20-HETE inhibits the proliferation of vascular smooth muscle cells via transforming growth factor-β

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Abstract 20-Hydroxyeicosatetraenoic acid (20-HETE), a cytochrome P450 arachidonic acid metabolite, has been shown to modulate the growth of vascular smooth muscle cells (VSMCs). We asked whether 20-HETE modulates the proliferation of R22D cells, a clonal VSMC from neonatal rats, by releasing transforming growth factor-β (TGF-β). Incubation of R22D cells with 20-HETE for 24 h attenuated [3H]thymidine incorporation in a concentration-dependent manner without causing the release of lactate dehydrogenase. 20-HETE also inhibited platelet-derived growth factor (PDGF)-induced [3H]thymidine incorporation in R22D cells and human VSMCs. At 5 μM, 20-HETE reduced [3H]thymidine incorporation by 34 ± 6%; anti-TGF-β neutralizing antibody, but not nonspecific IgG, completely reversed the attenuated [3H]thymidine incorporation induced by 20-HETE. In addition, 20-HETE attenuated fetal bovine serum- and PDGF-induced expression of cyclin D1, a downstream effector of receptor tyrosine kinases and G protein-coupled receptors (19). Blockade of the formation of 20-HETE attenuated norepinephrine-induced (18) and angiotensin II-induced (20) ERK 1/2 phosphorylation, suggesting that 20-HETE may serve as a second messenger of these growth factors.

Recent studies indicate that the secretion, activity, and clearance of transforming growth factor-β (TGF-β) can be regulated by long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) (21, 22). EPA inhibits platelet-derived growth factor (PDGF)-induced DNA synthesis by stimulating the secretion of TGF-β in mesangial cells (21). In addition, fatty acids may attenuate TGF-β activity and thus increase TGF-β activity (22). Although fatty acids have been shown to modulate the secretion, activity, and clearance of TGF-β, it is not known whether 20-HETE may modulate TGF-β availability and subsequently modulate cell growth.

In blood vessel walls, TGF-β1, the major isoform of TGF-β, is secreted in a latent, inactive form that binds to...
latent TGF binding protein (LTBP) with binding sites for extracellular matrix (ECM) (23). The matrix-bound latent molecule may be secreted and/or activated when LTBP is proteolytically cleaved by a protease such as plasmin or matrix metalloprotease (MMP) (23). In VSMCs, TGF-β may exert a growth-inhibitory effect by inducing cell cycle arrest at G1 phase (24), which may be attributable to the attenuation of cyclin D, cyclin E, and cyclin-dependent kinases (CDKs) 2, 4, and 6 (25, 26). In contrast, TGF-β may stimulate cell growth in VSMCs (27, 28). These discrepancies may be the result of differences in VSMC phenotype (27, 29) and the concentration of TGF-β in VSMCs (28). In this study, we tested the hypothesis that TGF-β may mediate the effect of 20-HETE on modulating cell proliferation in VSMCs. We demonstrated that TGF-β may mediate the inhibitory effect of 20-HETE on the growth of VSMCs from neonatal rat or human.

MATERIALS AND METHODS

Materials

20-HETE (in ethanol) purchased from Cayman Chemical Co. (Ann Arbor, MI) was dried under N₂ gas and then resuspended in the serum-free medium before use; an equal volume of serum-free medium was used as vehicle. Antibodies against cyclin D1, phospho-ERK 1/2, and phospho-MAPK/ERK kinase (MEK) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal IgGs against CDK4, β-actin, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Indomethacin and tryptose phosphate broth were purchased from Sigma-Aldrich (St. Louis, MO). Paxilline was from Alomone Labs (Jerusalem, Israel). PDGF B/B was purchased from Roche (Mannheim, Germany). Recombinant human TGF-β1, monoclonal anti-TGF-β (TGF-β1,2,3), mouse IgG isotype control, anti-human TGF-β RII antibody, and the TGF-β immunoassay kit were purchased from R&D Systems (Minneapolis, MN).

Cell culture

R22D cells, a primary culture of VSMCs isolated from neonatal rats, were established and selected for abundant production of elastin by Peter A. Jones (30). VSMCs with a heterogeneous phenotype were isolated from aortas of neonatal Sprague-Dawley rats as described previously (31). Human VSMCs were cultured from explants of thoracic aorta media. All cells were maintained under 5% CO₂ in minimum essential medium with penicillin-streptomycin (1%), tryptose phosphate broth (2%), and fetal bovine serum (FBS) (10% or 20% for cells from rat vs. human). In most of the experiments, cultured VSMCs were made quiescent by incubation with serum-deprived medium containing transferrin (5 µg/ml) and BSA (0.05%) for 24 h. In one experiment, R22D cells were seeded on a 24-well plate at 6,000 per well in growth medium overnight and then in serum-free medium for 24 h. PDGF (5 ng/ml) was added to each well in the presence or absence of 20-HETE (5 µM). After 1–6 days, cells from quadruplicate wells were trypsinized and counted using a hemocytometer.

RNA extraction and quantitative real-time PCR

Quiescent R22D cells were incubated for different time periods on six-well plates with 20-HETE, washed with phosphate-buffered saline, and extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Reverse transcription was performed using RevertAid H Minus Reverse Transcriptase (ThermoFisher, Waltham, MA) and cDNA was amplified in triplicate using SYBR Green PCR Master Mix (ThermoFisher) and CFX Connect Real-Time PCR Detection System (BioRad, Hercules, CA). The relative expression was calculated using the 2^(-ΔΔCt) method. TGF-β mediates the effect of 20-HETE
transcriptase reaction was carried out using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Total RNA was added to a reaction mixture containing oligo-deoxythymidine (0.5 μg/μl), dNTP (20 mM), dithiothreitol (0.1 M), Tris-HCl (250 mM, pH 8.3), KCl (375 mM), and MgCl₂ (15 mM). The reaction was conducted for 90 min at 37°C. Quantitative realtime PCR was carried out with a sequence detection system (ABI PRISM 7000; Applied Biosystems) in reaction mixture containing I × Smart Quant Green Master Mix (Protech Technology, Enterprise), RNA (50 ng), and the following primers: sense (5'-GCGCTGGATACCAACTTACTGCT-3') and antisense (5'-AGGCTCCAAATGTAGGGGCAGG-3') for TGF-β1, and sense (5'-GTAAACCCGTTGAAACCCATT-3') and antisense (5'-CATCCCAATCGTATGAGG-3') for 18S rRNA. The reactions were performed with preliminary denaturation for 10 min to activate DNA polymerase, followed by 40 cycles of denaturation at 95°C for 15 s, annealing/extension at 60°C for 1 min. All PCRs were done in duplicate on the same 96-well plate. For quantification, the threshold of cycle method was used to calculate relative fold changes normalized against the 18S RNA.

### Western blot

Confluent cells were cultured in serum-free medium for 24 h before incubation with 20-HETE at the indicated times. In some experiments, cells were exposed to FBS (10%) or PDGF (5 ng/ml) at the indicated times with or without administration of 20-HETE (5 μM). For cyclin D1 and CDK4, cells were exposed to 20-HETE (5 μM), 20-HETE plus PDGF (10%), or 20-HETE plus PDGF (5 ng/ml) in the presence or absence of anti-TGF-β antibody for 12 h. Total protein of each sample was loaded onto 10% SDS-polyacrylamide gels and separated by electrophoresis for 2 h. The protein samples were transferred to nitrocellulose membranes in a transfer buffer. The membranes were washed three times with TTBS solution (containing 1% Tween 20, Tris base 50 mM, and NaCl 150 mM), incubated overnight with antibody against cyclin D1, CDK4, phospho-ERK 1/2, phospho-MEK 1/2, GAPDH, and actin (1:5,000) at 4°C, and then incubated with peroxidase-conjugated secondary antibody (1:5,000) for 1 h. An ECL detection system (Perkin-Elmer, Boston, MA) was used for detection.

### [³H]thymidine incorporation

Cells were grown to confluence on 24-well plates before administration of concentrations of 20-HETE in growth or serum-free medium. In some experiments, quiescent cells were stimulated with PDGF (5 ng/ml) in the presence or absence of 20-HETE (5 μM). In other experiments, quiescent R22D cells were exposed to 20-HETE in the presence of TGF-β neutralizing antibody, anti-TGF-β RI antibody, or nonspecific IgG. After incubation for 18 h, 1 μCi/ml [³H]thymidine was added to the culture medium and incubated for an additional 6 h. Cells were then washed three times with ice-cold phosphate-buffered saline, precipitated with ice-cold 15% trichloroacetic acid overnight, lysed with 1 N NaOH, and incubated at room temperature for at least 30 min. The radioactivity of incorporated [³H]thymidine was determined by liquid scintillation counting (Tri-Carb 2900TR; Perkin-Elmer).

### Assay of TGF-β

Confluent R22D cells were made quiescent for 24 h before treatment with 20-HETE (5 μM) at the indicated times. Conditioned media were collected and frozen at −20°C until assay. The concentration of total TGF-β, (latent + active) in each sample was determined by a Quantikine TGF-β immunoassay kit from R&D Systems.

### Lactate dehydrogenase assay

Confluent R22D cells were made quiescent for 24 h before treatment with 20-HETE (5 or 10 μM) or H₂O₂ (10 mM) for 24 h. Conditioned media were collected and frozen at −20°C until assay of lactate dehydrogenase (LDH). The cytotoxic effects were determined by measuring LDH release with a CytoTox 96 NonRadioactive Cytotoxicity Assay kit from Promega.

### Analysis of data

All values are presented as means ± SEM and were analyzed with a t-test or ANOVA followed by Duncan’s post hoc test. Statistical significance was determined as P < 0.05.

### RESULTS

#### 20-HETE inhibited DNA synthesis in VSMCs

20-HETE inhibited [³H]thymidine incorporation in a concentration-dependent manner in R22D cells cultured in either serum-free medium (Fig. 1A) or in growth medium (Fig. 1B); at 5 μM, 20-HETE significantly inhibited

![Fig. 2. 20-HETE inhibited the PDGF-induced proliferation of R22D cells without causing lactate dehydrogenase (LDH) release. A: R22D cells were exposed to serum-free medium for 24 h before the addition of PDGF (5 ng/ml) in the presence or absence of 20-HETE (5 μM; n = 4). The results shown are representative of three experiments. B: Quiescent R22D cells were exposed to 20-HETE (5 or 10 μM) or H₂O₂ (10 mM) for 24 h. Percentage of LDH release is presented as means ± SEM of three experiments with quadruplet measurements in each experiment (n = 3). * P < 0.05 compared with the corresponding control group at time zero; ** P < 0.05 compared with the corresponding group without 20-HETE.](downloaded_from_jlr.org)
[^3]H][H]thymidine incorporation by 51 ± 3% and 22 ± 1%, from 32,513 ± 1,526 and 48,317 ± 2,165 cpm, respectively (n = 4; P < 0.05). Lower concentrations of 20-HETE (10^{-10} to 10^{-7} M) did not alter the thymidine incorporation in R22D cells (n = 4; data not shown). The inhibitory effect of 20-HETE on [^3]H][H]thymidine incorporation was also observed in aortic VSMCs (Fig. 1C); 20-HETE (5 μM) significantly inhibited [^3]H][H]thymidine incorporation by 26 ± 2% (n = 4; P < 0.05). In addition, coadministration of 20-HETE (5 μM) with PDGF (5 ng/ml)

![Graph A](image1.png)

**A**: Quiescent cells were treated with anti-TGF-β or nonspecific IgG (10 μg/ml) in the presence or absence of 20-HETE (5 μM) for 24 h before the determination of incorporated [^3]H][H]thymidine (n = 4). B: Quiescent cells were treated with anti-TGF-β RII antibody (20 μg/ml) in the presence or absence of 20-HETE (5 μM) for 24 h before the determination of incorporated [^3]H][H]thymidine (n = 4). C: Quiescent cells were incubated with 20-HETE (5 μM) or vehicle for 12 and 24 h before the determination of TGF-β1 secretion in the medium by ELISA (n = 3). D: Quiescent cells were exposed to 20-HETE (5 μM) for indicated time periods before harvest of the cells for quantitative real-time PCR analysis with TGF-β1 primers. E: Quiescent cells were pretreated with indomethacin (a cyclooxygenase inhibitor; 1 μM) or paxilline (a BK channel inhibitor; 100 nM) for 30 min, followed by the addition of 20-HETE (5 μM) for 24 h, before the determination of incorporated [^3]H][H]thymidine (n = 4). Values are means ± SEM. * P < 0.05 compared with the control value; # P < 0.05 compared with the indicated group. The results shown are representative of three experiments.
inhibited PDGF-induced [3H]thymidine incorporation by 33 ± 7% in R22D cells (Fig. 1D) and by 29 ± 5% in human VSMCs (Fig. 1E). In contrast to the inhibitory effect of 20-HETE on basal levels (Fig. 1A, D) in R22D cells, 20-HETE per se did not inhibit basal [3H]thymidine incorporation (117 ± 3 cpm) (Fig. 1E) in human VSMCs. In quiescent R22D cells, PDGF significantly increased the cell number with time, which was inhibited by coincubation with 20-HETE (5 μM) by 28% at day 5 (Fig. 2A). However, 24 h of exposure to 20-HETE did not alter the morphology of R22D cells (data not shown) or cause LDH release (Fig. 2B), whereas H2O2 (10 mM) induced 90 ± 2% LDH release in this preparation.

Anti-TGF-β antibody reversed 20-HETE-induced inhibition of DNA synthesis

Previous studies have indicated that long-chain polyunsaturated fatty acids may modulate cell growth by releasing TGF-β (21). We asked whether 20-HETE inhibited cell proliferation by releasing TGF-β. As illustrated in Fig. 3A, anti-TGF-β antibody exhibited no effect on basal [3H]thymidine incorporation in R22D cells; however, it completely reversed the inhibitory effect of 20-HETE on [3H]thymidine incorporation. Non specific IgG exhibited no effect on either basal [3H]thymidine incorporation or the inhibitory effect of 20-HETE (n = 4). In another experiment, anti-TGF-β RII antibody against TGF-β receptor type II (20 μg/ml) also significantly reversed the inhibitory effect of 20-HETE (i.e., a 23 ± 1% inhibition) on [3H]thymidine incorporation to the control level (n = 4; P < 0.05) (Fig. 3B). These results suggested that the inhibitory effect of 20-HETE may be mediated by TGF-β in R22D cells. As revealed by ELISA, the concentration of TGF-β1 in the condition medium collected from the cells treated with 20-HETE (5 μM) for both 12 and 24 h was higher than that of vehicle-treated cells (Fig. 3C). After 24 h of treatment with 20-HETE, TGF-β1 secretion was 45% higher than that of the vehicle group, which corresponds to 237 ± 26 pg/ml more secreted TGF-β1 in the medium. However, the mRNA expression of TGF-β1 was not affected by 3 to 24 h of exposure to 5 μM 20-HETE, as revealed by quantitative real-time PCR (Fig. 3D).

Previous work has shown that 20-HETE may be further converted by cyclooxygenase to biologically active metabolites (10–12). However, pretreatment of indomethacin, a cyclooxygenase inhibitor, did not affect the growth-inhibitory effect of 20-HETE (Fig. 3E). In addition, 20-HETE may induce vasoconstriction by inhibiting the Ca2+-activated K+ channel (8). Nevertheless, pretreatment with paxilline, a K+ channel inhibitor, did not affect the inhibitory effect of 20-HETE either (Fig. 3E).

To determine whether an additional 200 pg/ml TGF-β1 may exert an inhibitory effect on DNA synthesis in R22D
cells, the effect of TGF-β1 on [3H]thymidine incorporation was studied in these cells. As illustrated in Fig. 4A, TGF-β1 inhibited [3H]thymidine incorporation of R22D cells in a concentration-dependent manner, with significant inhibition first seen at 200 pg/ml. At 200 pg/ml, TGF-β1 significantly inhibited [3H]thymidine incorporation by 36 ± 3% (n = 4; P < 0.05). As illustrated in Fig. 4B, TGF-β1 exhibited a different pattern on [3H]thymidine incorporation in aortic VSMCs. Low concentrations of TGF-β1 (<200 pg/ml) stimulated [3H]thymidine incorporation, whereas high concentrations (>500 pg/ml) inhibited [3H]thymidine incorporation in aortic VSMCs.

20-HETE inhibited FBS- or PDGF-induced cyclin D1 expression

It has been established that TGF-β mediates the effect of 20-HETE on cell proliferation by the downregulation of cyclin D1 expression (26, 32). In a representative result illustrated in Fig. 5, 20-HETE attenuated FBS- and PDGF-induced cyclin D1 expression by 23% and 30%, which averaged 32 ± 7% (n = 4; P < 0.05) and 44 ± 5% (n = 3; P < 0.05) in repeated studies, respectively. Anti-TGF-β antibody exhibited no effect on basal cyclin D1 expression but completely reversed the inhibitory effect of 20-HETE on FBS- and PDGF-induced cyclin D1 expression (P < 0.05). Nevertheless, nonspecific IgG exhibited no effect on either basal cyclin D1 expression or the inhibitory effect of 20-HETE. In contrast, the expression of CDK4 or actin remained stable irrespective of different treatments.

20-HETE did not affect the phosphorylation of MEK or ERK 1/2

It has been reported that 20-HETE may mediate the mitogen-induced ERK 1/2 phosphorylation and may increase the kinase activity of ERK 1/2 (3, 17, 18). We asked whether 20-HETE inhibited DNA synthesis by inhibiting ERK 1/2 phosphorylation in R22D cells. As illustrated in Fig. 6A, 5 μM 20-HETE did not affect MEK or ERK 1/2 phosphorylation levels at 2–60 min after the addition of 20-HETE. FBS (Fig. 6B) or PDGF (Fig. 6C) induced a transient increase of ERK 1/2 phosphorylation, whereas pretreatment of 20-HETE did not affect the FBS- or PDGF-induced phosphorylation of MEK and ERK 1/2. In addition, 20-HETE did not cause the phosphorylation of p38 or JNK in R22D cells (n = 3; data not shown).

DISCUSSION

In the present study, we demonstrated that 20-HETE inhibited the proliferation of VSMCs from neonatal rat and human. To our knowledge, this is the first report dem-

![Fig. 6.](https://example.com/figure6.png)

Fig. 6. 20-HETE did not affect phosphorylation of MAPK/ERK kinase (MEK) or extracellular regulated protein kinase 1/2 (ERK 1/2) in R22D cells. A: Quiescent cells were incubated with 20-HETE (5 μM) for the indicated time periods, with FBS (10%; S)-induced increase in MEK and ERK 1/2 phosphorylation as positive controls. B, C: 20-HETE (5 μM) or an equal volume of serum-deprived culture medium (vehicle) was incubated with cells for 30 min before the addition of serum (10%; B) or PDGF (5 ng/ml; C). MEK and ERK 1/2 phosphorylation with time was analyzed using Western blot. The results shown are representative of three experiments.
onstrating the effects of 20-HETE on human VSMCs and that 20-HETE may act as a growth inhibitor. The growth-inhibitory effect of 20-HETE appears to be mediated by TGF-β, because TGF-β neutralizing antibody and anti-TGF-β RI antibody reversed the effect of 20-HETE on DNA synthesis in R22D cells. In addition, TGF-β inhibited DNA synthesis by the attenuation of cyclin D1 expression (26, 32). The finding that TGF-β neutralizing antibody reversed the inhibition of cyclin D1 expression induced by 20-HETE further supports the idea that TGF-β mediates the effect of 20-HETE. Furthermore, the 20-HETE-induced increase in TGF-β1 concentration in the medium appears to be high enough to inhibit DNA synthesis in VSMCs, as demonstrated by the exogenous addition of known concentrations of authentic TGF-β1.

It is unclear how 20-HETE increases TGF-β secretion into the culture medium. Nevertheless, it is unlikely that 20-HETE increases the transcription of TGF-β, because TGF-β mRNA expression was not affected by 20-HETE. Alternatively, the secretion of TGF-β may be increased by an enhanced MMP activity (33). In fact, TGF-β may be released by a polyunsaturated fatty acid such as EPA (21); however, EPA has been shown to inhibit, not increase, MMP activity (34). Overall, it seems unlikely that 20-HETE increased MMP activity to enhance the secretion of TGF-β, because most polyunsaturated fatty acids either inhibit or exert no effect on MMP activity (34). Alternatively, interference with the interaction of TGF-β, LTBP, and ECM may cause an enhanced secretion of TGF-β (23). Although fatty acids have been demonstrated to inhibit the complex formation of TGF-β and a plasma protein (22), it remains to be determined whether 20-HETE may exert an inhibitory effect on the complex formation of TGF-β with LTBP, decrease the sequestration of latent TGF-β complex into ECM, and subsequently increase the TGF-β concentration in the culture medium. However, we cannot rule out other possibilities for the modulation of TGF-β release from ECM, because fatty acids may modulate the composition of ECM (35–37).

In contrast to our finding that 20-HETE inhibited DNA synthesis in VSMCs, a previous study demonstrated that 20-HETE may increase DNA synthesis in VSMCs from aorta of adult rats (17). Because TGF-β is a pleiotropic growth factor that may either promote (28) or inhibit (24) the growth of VSMCs, TGF-β may play a role in causing the pleiotropic effects of 20-HETE. In aortic VSMCs, we have shown that lower concentrations of TGF-β1 stimulate but higher concentrations inhibited DNA synthesis (Fig. 4B), consistent with a previous finding in VSMCs (28). However, 20-HETE did not stimulate growth in the aortic VSMC preparation (Fig. 1C), probably because that exogenously added authentic TGF-β may not fully mimic the kinetics and the process of TGF-β release in response to 20-HETE. Nevertheless, the amount of TGF-β secreted may be a switch in the regulation of VSMC growth by 20-HETE. Furthermore, the effect of 20-HETE on cell proliferation may depend on VSMC phenotype (27). It has been demonstrated that TGF-β may exert a concentration-dependent growth-inhibitory effect in spindle-shaped cells but a growth-stimulatory effect at low concentrations in the epithelioid phenotype (27). 20-HETE may exert different effects on DNA synthesis, depending on both the amount of TGF-β secreted and the phenotype of VSMCs.

In conclusion, our study demonstrates that the growth-inhibitory effect of 20-HETE may be mediated by enhancing TGF-β secretion in VSMCs. Our results provide a novel mechanism of 20-HETE in the regulation of VSMC proliferation.

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REFERENCES


