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Tumor-promoting and pro-angiogenic effects of roxarsone via VEGFR2/PLCγ/PKC signaling

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ABSTRACT

Roxarsone is an organoarsenic feed additive used in livestock and poultry production that is released into the environment, where it poses a risk to human health. It is known to have a tumor-promoting effect that is brought about by pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and it receptors (VEGFR). However, little information is available about the other signaling molecules that could be involved. This study aims to investigate the role of PLCγ/PKC signaling in roxarsone-induced angiogenesis in a mouse B16-F10 melanoma xenograft model and rat vascular endothelial cells (ECs). Results showed treatment with 5 mg/kg and 25 mg/kg roxarsone resulted in an obvious increase in the weight and volume of B16-F10 xenografts and PLCγ/PKC phosphorylation in a dose-dependent manner in C57BL/6 mice. SU5416, a VEGFR2 inhibitor, significantly attenuated the tumor growth induced by roxarsone. Further, 1.0 μmol/L roxarsone treatment in rat ECs was observed to significantly increase the optical density rate in the MTT assay, the number of BrdU-positive cells in the proliferation assay, the migration distance in the scratch test, and the number of meshes formed in the tube formation assay. In addition, treatment with 1.0 μmol/L roxarsone was associated with significantly higher phosphorylation of PLCγ/PKC than the control treatment. U73122, a PLCγ inhibitor, was found significantly to combat the effects of 1.0 μmol/L roxarsone on the ECs. Roxarsone is capable of promoting the growth of mouse B16-F10 xenografts and tube formation in vascular ECs. Moreover, VEGFR2/PLCγ/PKC signaling may play a regulatory role in *in vivo* and in vitro roxarsone-induced angiogenesis.

1. Introduction

Roxarsone (3-nitro-4-hydroxy phenyl arsenic acid), an organic arsenic compound, is widely used as a feed additive for ensuring proper weight gain, feed efficiency and pigmentation in chicken and pigs. In fact, the aromatic organoarsenic additives such as roxarsone were banned in Europe in 1999 [1] and in the United State of Amercian in 2013 [2]. However, these compounds are still in widespread use in many developing countries (e.g., China, India, Brazil and Argentina etc.) [3–5]. Only a small amount of roxarsone is absorbed by the animal, which means that the vast majority of roxarsone is excreted unchanged via the feces [6]. Roxarsone was detected as the major arsenic species in chicken tissues. The occurrence of roxarsone in the chicken tissues is indicative of food-borne residues resulting from its administration during farming. It was strong evident that the use of arsenic-based drugs such as roxarsone contributes to dietary iAs exposure in consumers of conventionally produced chickens [7,8]. In environmental systems, previous studies have shown that its parent form in animal feces can gradually transform into dimethyl arsenic and monomethylarsonic acid, and more toxic As (V) and As (III) for many months [5,9]. Thus, the ROX residue in chicken and swine products or use of animal manure as an organic fertilizer may result in an increase in the level of arsenic in the environment or foods derived from animals, and a subsequent increase in the risk of arsenic exposure in humans. Many studies have shown that exposure to various forms of arsenic can cause serious health problems, including skin lesions, cardiovascular diseases, neuronal disorders, and cancers [10–12]. Further, in our previous work, we had demonstrated that roxarsone enhanced the growth of MCF-7 xenograft tumors [13].

Our previous work has demonstrated that a pro-angiogenic mechanism may be responsible for the tumor-promoting effect of roxarsone [13,14]. In agreement with our previous findings, it has been reported that angiogenesis plays crucial roles in the initiation of carcinogenesis and tumor progression, vascular diseases and various

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ischemic and inflammatory diseases [15,16]. Angiogenesis is regulated by a variety of factors, among which the positive regulators of angiogenesis include the family of vascular endothelial growth factors (VEGFs) and fibroblast growth factors [17]. For instance, it is well established that VEGF is capable of promoting the in vitro proliferation and migration of endothelial cells (ECs) and in vivo angiogenesis [18], and similar in vitro findings have been reported by us too [13,14]. Furthermore, VEGF secreted by a variety of tumor cells acts on ECs via the paracrine system, and this is associated with tumor angiogenesis, metastasis and poor prognosis [19–21]. In addition to VEGF, certain signaling proteins, such as the Src family tyrosine kinases, the Ras pathway proteins, phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC)γ1, are also believed to be involved in the angiogenesis-induced promotion of tumor growth [22].

The signaling enzymes required for angiogenesis have been investigated in physiological and pathophysiological conditions. For instance, a genetic study showed that PLCγ deletion was associated with deficiency in vasculogenesis and subsequent mortality during embryogenesis, which was indicative of the essential role played by PLCγ in angiogenesis [23,24]. Moreover, PLCγ1 plays a role in capillary tube formation via its effect on EC migration, while PLCγ2 plays a role in EC proliferation and survival; thus, PLCγ1 and PLCγ2 have distinct functions in the case of ECs [22]. In addition, mice lacking PLCβ3 or PLCε, members of the mammalian PLC subfamilies, exhibit marked attenuation of tumor angiogenesis and significant resistance to tumorigenesis [25,26]; thus, PLC proteins may play a crucial role in tumorigenesis via the augmentation of angiogenesis. Recent results show that Casitas B-lineage lymphoma, identified as a negative regulator of angiogenesis, prevents tumor growth by inhibiting PLCγ1 tyrosine phosphorylation and promoting its ubiquitylation [27,28]; these findings also show that PLC is a key molecule in the angiogenic switch. Based on all these findings, it is possible that the PLCγ/PKC pathway, which is present downstream of the VEGF/VEGFR signaling pathway, is important for the angiogenesis-inducing effect of roxarsone, and that it may also be involved in roxarsone-induced tumor growth.

In this study, we have investigated whether roxarsone induces angiogenesis via PLCγ/PKC signaling. To this end, we have studied the angiogenic effects of roxarsone in primary cultured rat aorta ECs and a xenograft model of cells of the melanoma cell line B16-F10 in C57BL/6 mice. We noted that inhibition of PLCγ attenuated roxarsone-induced proliferation, migration and tube formation in ECs and that roxarsone-induced tumor growth involved PLCγ/PKC signaling. Our results indicate the role of PLCγ/PKC signaling in roxarsone-induced angiogenesis.

2. Material and methods

2.1. Experimental animals, chemicals and antibodies

Male C57BL/6 mice (20 ± 2 g, 6–8 w old) and male Wistar rats (200–250 g) were purchased from the Center of Comparative Medicine, Yangzhou University, China. The animals were housed at a temperature of 22 °C and humidity of 55% ± 5% with a 12-h/12-h light/dark cycle. Food and water were provided *ad libitum*.

Roxarsone was purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA). The 1.0 mM stock solution that was obtained was dissolved in 5 mL methanol and then diluted to 50 mL with deionized water. Then, 0.1, 1.0 and 10.0 μM roxarsone working solutions were made by further diluting the stock solution with Dulbecco's modified Eagle medium (DMEM) medium.

Sodium heparin and trypsin were purchased from Sigma-Aldrich. DMEM, penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). Recombinant rat VEGF165 was purchased from Peprotech Co. (Rocky Hill, NJ, USA). The VEGFR2 inhibitor SU5416 and PLCγ inhibitor U73122 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Growth Factor Reduced Matrigel®TM Matrix was purchased from BD Biosciences (San Jose, CA, USA). Rabbit monoclonal PLCγ, phospho-PLCγ, PKC and phospho-PKC antibodies were from Cell Signaling Technology (Danvers, MA, USA). Rabbit 5-bromo-2-deoxyUridine (BrdU) and mouse monoclonal BrdU antibody were purchased from Boster Biotechnology (Wuhan, China).

2.2. Cell culture

B16-F10 melanoma cells were gifted by the Research Group of Laboratory Animals of Yangzhou University, and maintained in DMEM containing 10% FBS and penicillin/streptomycin.

The Wistar rats were anaesthetized using 2% thiopental sodium and sacrificed, and ECs were isolated from the thoracic aorta and cultured in DMEM supplemented with 15% (v/v) FBS, 100 μg/mL sodium heparin, 4 ng/mL VEGF and 100 U penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere as reported previously [14]. The cells were subcultured once they had formed a monolayer (after approximately 6 days of incubation). For the following assays, the ECs were digested with 2% trypsin, briefly centrifuged at 1000 rpm for 10 min and resuspended at the required density in DMEM.

2.3. Mouse B16-F10 xenograft studies

C57BL/6 male mice were housed at a temperature of 22 ± 2 °C and humidity of 55% ± 5% with a 12-h light/12-h dark cycle for one week of acclimatization. Then, 1×10^5 B16-F10 cells in 0.3 mL non-supplemented DMEM were injected subcutaneously into the external surface of the right ribs of the mice. The animals with visible tumors after 7 days or so were intragastrically administered PBS, roxarsone (1 mg/kg, 5 mg/kg or 25 mg/kg), 10 mg/kg SU5416 or 10 mg/kg SU5416 plus 5 mg/kg roxarsone once a day for one week. During this week of administration, the body weight and feed and drink intake of the animals were assessed. The length (L) and width (W) of the tumors were measured using Vernier calipers once a day, and tumor volume was calculated using formula $1/2\,L \times W^2$. After the animals were sacrificed at one week after administration, the tumors were excised, weighed, fixed overnight in neutral buffered formalin, embedded in paraffin, and sectioned. The sections were then stained with hematoxylin and eosin (HE) and photographed using a Leica light microscope (Wetzlar, GER) at 200 × magnification.

2.4. Immunohistochemical staining for CD31

Mouse tumor sections were de-paraffinized using traditional methods and then incubated in 3% peroxide for 10 min to inactivate endogenous peroxidase. Antigen retrieval was accomplished in 0.1 M sodium citrate that was brought to a boil in a microwave oven at high power, and then heating was continued at 30% power for 10 min. Tissues were blocked with 5% BSA and incubated overnight at 4 °C with antibodies against CD31 (1:100 dilution). Then, the tissues were subject to coloration with the SABC-AP three-step detection kit according to the manufacturer's instructions (Boster, Wuhan, China). Sections were then photographed using Leica microscope at 200 × magnification.

2.5. Cell viability by the MTT assay

Cells were plated in 96-well plates at 2×10^3 cells per well, and incubated overnight in DMEM containing reduced serum and growth factor (1:5 dilution of complete DMEM with non-supplemented DMEM). Then, cells were treated with PBS, 5 ng/mL VEGF, roxarsone (0.1, 1.0 or 10.0 μM), 20.0 μM U73122, or 20.0 μM U73122 plus 1.0 μM roxarsone. After 24 h of incubation, a final concentration of 0.4 mg/mL MTT was added in the medium. The reduced formazan after 4 h was solubilized with 150 μL dimethyl sulfoxide by shaking for 10 min, and absorbance was measured at 570 nm in a microplate reader (Multiskan