Isookanin Inhibits PGE2-Mediated Angiogenesis

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Inflammation is increasingly recognized as a critical mediator of angiogenesis, and unregulated angiogenic responses often involve human diseases. The importance of regulating angiogenesis in inflammatory diseases has been demonstrated through some successful cases of anti-angiogenesis therapies in related diseases, including arthritis, but it has been reported that some synthetic types of antiangiogenic drugs have potential side effects. In recent years, the importance of finding alternative strategies for regulating angiogenesis has begun to attract the attention of researchers. Therefore, identification of natural ingredients used to prevent or treat angiogenesis-related diseases will play a greater role. Isookanin is a phenolic flavonoid presented in Bidens extract, and it has been reported that isookanin possesses some biological properties, including antioxidative and anti-inflammatory effects, anti-diabetic properties, and an ability to inhibit α-amylase. However, its antiangiogenic effects and mechanism thereof have not been studied yet. In this study, our results indicate that isookanin has an effective inhibitory effect on the angiogenic properties of microvascular endothelial cells.

isookanin anti-angiogenesis HMEC-1 cell prostaglandin E2 (PGE2) cell cycle arrest ERK1/2 and CREB signaling pathway

1. Introduction

Angiogenesis is the formation of new capillaries from existing vasculature and is an integral biological process that occurs throughout life in a number of physiological and pathological processes [1]. It is known that angiogenesis is essential for reproduction, development, and wound repair, where it is highly regulated. However, many diseases are caused by unregulated angiogenesis, including cancer, rheumatoid arthritis, inflammatory bowel disease (IBD), and diabetic retinopathy [2][3]. In addition, angiogenesis is also involved in various skin diseases including psoriasis, telangiectasias, and rosacea, where various types of blood vessel growth can be observed in diseased skin [4].

Many potent proangiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin, and transforming growth factor-β (TGF-β) have been found to be involved in angiogenesis [2]. Abnormal angiogenesis caused by these stimulants is accompanied by a series of endothelial cell behavioral changes associated with increased cell proliferation, cell migration and invasion, and the formation of new tubular structures [5]. Angiogenesis inhibitors targeting this set of mechanisms have been studied and applied in clinical trials, where the data have strongly suggested that suppressing aberrant angiogenesis may be a promising treatment for angiogenesis-related diseases [6][7].

Meanwhile, recent clinical and basic scientific studies indicate that angiogenic inducers involve (many) other factors, including inflammatory mediators [8]. Among the many inflammatory mediators, prostaglandins (PGs) are representative and are naturally occurring lipid compounds derived from the cyclooxygenase (COX)-mediated metabolism of arachidonic acid [9]. Prostaglandins link the interactions between various immune regulatory cells and are thought to play a key role in regulating the inflammatory response [10]. Prostaglandin E_2 (PGE_2) is the most abundant prostanoid in humans and the most important COX-2 mediated product found to date. PGE_2 is involved in regulating many different fundamental biological functions, and its role in inflammation and immune response has been well established in previous studies [11][12].

To date, PGE_2 has been the most researched inflammatory mediator in immune cells, and in recent years, a lot of attention has been paid to the other roles of PGE_2 [13]. However, the mechanisms and effects of PGE_2 on endothelial cells and angiogenesis have been less studied [14]. PGE_2 affects target cells by binding four cognate receptors called EP1, EP2, EP3, and EP4, which belong to a large family of G protein-coupled receptors (GPCRs). Ligand binding of different EP receptors leads to different actions through activation of separate downstream signaling pathways [15]. Some prior studies have confirmed that PGE_2 and EP receptors have a direct role in angiogenic gene expression and angiogenic cell behavior [14][16][17][18][19].

The importance of angiogenesis in inflammatory disease processes has been demonstrated by successful cases of antiangiogenic therapeutic trials for diseases such as asthma [20], rheumatic diseases [21], and dermatosis [22]. These treatments are commonly used FDA-approved drugs that target angiogenic factors or angiogenic factor signaling cascades [6]; however, these drugs are known to have some clinical limitations and some side effects associated with their administration [23][24].

Therefore, the importance of finding alternative strategies for controlling angiogenesis has begun to attract people's attention, and the identification of natural compounds will play an important role in finding therapeutics for the prevention and treatment of angiogenesis-related diseases [25]. Isookanin ($C_{15}H_{12}O_6$, molecular weight: 288.3) is a phenolic flavonoid presented in *Bidens* extract [26]. It has been reported that isookanin possesses some biological properties, including antioxidative [27][28] and anti-diabetic properties [27], anti-inflammatory effects [29] and an ability to inhibit α-amylase [26]. However, the antiangiogenic effects and mechanism thereof have not been studied yet.

2. Current Insights

Inflammatory mediators are well known for their role in inducing an appropriate immune response to external stimuli, and recently, they have also been shown to play an important role in the body's response to vascular disease [30]. Previous studies have shown that the inflammatory response enhances angiogenesis and tumor growth [31]. Prostaglandins, especially PGE_2, have been demonstrated to play an important role in immune regulation, and according to recent reports, PGE_2 also plays a role in promoting the assembly of new blood vessels in angiogenesis [14]. The development of anti-angiogenesis agents has attracted great interest due to the high prevalence of angiogenesis-related diseases. The importance of antiangiogenic agents in the treatment of cancer is well established [32], and their role in the treatment of chronic inflammatory disorders is also gaining more

support. In cases such as rheumatoid arthritis [33] and human IBD [34], it has been demonstrated that angiogenesis plays an important role in the pathophysiology of these chronic inflammatory diseases. There are several types of synthetic drugs for anti-angiogenesis therapy, but considering their side effects, it is worth investigating bioactive active compounds derived from natural products for the prevention and treatment of angiogenesis-related diseases [35]. Currently, a large variety of polyphenolic compounds from natural sources have drawn considerable attention for their multiplex biologic properties. Many natural compounds—for example, resveratrol, curcumin, or quercetin can target a wide variety of molecules, implying that natural products might be effective inhibitors [36]. Isookanin is a phenolic flavonoid component contained in some plants, such as *Asteraceae* [26] and *Leguminosae* [28], and its anti-inflammatory efficacy and mechanism of action have already been revealed in our previous research [29].

In this study, we investigated the role that isookanin plays in the process of PGE_2-induced angiogenesis. The results of the LDH experiment show that isookanin has no cytotoxicity, and the results of MTT cell viability confirm that isookanin can reduce the proliferation of PGE_2-induced HMEC-1 cells in a concentration-dependent manner, while not causing cell damage. In addition, we confirmed that isookanin inhibited the migration and tube formation of HMEC-1 cells induced by PGE_2. These results support the potential of isookanin as an antiangiogenic agent. In addition, isookanin induces the S phase arrest of HMEC-1 cells through downregulating the expression of cell cycle-related proteins. Moreover, the flow cytometric data of cell cycle assay did not detect the increase of sub-G1 cells after isookanin treatment compared with the control group. This indicates that the antiangiogenic activity of isookanin is due to the inhibition of cell proliferation, not the induction of cell death.

PGE_2 affects target cells by activating cognate receptors EP1-4 that belong to the G protein-coupled receptor superfamily of cell surface-expressed receptors [15]. The receptors demonstrate distinct, as well as opposing, effects on intracellular signaling events. The EP1 receptor couples to Gq and mediates a rise in intracellular calcium concentration, and the EP3 receptor couples to Gi, reducing cAMP concentration. Both EP2 and EP4 couple to Gs, leading to increased synthesis of cAMP and consequent activation of PKA [14]. Subsequent PKA activation is involved in multiple signaling, including activation of the cAMP response element (CREB) [37]. The transcription factor CREB plays a key role in controlling cell growth, survival, and cell cycle progression, and it plays an important role in the development of many cell types, including vascular smooth muscle cells [38], adipocytes [39], glioma cell [40], and vascular endothelial cells [41]. Multiple intracellular signaling kinases can induce the phosphorylation of CREB, including extracellular signal regulated kinases (ERKs) [40][42]. Moreover, the ERK signaling cascade is a central MAPK pathway that plays a role in the regulation of various cellular processes, such as proliferation, differentiation, survival, and apoptosis under some conditions [43]. For example, Pozzi et al. [44] demonstrated an EP4-mediated PI3K/ERK signaling pathway in mouse colon carcinoma cells. Activation of the ERK1/2 pathway is required for the growth, proliferation, and migration of endothelial cells, and this pathway is considered an important target for antiangiogenic agents [45][46].

In recent studies, it is known that PGE_2 can promote cell growth by activating multiple signaling pathways involved in cell proliferation and that both MAPK and cAMP/PKA pathways are required for PGE_2-induced cell proliferation [40][47]. In this study, PGE_2 was shown to increase phosphorylation of ERK1/2 and CREB; therefore, PGE_2-induced proliferation is associated with activation of ERK1/2 and CREB in vascular endothelial cells. In addition, isookanin

exhibits the efficacy of inhibiting phosphorylation of both ERK 1/2 and CREB induced by PGE_2. These results suggest that the mechanism by which isookanin inhibits the proliferation of vascular endothelial cells is by inhibition of ERK1/2 and CREB phosphorylation. However, these results should be confirmed by further experiments to determine whether isookanin inhibits CREB activation through ERK1/2 dependence or acts directly on CREB.

PGE_2 may induce angiogenesis by acting indirectly on a variety of cell types to produce proangiogenic factors. Indeed, PGE_2 stimulation of airway smooth muscle, endometrium, or colon cancer cells leads to increased production of VEGF, bFGF, or CXCL1 that, in turn, act on target endothelial cells to promote the angiogenesis [48][49][50]. In addition, previous studies have reported that PGE_2 acts directly on endothelial cells, promoting the creation of new blood vessels through activation of G protein-coupled receptor EP4 and its downstream pathway [14]. Of all the PGE_2 receptors, the roles of the EP4 receptor in health and disease have received much attention [51]. Chell et al. [52] reported that EP4 receptor protein expression was increased in colorectal cancers, as well as in adenomas when compared with normal colonic epithelium. EP receptor overexpression—and thus upregulation of various signaling cascades associated with angiogenic tumorigenesis, PGE_2 modification of the tumor microenvironment, and evasion of the immune system—is regulated through the EP receptors [53]. Therefore, the direct effect of isookanin on the expression of the EP receptor was confirmed and is presented in Supplementary Materials. The result shows that isookanin effectively inhibited the expression of EP4 (Figure S1 in Supplementary Materials). These results suggest that isookanin possibly regulates downstream signaling by inhibiting the expression of EP4, and thereby inhibiting angiogenesis. However, to confirm the effect between the suppression of EP4 expression and downstream signaling, additional experiments are required. In addition, recent studies have reported that several specific receptors may be involved in the angiogenesis of PGE_2. Finetti et al. [54] reported that PGE_2 primes the angiogenic switch through a synergistic interaction with the fibroblast growth factor-2 pathway. There is also a report claiming that EP2 receptor agonists stimulate angiogenesis to promote adipogenesis [55]. Based on these studies, it can be expected that several specific receptors, including EP4, are the major receptors responsible for PGE_2-induced angiogenesis. To identify the role of specific receptors for PGE_2 in an angiogenesis model, further studies using specific EP antagonists or gene silencing are required.

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