20-Hydroxyeicosatetraenoic Acid (20-HETE) Stimulates Migration of Vascular Smooth Muscle Cells

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Key Words

20-Hydroxyeicosatetraenoic Acid • Migration • Vascular smooth muscle • Second messengers • Cyp4A • PDGF-BB

Abstract

Aim: We tested the hypothesis that 20-HETE production contributes to platelet derived growth factor (PDGF)-BB stimulated migration of VSMC in a cell culture model. **Methods:** Studies were performed with A10 cells which are a rat vascular smooth muscle derived cell line. Migration was determined using a Boyden chamber chemotactic assay. **Results:** Pre-treatment of cells with two doses of 20-HETE (100 and 500 nM) significantly increased PDGF-BB stimulated VSMC migration by 34-58% of control; whereas, prior incubation of cells with inhibitors of 20-HETE production, 17-ODYA (1-25 M) or HET0016 (100 nM), significantly decreased PDGF-BB stimulated migration by 40-90%. 20-HETE mediated increase in PDGF-BB migration was completely prevented by the 20-HETE antagonist, WIT-002. In order to determine what second messenger pathways are involved in the 20-HETE mediated stimulation of

VSM migration, experiments were performed with specific inhibitors of tyrosine kinase (tyrphostin 25, 10 μ M), mitogen-activated extracellular signal-regulated kinase (MEK, PD98059, 20 μ M and U0126, 10 μ M), protein kinase C (Myr-PKC, 50 μ M), and phosphoinositide 3-kinases (PI3Ks) (wortmannin, 50 nM). Blockade of MEK and PI3K all abolished the increase in 20-HETE mediated migration. **Conclusion:** 20-HETE stimulates PDGF-mediated VSM migration acting through pathways that involve MEK and PI3K.

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Introduction

20-Hydroxyeicosatetraenoic Acid (20-HETE) is a cytochrome P450_{4A} derived metabolite of arachidonic acid (AA) endogenously produced by vascular smooth muscle cells (VSMC) [1]. 20-HETE has been extensively characterized as a potent vasoconstrictor acting to inhibit K⁺-channel activity in VSMC [2, 3]. Activation of 20-HETE production is believed to be a critical step in the mitogenic actions of several hormones including norepinephrine, angiotensin II and epidermal growth factor (EGF) [4-6]. Previous studies in rat aortic VSMCs have demonstrated the ability of 20-HETE to significantly

increase [³H]thymidine incorporation through the mitogen-activated protein kinase (MAPK) pathway [4]. It is believed that 20-HETE activates MAPK through activation of Ras since the proliferative actions of 20-HETE can be significantly attenuated by Ras farnesyl transferase inhibitors [7]. This pathway has recently been shown to mediate angiotensin II-induced neointimal formation following balloon injury [8].

Recent studies have indicated that 20-HETE is an important mediator of vascular endothelial growth factor (VEGF) mediated angiogenesis and vessel sprouting. Specific inhibition of 20-HETE production with HET0016 significantly decreased VEGF mediated angiogenesis in skeletal muscles of the rat [9]. Additional studies by Jiang et.al., clearly demonstrated an increase in sprouting from renal arterial microvessels transduced with an adenovirus containing the CYP4A1 isoform under the control of a VSMC specific promoter [10]. These studies suggest that 20-HETE may be a critical factor in angiogenesis; however, its role in the migration of VSMC is not known. The current studies were designed to examine the role of 20-HETE in VSMC migration and to identify what second messenger pathways are essential for this process.

Materials and Methods

Cell Culture and Reagents

Experiments were performed on A10 cells (ATCC) from passage 3-6. A10 cells were grown in DMEM supplemented with 4.5 g/L glucose, 10% FBS, 100 U/mL penicillin and 100 g/mL streptomycin. 20-HETE, 15(S)-HETE and 17-ODYA were obtained from Cayman Chemical Company, Ann Arbor, MI and received dissolved in ethanol. Platelet-derived growth factor (PDGF-BB) was from Research Diagnostics, Inc., Flanders, NJ. The myristoylated-PKC peptide inhibitor and the mitogen-activated extracellular signal-regulated kinase (MEK) inhibitors, U0126 and PD 98059, were obtained from Promega, Madison, WI. The tyrosine kinase inhibitor Tyrphostin 25 was from BIOMOL, Plymouth Meeting, PA. HET0016 was a gift from Taisho Pharmaceutical Co., Ltd., Saitama, Japan. WIT-002 (20-hydroxyeicosa-6(Z),15(Z)-dienoic acid) was obtained from Dr. Richard Roman, Medical College of Wisconsin, Milwaukee, WI.

Reverse transcription-PCR

RNA was isolated from A10 cells (P8) or rat liver using a commercially available kit specifically designed for rt-PCR according to manufactures' guidelines (RNAqueous, Ambion, Austin, TX). RNA was eluted in a 60 l volume with nuclease-free water. 1 g of RNA in a 10 l volume was then used in a reverse transcription reaction using random hexamer primers with AMV reverse transcriptase in a 20 l final volume. In order to control for amplification of genomic DNA, reactions lacking

AMV reverse transcriptase were run in parallel. PCR was performed on 1 l of the reverse transcription reaction in a 25 l volume using standard PCR reaction conditions. All CYP4A isoforms and GAPDH were amplified from the same reverse transcription reaction. The primer sequences for the rat CYP4A isoforms were previously published [11]. The sequence of the rat beta-actin primers are: beta-actin +: 5'-CCA CCA ATC CAC ACA GA; beta-actin -: 5'-TCG CTG ACA GGA TGC A. PCR reactions were cycled for 45 cycles for A10 cells and 35 cycles for liver. PCR products were separated on 1.2% agar gels and visualized under ultraviolet illumination. Images were captured using a gel documentation system with software supplied by the manufacturer (Gel Doc 2000, Bio-Rad, Hercules, CA). Experiments were repeated from 3 separate preparations of A10 cell RNA.

Western Blots

Western Blots for Cyp4A proteins were performed A10 cells using cell lysates prepared by brief sonication of cells followed by centrifugation at 3,000 x g for 10 min at 4°C. Samples of 100 g of cell lysate and 20 g of rat liver microsomes were boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA) for 5 min and electrophoresed on 7.5% SDS-polyacrylamide gels and transferred onto nitrocellulose membrane. Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) for 2 hours at room temperature, then incubated with goat anti-rat polyclonal antibody as well as a mouse anti-actin antibody (Gentest, 1:5,000) overnight at 4°C. The membranes were then incubated with either Alex 680 donkey anti-goat IgG (Molecular Probes) or IRDye 800 donkey anti-mouse IgG (Rockland, Gilbertsville, PA) for 1 hour at room temperature. The membranes were then visualized using an Odyssey infrared imager (LI-COR, Lincoln, NE) which allows for the simultaneous detection of two proteins. Densitometry analysis was performed using Odyssey software (LI-COR, Lincoln, NE).

Migration Assay

The protocol for A10 cell migration is outlined in Figure 1. A10 cell migration was examined in transwell cell culture chambers using a polycarbonate membrane with 8-µm pores in 24-well plates (Costar Corp, Cambridge, MA) as previously described [12]. A10 cells were grown to a confluency of 70%, trypsinized and suspended in DMEM/ 0.4% FBS to a concentration of 3.0×10^5 cells/ml. Cells were then pretreated with either 20-HETE (100 or 500 nmol/L) or the specific CYP4A inhibitors, 17-ODYA (0.5-10 mol/L) or HET0016 (10 or 100 nmol/L) (dissolved in ethanol) for 1 hour at 37°C. Control cells were treated with vehicle (ethanol). In additional experiments utilizing inhibitors of MEK, tyrosine kinase, PKC, PI3K, and the 20-HETE receptor antagonist, WIT-20, cells were incubated for 15 min at 37°C with each specific inhibitor prior to exposure to 20-HETE (500 nM). Cells were then incubated for an additional 45 min at 37°C. After a 1 hour incubation period, DMEM/ 0.4% FBS (0.6 ml) with or without PDGF-BB (20 µg/ml), was then added to the lower compartment and 0.1-ml cell suspension (final: 30,000 cells/well) was then added to the upper compartment and the cells incubated at 37°C. After 4 h, the top surface of the filters were scraped and rinsed with PBS to wash

Fig. 1. Overview of migration assay. A10 cells were grown to a confluency of 70%, trypsinized and counted. In experiments examining the effect of inhibitors of second messenger systems, cells were preincubated for 15 min at 37° with the inhibitors. Cells were then incubated with 20-HETE, 17-ODYA or HET0016 for 1 hour at 37°. After this time, cells were placed in a modified Boyden chamber with PDGF and incubated for 4 hours at 37°C. After this time, cells on the bottom of the membrane were fixed, stained, and counted.

away non-migrating cells. The filters were then fixed with methanol (10 min at 4°C) and the remaining cells counterstained with hematoxylin. The number of VSMCs per 320× high power field (HPF) that migrated to the lower surface of the filters was determined microscopically. Three filters were used for each different drug treatment and cell numbers from four randomly chosen HPFs were counted per filter and averaged to obtain the number of migrating cells. Data are expressed as percent of the amount of cells that migrated under control conditions. Experiments were repeated 3-4 times as indicated.

Statistical Analysis

Mean ± SEM are presented. Means were compared using an unpaired *t* test for two groups or analysis of variance (ANOVA) followed by Student Newman-Keuls post-hoc test where appropriate. A value of *P* < 0.05 was considered to be statistically significant.

Results

CYP4A expression in A10 cells

The CYP4A1 isoform was the only isoform detected in A10 cells by rt-PCR using primers specific for all 4 rat CYP4A isoforms (Figure 2A). All 4 isoforms were amplified from rat liver. A single band in A10 cell lysates was detected by Western blot. This band migrated at the same molecular weight as CYP4A proteins in the liver (Figure 2B).

20-HETE increases PDGF-BB stimulated migration

We examined the effect of 20-HETE on PDGF-BB stimulated migration using a Boyden chamber chemotactic assay (Figure 3A). Two concentrations of 20-HETE were used, 100 and 500 nM, and the number of migrating A10 cells compared to control cells. 20-HETE increased PDGF-BB stimulated migration by 34 ± 10 and 58 ± 7%

of control at 100 and 500 nM concentrations (Figure 3B). Migration of A10 cells was not observed in the absence of PDGF-BB even when 20-HETE was added (data not shown).

Inhibition of 20-HETE decreases PDGF-BB stimulated migration

In order to determine the role of endogenous 20-HETE in PDGF-BB stimulated migration, A10 cells were preincubated with two different inhibitors of 20-HETE synthesis, 17-ODYA and HET0016 [13, 14]. 17-ODYA at a concentration of 0.5 M had no effect on migration; however, at higher concentrations significant inhibition of PDGF-BB stimulated migration was observed (Figure 4). 17-ODYA at a 25 M concentration was able to reduce PDGF-BB stimulated migration to 12 ± 9% as compared to control (Figure 4). HET0016 was able to inhibit PDGF-stimulated migration at lower concentrations than 17-ODYA. Migration was reduced to 46 ± 18% by 100 nM HET0016 (Figure 4).

Antagonism of 20-HETE mediated increase in PDGF-BB migration

Previous studies have reported that the vasoconstrictor actions of 20-HETE can be antagonized by regioisomers of other HETEs and closely related analogs which by themselves are inactive [15]. In order to determine if we could antagonize 20-HETE stimulation of PDGF-BB migration, experiments were performed in the presence of 15(S)-HETE and WIT-002, a specific antagonist of 20-HETE. Pretreatment of A10 cells with 15(S)-HETE (500 nM) alone was able to stimulate PDGF-BB migration to similar levels as those observed with 20-HETE (Figure 5). Treatment of A10 cells with 20-HETE and 15(S)-HETE increased migration as

Fig. 2. rt-PCR analysis of CYP4A isoform expression in A10 cells and rat liver. M= molecular weight marker. B) Western Blot analysis of CYP4A protein in rat liver and A10 cells. M= molecular weight marker.

compared to control; however, the levels of migration were significantly attenuated when compared to those of either metabolite alone (Figure 5). WIT-002 (10 M) did not have any effect on migration in control A10 cells but was able to completely block the effect of 20-HETE to stimulate PDGF-BB mediated migration (Figure 5).

20-HETE stimulates PDGF-BB migration through MEK and PI3 kinase pathways

In order to determine the potential second messenger pathways responsible for 20-HETE stimulation of PDGF-BB mediated migration, experiments were performed in the presence of inhibitors of tyrosine kinase (tyrphostin 25), MEK (PD98059 and U0126, 10), protein kinase C (Myr-PKC), and phosphoinositide 3-kinases (PI3Ks) (wortmannin). Inhibition of tyrosine kinases decreased the basal level of migration to PDGF-BB but did not have any effect on 20-HETE stimulation of migration (Figure 6). Neither MEK inhibitor had any effect on basal migration but both completely attenuated 20-HETE stimulation of PDGF-BB migration (Figure 6). Similar results for migration under basal conditions and in response to 20-HETE were also observed for the PI3K inhibitor wortmannin (Figure 6). Blockade of protein kinase C completely inhibited PDGF-BB mediated migration and

this effect was not influenced by addition of 20-HETE (Figure 6).

Discussion

Proliferation and migration of VSMC are critical processes involved in neointimal thickening and the progression of restenosis after angioplasty [16]. 20-HETE has been demonstrated to be an important mitogen in VSMC mediating the proliferative actions of norepinephrine via small GTP binding proteins [4, 7]. 20-HETE also plays a critical role in angiotensin II-induced neointimal formation following balloon injury suggesting that it may be involved in restenosis [8]. Our findings that 20-HETE mediates PDGF-BB mediated smooth muscle cell migration lends further support for a pivotal role of 20-HETE in the regulation of VSMC function. Since proliferation and migration of VSMC are important components of restenosis and atherosclerosis, investigation of the therapeutic potential of inhibition of 20-HETE synthesis in fighting the progression of these diseases may be warranted.

Our results demonstrate the inhibition of 20-HETE synthesis with either 17-ODYA or HET0016 is able to

Fig. 3. Representative images of PDGF-BB migration of A10 cells using a Boyden chamber chemotactic assay in control cells and cells pre-incubated with 100 and 500 nM 20-HETE. Cells on the bottom of membrane were stained with hematoxylin and counted. B) Effect of 20-HETE on PDGF-BB mediated A10 cell migration. Dashed line represents control PDGF stimulated migration. * = $p < 0.05$ as compared to control cells, $n = 4$.

Fig. 4. Inhibition of 20-HETE synthesis decreases PDGF-BB migration in A10 cells. Cells were preincubated with different doses of P450 inhibitors, 17-ODYA or HET0016 as described in the methods. Dashed line represents control PDGF stimulated migration. * = $p < 0.05$ as compared to control cells, $n = 4$.

significantly attenuate PDGF-BB mediated VSMC migration. Although the ability of PDGF-BB to directly stimulate 20-HETE synthesis in A10 cells was not examined in the current study, previous studies have demonstrated epidermal growth factor (EGF), a closely related growth factor, can directly stimulate 20-HETE production in cultured proximal tubule cells [6]. This raises

the possibility PDGF-BB may also directly stimulate 20-HETE production in VSMC. Future experiments directly examining the ability of PDGF-BB to stimulate 20-HETE production are needed to address this question.

In the present study, 20-HETE stimulation of PDGF-BB migration was attenuated by co-incubation with 15(S)-HETE and abolished by WIT-002. 15(S)-HETE

Fig. 5. Effects of 20-HETE, 15(S)-HETE and WIT-002 on PDGF-BB migration in A10 cells. Cells were preincubated with 15(S)-HETE (500 nM) or WIT-002 (10 M) prior to treatment with 20-HETE or vehicle (ethanol). Dashed line represents control PDGF stimulated migration. * = $p < 0.05$ as compared to control cells. † = $p < 0.05$ as compared to 20-HETE, $n = 4$.

Fig. 6. Effect of inhibition of tyrosine kinase (tyrphostin 25, 10 M), mitogen-activated extracellular signal-regulated kinase (MEK, PD98059, 20 M and U0126, 10 M), protein kinase C (Myr-PKC, 50 M), and phosphoinositide 3-kinases (PI3Ks) (wortmannin, 50 nM) on migration in A10 cells with and without 20-HETE. Dashed line represents control PDGF stimulated migration. * = $p < 0.05$ as compared to control cells. † = $p < 0.05$ as compared to 20-HETE, $n = 3$.

was able to stimulate A10 migration to levels similar as those observed with 20-HETE which was consistent with previous findings [17]. However, when added in combination with 20-HETE the level of migration was significantly attenuated. The mechanism responsible for this observation is not known but could be due to differences in second messenger pathways utilized by

15(S)-HETE and 20-HETE or an antagonistic effect due to interaction with membrane receptors. It is also possible that this effect was a due to the dose-response characteristics of 15(S)- and 20-HETE since the concentration of both of these metabolites used was in the upper range of physiological levels. Our finding that WIT-002, which had no effect on PDGF-BB migration

on its own, completely attenuated the effects of 20-HETE on PDGF-BB mediated migration further supports the existence of a 20-HETE receptor in VSMC. Previous studies in the renal vasculature have also demonstrated that closely related, inactive analogs such as 15(S)-HETE and WIT-002 can attenuate the vasoconstrictor actions of 20-HETE [15]. The idea of a classical seven transmembrane domain receptor for 20-HETE is controversial given the fact that 20-HETE is a hydrophobic lipid that has been reported to inhibit ion channel activity in detached membrane patches [3]. However, there is evidence for an 11-12 epoxyeicosatrienoic acid (EET) receptor in vascular smooth muscle and mononuclear cells [18-20]. It is possible that these putative receptors could be part of a larger family of undiscovered fatty-acid receptors which are active in a variety of cells. Further experiments will be required in order to identify this receptor and its role in 20-HETE stimulation of PDGF-BB mediated migration in VSMC.

Our results demonstrate that inhibition of MEK with two different agents is able to block the stimulatory actions of 20-HETE on PDGF-BB mediated migration. In contrast, inhibition of tyrosine kinase reduced basal migration but had no effect on 20-HETE stimulation of PDGF-BB mediated migration in A10 cells. The effect of these pathways on 20-HETE mediated migration is opposite of that previously described for inhibition of K⁺-channel activity by 20-HETE in VSMC [21]. 20-HETE works through tyrosine kinases upstream of MEK to inhibit K⁺-channel activity and cause vasoconstriction. However, our data suggests that 20-HETE is able to activate MEK independent of tyrosine kinase to stimulate PDGF-BB migration in A10 cells. Blockade of protein kinase C was able to completely inhibit PDGF-BB mediated migration in A10 cells and 20-HETE had no effect on this response. This result is consistent with a previous report which also found that inhibition of protein kinase C blocked PDGF-BB migration in vascular smooth muscle cells [22].

To date, little is known about the interaction of P450 monooxygenase metabolites with the PI3 kinase pathway. In pulmonary artery smooth muscle, 20-HETE acts through the PI3-kinase-Akt pathway to stimulate NO release leading to relaxation [23]. The PI3-kinase pathway is also important for the mitogenic actions of EETs in renal epithelial cells [24]. We found that blockade of the PI3-kinase pathway completely inhibited the effects of

20-HETE on PDGF-BB migration in A10 cells.

These studies complement the growing evidence for a role of 20-HETE in angiogenesis. Specific over-expression of 20-HETE in renal interlobar arteries increases sprouting of neovessels [10] and inhibition of 20-HETE synthesis significantly reduces the angiogenic response to VEGF in skeletal muscle and the cornea [9, 5]. Since growth of new blood vessels is dependent on formation of both capillary-like tubes by endothelial cells as well as subsequent infiltration by VSMC, 20-HETE may be an important mediator of each of these processes in vivo. The angiogenic properties of 20-HETE make it a potential target for the development of novel anti-angiogenic therapies to limit the growth of tumors. This view is supported by studies which have demonstrated that blockade of 20-HETE synthesis with HET0016 decreases both size and vascularization of gliosarcomas in rats [25, 26].

Conclusion

In summary, we found that 20-HETE augments PDGF-BB mediated migration in A10 cells and that inhibition of endogenous 20-HETE synthesis decreases PDGF-BB mediated migration. Both 15(S)-HETE and WIT-002, an inactive antagonist of 20-HETE, attenuate the effects of 20-HETE on PDGF-BB migration. The effect of 20-HETE on PDGF-BB migration in A10 cells is mediated through MEK and PI3 kinase pathways. The results of these studies when added to the previously described functions of 20-HETE on neointimal formation and angiogenesis suggest that 20-HETE may be a novel target in VSMC proliferative disorders such as restenosis and atherosclerosis.

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