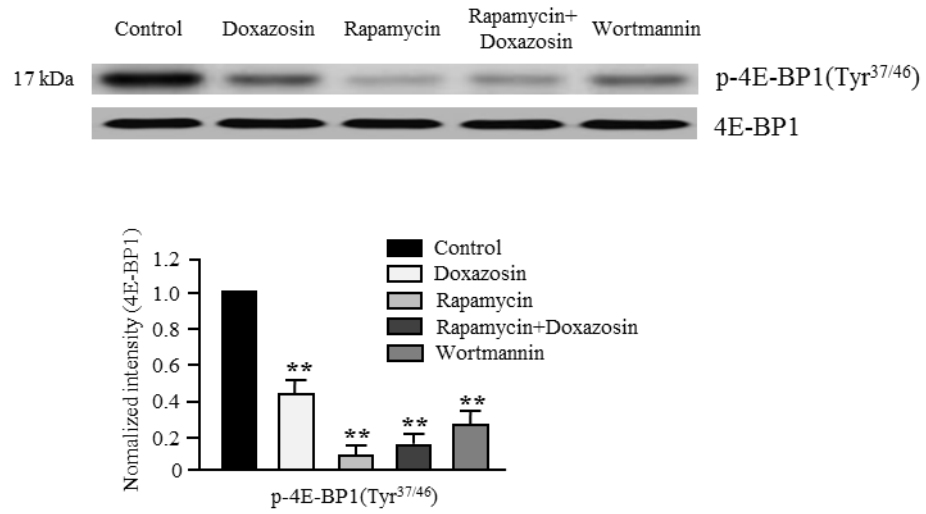


Figure 31. Doxazosin down-regulates 4E-BP1 phosphorylation. Cells were treated with doxazosin, rapamycin as an mTOR inhibitor, rapamycin plus doxazosin, and wortmannin as a PI3K inhibitor, respectively. Immunoblotting for un-phosphorylated 4E-BP1 was used as a loading control. **P<0.01 compared to control.

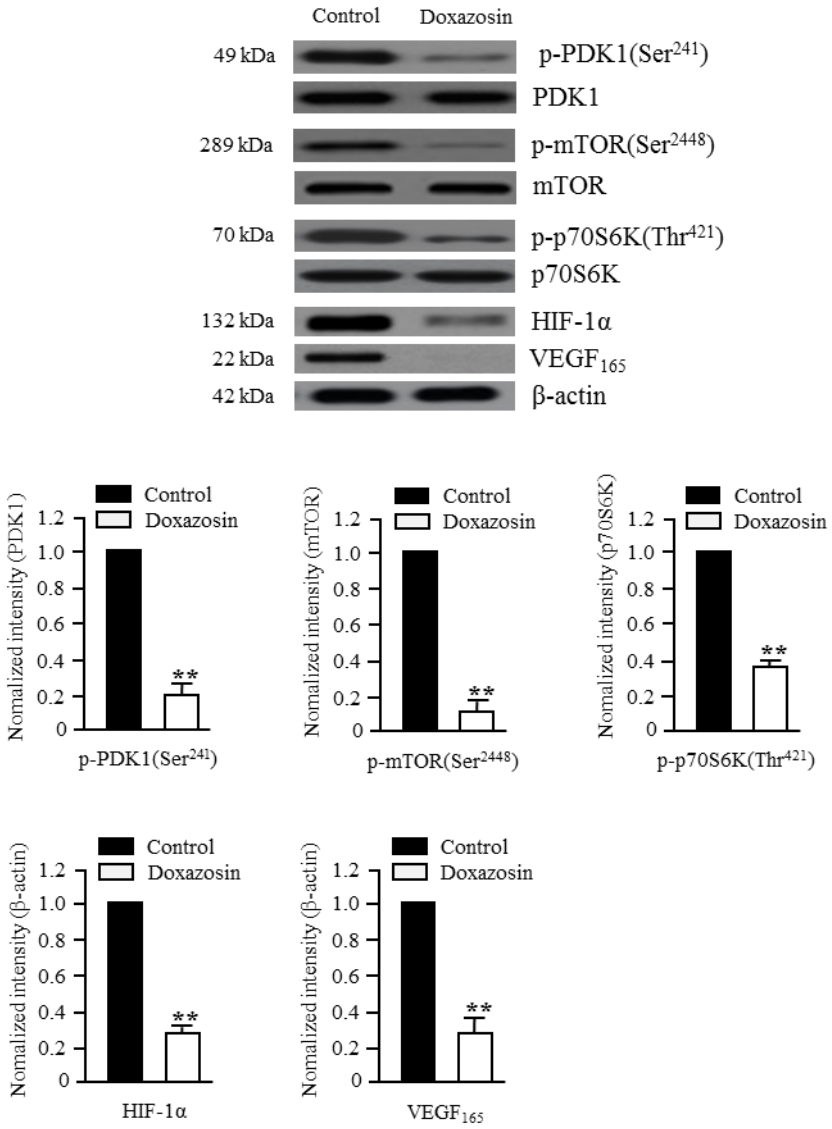
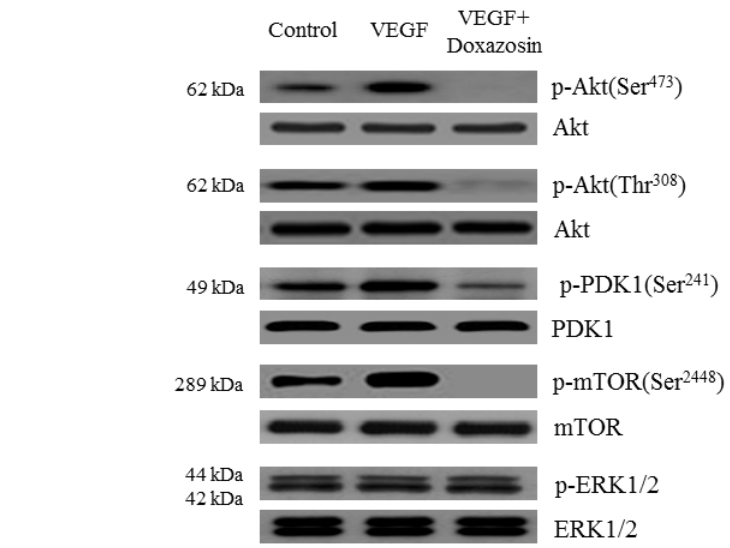


Figure 32. Doxazosin down-regulates mTOR target pathway phosphorylation and HIF-1α and $VEGF_{165}$ expression.

After doxazosin treatment, cells were collected for Western blot analysis for the phosphorylated and non-phosphorylated proteins (PDK1, mTOR, 70S6K). β-actin served as a loading control for HIF-1α and $VEGF_{165}$. **P<0.01 compared to control.


Control
VEGF
VEGF+
Doxazosin
62 kDa
p-Akt(Ser473)
Akt
62 kDa
p-Akt(Thr308)
Akt
49 kDa
p-PDK1(Ser241)
PDK1
289 kDa
p-mTOR(Ser2448)
mTOR
44 kDa
42 kDa
p-ERK1/2
ERK1/2

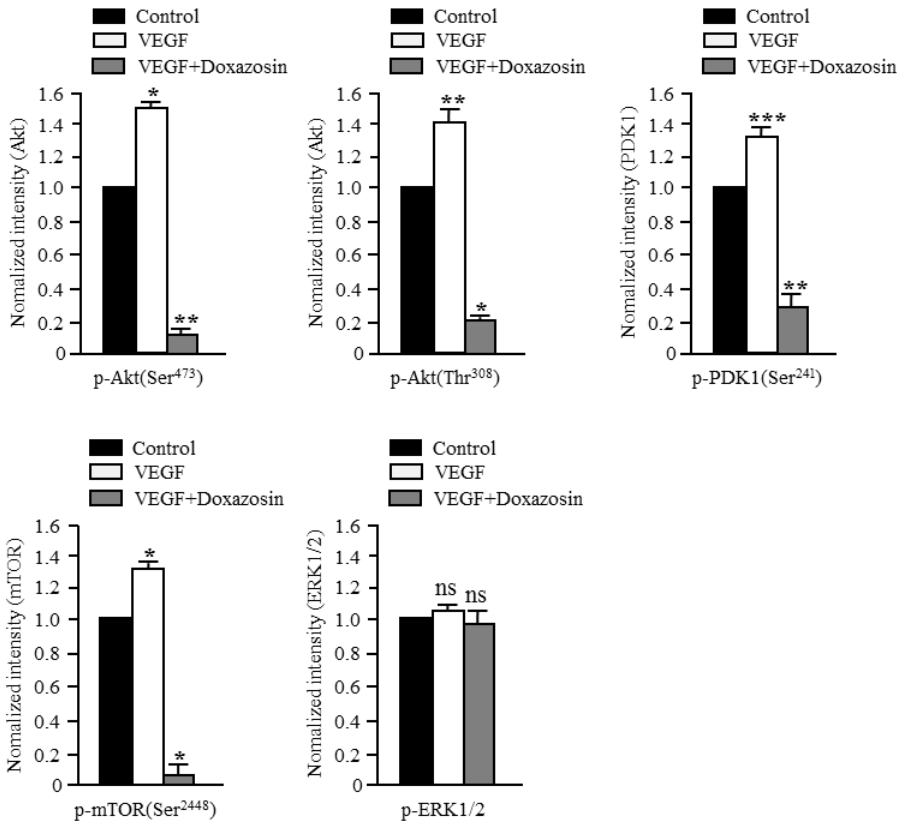

Control
VEGF
VEGF+Doxazosin
Nomalized intensity (Akt)
p-Akt(Ser473)
Nomalized intensity (Akt)
p-Akt(Thr308)
Nomalized intensity (PDK1)
p-PDK1(Ser241)
Nomalized intensity (mTOR)
p-mTOR(Ser2448)
Nomalized intensity (ERK1/2)
ns
p-ERK1/2

Figure 33. Doxazosin suppressed VEGF-dependent Akt, PDK1, and mTOR phosphorylation.

After treatment with doxazosin, cells were harvested for immunoblotting analysis with antibodies specific for phosphorylated or non-phosphorylated proteins indicated for Akt phosphorylation, and the phosphorylation of down-stream components, including PDK1, mTOR, and ERK1/2. *P<0.05 compared to control; **P<0.01 compared to control.

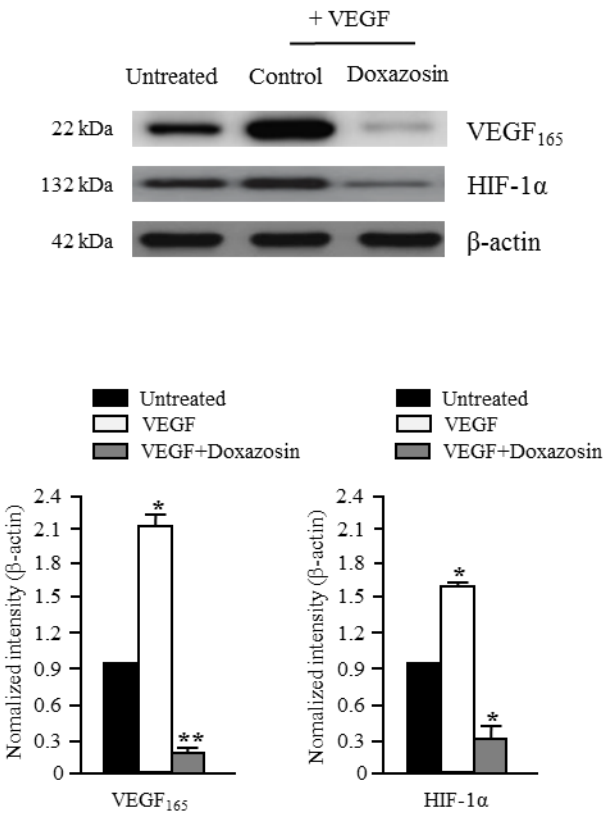


Figure 34. Doxazosin inhibits VEGF-induced HIF-1α and VEGF in HUVECs.

Cells were incubated with 10 ng/ml VEGF and then control-treated or treated with doxazosin in HUVECs. HIF-1α and VEGF protein expression was then developed by Western blot analysis. Three independent experiments were conducted in triplicate. *P<0.05 compared to control; **P<0.01 compared to control.

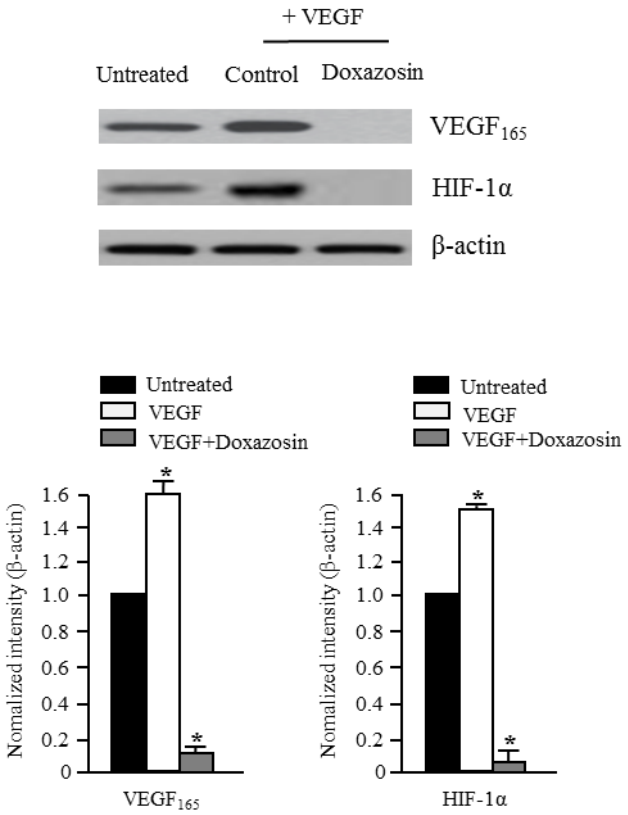


Figure 35. Doxazosin inhibits VEGF-induced HIF-1 α and VEGF in ovarian carcinoma cells.

SKOV-3 cells were incubated with 10 ng/ml VEGF and then control-treated or treated with doxazosin in ovarian carcinoma cells. HIF-1 α and VEGF protein expression was then developed by Western blot analysis. Three independent experiments were conducted in triplicate. *P<0.05 compared to control.

Doxazosin inhibits VEGF-induced VEGFR-2 phosphorylation and VEGFR-2-dependent transcription

VEGFR-2 is a key signal transducer during VEGF-induced endothelial cell vascular development and pathological angiogenesis. We next assessed the effect of doxazosin on VEGF-induced VEGFR-2 phosphorylation in SKOV-3 cells and HUVECs (data not shown) to assess the biological and functional relevance of the direct relationship between VEGFR-2 and doxazosin. As shown in Figure 36, treatment with doxazosin suppressed VEGF-induced VEGFR-2 phosphorylation in SKOV-3 carcinoma cells. The effect of doxazosin on VEGFR-2 transcription was then evaluated using a luciferase reporter assay system and a construct containing the VEGFR-2 promoter fused to *luciferase*. Luciferase activity decreased in response to treatment with doxazosin in a dose-dependent manner (Figure 37), suggesting that doxazosin regulates VEGFR-2 activity. These results suggest that treatment with doxazosin decreases VEGFR-2 transcriptional activity.

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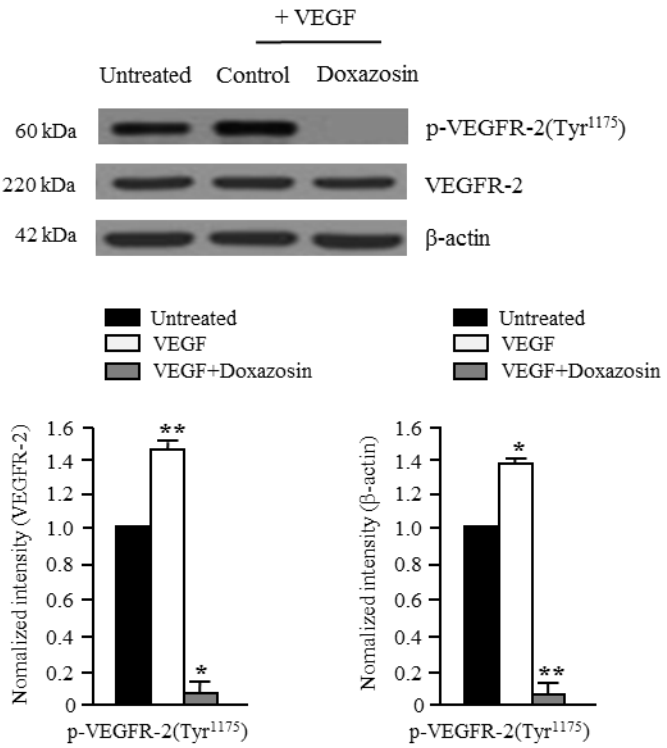

Figure 36. Doxazosin inhibits VEGF-induced VEGFR-2 phosphorylation and VEGFR-2-dependent transcription.

Cells were incubated with 10 ng/ml VEGF and then control-treated or treated with doxazosin. Total cell lysates were prepared and detected for VEGFR-2 phosphorylation levels through immnunoblot analysis. Unphosphorylated VEGFR-2 and β-actin were used as the loading control. *P<0.05 compared to control; **P<0.01 compared to control.

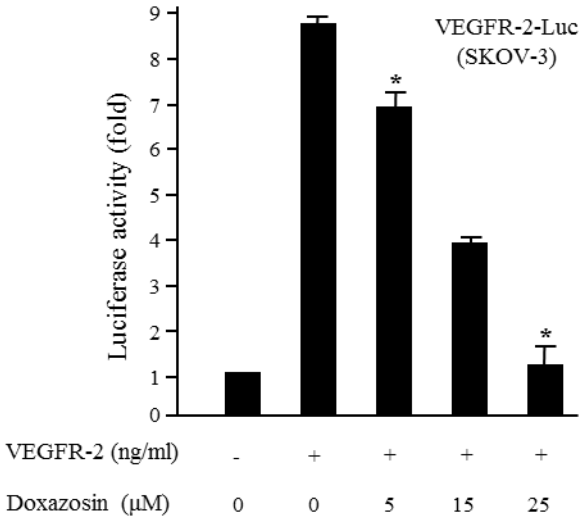

Figure 37. Suppression of VEGFR-2-dependent transcription by doxazosin.

Cells were co-transfected with 500 ng of VEGFR-2-Luc, 500 ng of a VEGFR-2 expression plasmid (pcDNA3.1/VEGFR-2), and increasing concentrations of doxazosin 0, 5, 15, and 25 μM). Assays were performed as described previously and the VEGFR-2-Luc and VEGFR-2 expression plasmid were provided by Dr. S.B. Rho (Rho *et al.,* 2012). The values are presented as the mean±SD. *P<0.05 compared to control.

Doxazosin inhibits tumor growth by antiangiogenic activity *in vivo*

We next determined whether doxazosin has direct effects on angiogenesis and tumor cell growth *in vivo*. SKOV-3 ovarian cancer cells were injected subcutaneously into nude mice (10/group), and the tumors were allowed to grow for 12 days until they reached a mean volume of 100 mm^3. The mice were treated orally with control or doxazosin, and tumor growth and morphology was evaluated every 3 days for 24 days. Doxazosin-treated tumors weighed ~75% less than those from control mice (Figure 38, *upper panel*). Hematoxylin and eosin (H&E) staining revealed that tumors in the control group were high-grade carcinoma with an irregular cell distribution. In contrast, doxazosin-treated tumors exhibited large areas of late-apoptotic or necrotic cells (Figure 38, *lower panel*). Tumors treated with doxazosin had significantly reduced cell proliferation protein (including cyclin D1, survivin) and enhanced apoptotic cell death proteins, as determined by westernblotting for PCNA and PARP. In addition, VEGF expression and the phosphorylation of VEGFR-2 were decreased in doxazosin treated tumors (Figure 39). Moreover, anti-CD31 (PECAM-1) staining in the endothelial cells of doxazosin-treated mice revealed a ~2.5-fold reduced number of blood vessels compared to the control (Figure 40). These

results suggested that doxazosin treatment is capable of decreasing tumor growth by suppressing angiogenesis *in vivo*.

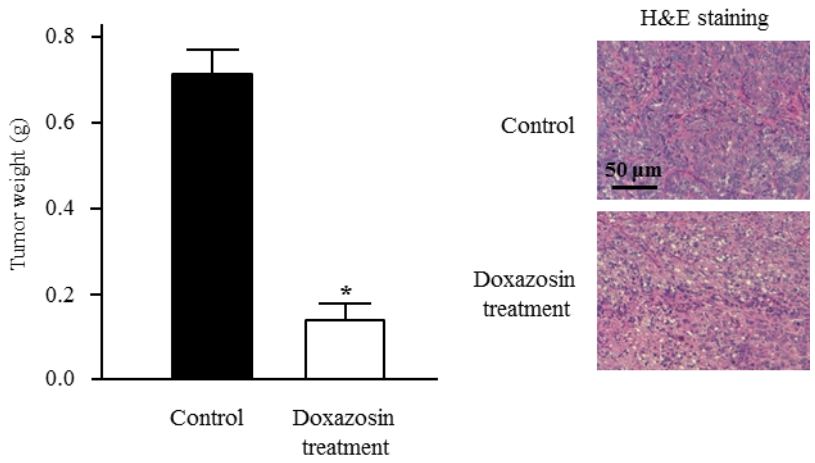

Figure 38. Doxazosin suppressed tumor growth .

SKOV-3 ovarian carcinoma cells injected subcutaneously in nude mice. Mean tumor weight was monitored at the end of the experiments. *P <0.05 compared to control group.

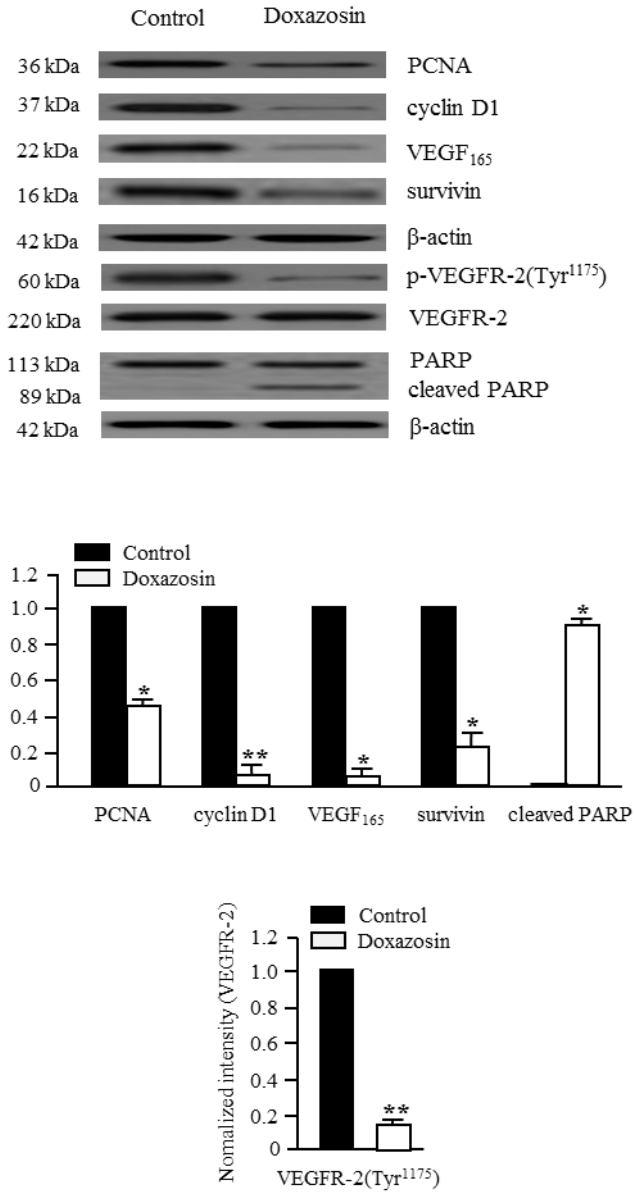

Figure 39. Doxazosin suppressed tumor growth related protein.

Tumor tissues were evaluated using immunoblot analysis with indicated cell proliferate protein PCNA, cyclin D1, VEGF, survivin, p-VEGFR-2 and cell death protein PARP antibodies. *P<0.05 compared to control; **P <0.01 compared to control.

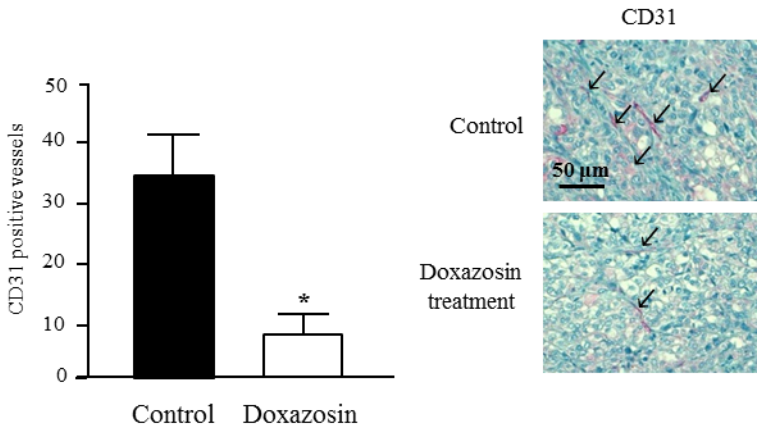

Figure 40. Doxazosin suppressed tumor growth by inhibiting angiogenesis *in vivo* .

CD31 staining was performed to visualize the blood vessels (arrows) in tumor tissues. Frozen sections of the tumors were stained for endothelial cells using an anti-CD31 (PECAM-1) antibody. *P<0.05 compared to control group.

Discussion

In this study, we identified a novel biological function of doxazosin as a powerful anti-angiogenic modulator that functions via the Akt/mTOR signaling pathway. Quinazoline-derived α1-adrenoceptor antagonists exert anticancer activity in prostate cancer. In addition, the induction of apoptotic cell death and inhibition of angiogenesis by doxazosin have been reported widely (Ferrara *et al.,* 2000; Meyer *et al.,* 2003; Meyer *et al.,* 2004; Kirby *et al.,* 1997; Benning *et al.,* 2002). Nevertheless, its precise molecular effects have not yet been reported in ovarian cancer. The phosphorylation of VEGFR-2 plays an important role in promoting VEGF-induced tumor angiogenesis. During cancer progression, angiogenesis is typically regulated by two key factors: VEGF, and HIF transcription factors. HIF proteins commonly enhance the expression of VEGF, whereas HIF-1α expression is enhanced during hypoxia (Ferrara *et al.,* 2000). Activated HIF-1α promotes the proliferation, migration, and invasion of endothelial cells, as well as tube formation in cancer. The upregulation of VEGF is commonly seen in most aggressive solid tumors, including ovarian, colon, lung, and uterine tumors; it is closely associated with tumor progression and a poor prognosis (Partin *et al.,* 2003; Keledjian *et al.,* 2003; Garrison

et al., 2006; Jiang *et al.,* 2008). However, the current study revealed that doxazosin could decrease both HIF-1α and VEGF expression during angiogenesis (Figures 32, 34 and 35). Treatment with doxazosin also suppressed VEGF-induced HUVEC proliferation significantly compared to the VEGF-treated control (Figure 21). Subsequently, we confirmed that doxazosin dramatically reduced the phosphorylation of PI3K and mTOR, as well as VEGFR-2 protein expression (Figures. 28, 32, and 36). These results clearly suggest that doxazosin inhibits endothelial cell angiogenesis during tumorigenesis.

PI3K/Akt signaling plays a vital role in the biological functions of human malignant tumors. Recent studies have reported that suppressing PI3K might be beneficial to inhibit tumor angiogenesis (Tahmatzopoulos *et al.,* 2004; Olson *et al.,* 1994; Paley *et al.,* 1997; Ishigami *et al.,* 1998). PI3K also regulates the signaling pathways that are involved in cell growth and/or apoptotic cell death (Jiang *et al.,* 2008). Therefore, the anti-apoptotic events that are modulated by Akt begin with PI3K. Akt is activated by PI3K, which recruits Akt to the cell membrane and allows its phosphorylation by PDK1 (Ohta *et al.,* 1999). The current study revealed that doxazosin decreased the phosphorylation of both PDK1 and Akt (Figures 29 and 32). In addition, doxazosin decreased the VEGF-induced phosphorylation of the mTOR signaling mediator

p70S6K and 4E-BP1. In contrast, VEGF-induced ERK1/2 phosphorylation was unaffected by doxazosin (Figure 33). Collectively, these results suggest that doxazosin could block VEGFR-2 transcriptional activity by suppressing VEGFR-2 phosphorylation via VEGF-dependent Akt/mTOR signaling.

In summary, our data demonstrate that doxazosin could suppress endothelial cell functions, including cell proliferation, migration, invasion, and capillary-like tubule formation, by suppressing VEGFR-2 phosphorylation and inhibiting Akt/mTOR signaling. It also suppresses the expression of HIF-1α and VEGF in ovarian carcinoma cells. These data supply additional evidence to support a role for doxazosin as a potent modulator of the biological and physiological mechanisms relevant to angiogenesis. The potential of doxazosin as an ovarian cancer treatment should be assessed in future studies.

GENAERAL DISCUSSION AND CONCLUSION

Our studies were planned to analyze the mechanism of the effects of doxazosin on JAK/STAT signaling induced by IFN-α or IFN-γ stimulation in ovarian carcinoma cells. Interferons are multifunctional cytokines. They are major modulators of the innate and adaptive immune systems that signal through the JAK/STAT pathway by activation of STAT1/2 (Pestka *et al.,* 2004). In this study, our results strongly indicate a new crucial molecular mechanism of doxazosin, which is a key regulatory factor that can target the JAK/STAT signaling cascade. Pre-treatment with IFN-α or IFN-γ significantly increased doxazosin-induced apoptotic cell death and up-regulated doxazosin-modulated cell growth inhibition. The cleavage of PARP which can be activated by doxazosin alone was also increased by IFN-α and IFN-γ. To explain the biological mechanism of cell proliferation suppression by doxazosin in terms of cell cycle changes, we observed both inhibition of cell cycle-regulatory proteins such as cyclin D1 and CDK4 and expression of p21 in doxazosin-treated carcinoma cells. As indicated in Chapter 1, cells treated with doxazosin plus IFN-α or doxazosin plus IFN-γ were arrested in the G_1 phase. The greatest pro-apoptotic effect occurred with the combination of

doxazosin and IFN-α. These results indicate that the activation of apoptosis contributed to the anti-tumor effect of doxazosin plus IFN-α. The expression of cyclin D1 and CDK4, which are relevant for the transition from the G_1 to the S phase, was suppressed in a time-dependent manner. The present study also demonstrated that doxazosin significantly down-regulates the phosphorylation of JAK/STAT in SKOV-3 cells. JAK and STAT inhibitor treatment completely restored the doxazosin-reduced phosphorylation of JAK1/2 and STAT1/3. Consistent with the results from carcinoma cells, inhibitors of JAK and STAT also strongly inhibited doxazosin-reduced JAK and STAT activity. Also, doxazosin and IFN-α or IFN-γ additively suppressed activation of the JAK/STAT pathway, thereby affecting downstream signaling components, which include PI3K, mTOR, 70S6K, and PKCδ activation. We further identified a novel biological function of doxazosin as a powerful anti-angiogenic modulator that functions via the Akt/mTOR signaling pathway. The phosphorylation of VEGFR-2 plays an important role in promoting VEGF-induced tumor angiogenesis. Activated HIF-1α promotes the proliferation, migration, and invasion of endothelial cells, as well as tube formation in cancer. VEGF is frequently upregulated in most aggressive solid tumors, including ovarian, colon, lung, and uterine tumors, and is closely associated with tumor progression and poor prognosis (Ferrara

et al., 2000; Partin *et al.,* 2003, Keledjian *et al.,* 2003; Garrison *et al.,* 2006; Jiang *et al.,* 2008). However, the current study revealed that doxazosin decreased both HIF-1α and VEGF expression during angiogenesis. Treatment with doxazosin also significantly suppressed VEGF-induced HUVEC proliferation compared to the VEGF-treated control. Subsequently, we confirmed that doxazosin dramatically reduced the phosphorylation of PI3K and mTOR, as well as VEGFR-2 protein expression. These results clearly suggest that doxazosin inhibits angiogenesis during tumorigenesis. Recent studies suggest that suppressing PI3K might inhibit tumor angiogenesis (Tahmatzopoulos *et al.,* 2004; Olson *et al.,* 1994; Paley *et al.,* 1997; Ishigami *et al.,* 1998). Akt is activated by PI3K, which recruits Akt to the cell membrane and allows its phosphorylation by PDK1 (Ohta *et al.,* 1999). In the current study, doxazosin decreased the phosphorylation of both PDK1 and Akt. It also decreased the VEGF-induced phosphorylation of the mTOR signaling mediator p70S6K and 4E-BP1. By contrast, VEGF-induced ERK1/2 phosphorylation was not affected by doxazosin. Collectively, these results suggest that doxazosin blocked VEGFR-2 transcriptional activity by suppressing VEGFR-2 phosphorylation via VEGF-dependent Akt/mTOR signaling. These findings provide the first detailed information on the biological pro-apoptotic mechanisms of doxazosin in

ovarian carcinoma cells. Our data demonstrate that doxazosin suppressed endothelial cell functions, including cell proliferation, migration, invasion, and capillary-like tubule formation, by suppressing VEGFR-2 phosphorylation and inhibiting Akt/mTOR signaling. These data provide additional evidence to support a role for doxazosin as a potent modulator of biological and physiological mechanisms relevant to angiogenesis. Doxazosin combination therapies may be a useful approach for more advanced ovarian cancers and patients with recurrence.

국문초록

JAK/STAT 인산화 및 VEGFR-2/Akt/mTOR 신호전달 억제를 통한 독사조신의 난소암 억제 기전 규명

박미선

지도교수: 김대용

서울대학교 대학원 수의학과 수의병리학 전공

독사조신은 남성의 전립선비대증과 고혈압에 사용되는 약물로 alpha1-adrenergic receptor 에 작용하는 것으로 알려져 있으며 전립선 세포 과증식에 대한 처리시 이를 억제하는 효과를 보이는 것으로 확인된 바 있었다. 그러나 그 정확한 기전은 아직 잘 알려져 있지 않음으로, 본 연구에서는 여성의 난소암 세포주와 동물모델을 사용하여 해당 물질의 난소암 성장억제 효과를 확인하고 자 한다. 독사조신은 처리농도 및 처리시간에 따라 점차적으로 난소암 세포주의 성장억제를 유발하였으며, 이러한 효과는 독사조신, IFN-α 또는 IFN-γ 단독과 비교해 볼 때 독사조신과 IFN 을 함께 사용하는 경우에서 더욱 활성화되었다. 세포사멸과 관련있는 caspase-3 및 PARP 의 활성화 증가와 함께 세포분열과 관련있는 cyclin D1 과 CDK 4 의 감소를 확인하였다. 이러한 독사조신의 효과는 JAK 와 STAT 특이적인 억제제에 의해 방해되었다. 본 연구에서는 IFN 과 독사조신의 처리를 통해 JAK 과 STAT 의 인산화를 조절할 수 있었으며, 그 결과 JAK/STAT 신호전달체계 하위과정인 PI3K, mTOR, p70S6K 과 P

KCδ를 조절한다는 것을 알 수 있었다. 난소암 이종이식 동물모델을 사용한 실험에서는 독사조신 처리에 의해 종양 성장이 억제되는 것을 확인할 수 있었다. 독사조신에 의한 난소암 혈관신생억제에대한 효과를 확인하기 위해서 HUVECs, 난소암 세포주와 난소암 세포주 이종이식 동물모델을 이용한 연구를 수행한 연구에서 독사조신 처리는 VEGF 에의한 HUVECs 의 migration, proliferation 와 tube formation 을 억제하였다. 독사조신은 VEGF 및 HIF-1α의 발현을 억제하고, VEGFR-2 전사와 VEGFR-2 인산화를 억제하였다. VEGF 처리후, PI3K, Akt, PDK1, mTOR 의 인산화를 억제하였으나, ERK1/2 인산화는 억제하지 않았다. 그러므로 독사조신은 VEGFR-2/Akt/mTOR 의 신호 전달 체계를 통해 혈관신생을 억제하는 것으로 확인되었다. 난소암 이종이식 동물모델을 사용한 실험에서는 독사조신 처리에 의해 혈관생성이 억제되어 있었으며, VEGFR-2 의 인산화가 감소되어 있었다. 본 논문은 독사조신의 JAK/STAT 기전 인산화 억제를 통한 난소암세포 성장억제효과와 VEGFR-2 신호전달체계를 통한 혈관신생 억제효과를 지니고 있음을 처음으로 밝혔다. 위와 같은 결과를 활용한다면 난소암 항암치료에 대한 새로운 전략을 제안할 수 있다고 본다.

Keywords: 독사조신, 난소암, IFN, JAK, STAT, Akt, mTOR, VEGFR-2

Student Number: 2003-31068

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