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 [^{3}H]thymidine incorporation in a concentration-dependent manner without causing the release of lactate dehydrogenase. 20-HETE also inhibited platelet-derived growth factor (PDGF)-induced [^{3}H]thymidine incorporation in R22D cells and human VSMCs. At 5 μM, 20-HETE reduced [^{3}H]thymidine incorporation by 34 ± 6%; anti-TGF-β neutralizing antibody, but not nonspecific IgG, completely reversed the attenuated [^{3}H]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 TGF-β_1, which was reversed by anti-TGF-β antibody. Further studies demonstrated that 20-HETE may increase TGF-β release to a level high enough to inhibit [^{3}H]thymidine incorporation without altering the steady-state mRNA level of TGF-β. Nevertheless, pretreatment of indomethacin (a cyclooxygenase inhibitor) or paxilline (a potassium channel inhibitor) did not affect the inhibitory effect on DNA synthesis induced by 20-HETE. These results demonstrate for the first time a growth-inhibitory effect induced by 20-HETE, which may be mediated by TGF-β.—Liang, C-J., H. E. Ives, C-M. Yang, and Y-H. Ma. **20-HETE inhibits the proliferation of vascular smooth muscle cells via transforming growth factor-β. *J. Lipid Res.* 2008.** 49: **66–73.**

Supplementary key words 20-hydroxyeicosatetraenoic acid • cytochrome P450 • platelet-derived growth factor • DNA synthesis

20-Hydroxyeicosatetraenoic acid (20-HETE) is a cytochrome P450 metabolite of arachidonic acid (1) that plays an important role in the regulation of vascular (2–4) and renal (5–7) function. 20-HETE may act as a potent vasoconstrictor by inhibiting Ca^{2+}-activated K^+ channels (8) and increasing the conduction of L-type Ca^{2+} channels (9). In contrast, 20-HETE may be converted by cyclooxygenase to prostaglandins and act as a vasodilator (10–12). In kidney, 20-HETE induces diuresis by inhibiting Na^+-K^+-ATPase in proximal tubules (13) and $Na^+/K^+/Cl^-$ cotransporter in the thick ascending limb of Henle's loop (5–7).

In addition, 20-HETE may regulate cell growth. It has been demonstrated that 20-HETE stimulated DNA synthesis in epithelial cells (14), mesangial cells (15), fibroblasts (16), and vascular smooth muscle cells (VSMCs) (17). In cultured VSMCs from adult rabbit (18) and rat (17) as well as in isolated renal arterioles (3), 20-HETE induces the phosphorylation of extracellular regulated protein kinase 1/2 (ERK 1/2), a mitogen-activated protein kinase that plays a pivotal role in the proliferation induced by the activation 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 clearance 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-β_1, the major isoform of TGF-β, is secreted in a latent, inactive form that binds to

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Abbreviations: CDK, cyclin-dependent kinase; ECM, extracellular matrix; EPA, eicosapentaenoic acid; ERK 1/2, extracellular regulated protein kinase 1/2; FBS, fetal bovine serum; 20-HETE, 20-hydroxyeicosatetraenoic acid; LDH, lactate dehydrogenase; LTBP, latent TGF binding protein; MEK, MAPK/ERK kinase; MMP, matrix metalloprotease; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; VSMC, vascular smooth muscle cell.

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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 N_2 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-β_1, monoclonal anti-TGF-β (TGF-$\beta_{1,2,3}$), 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% CO_2 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

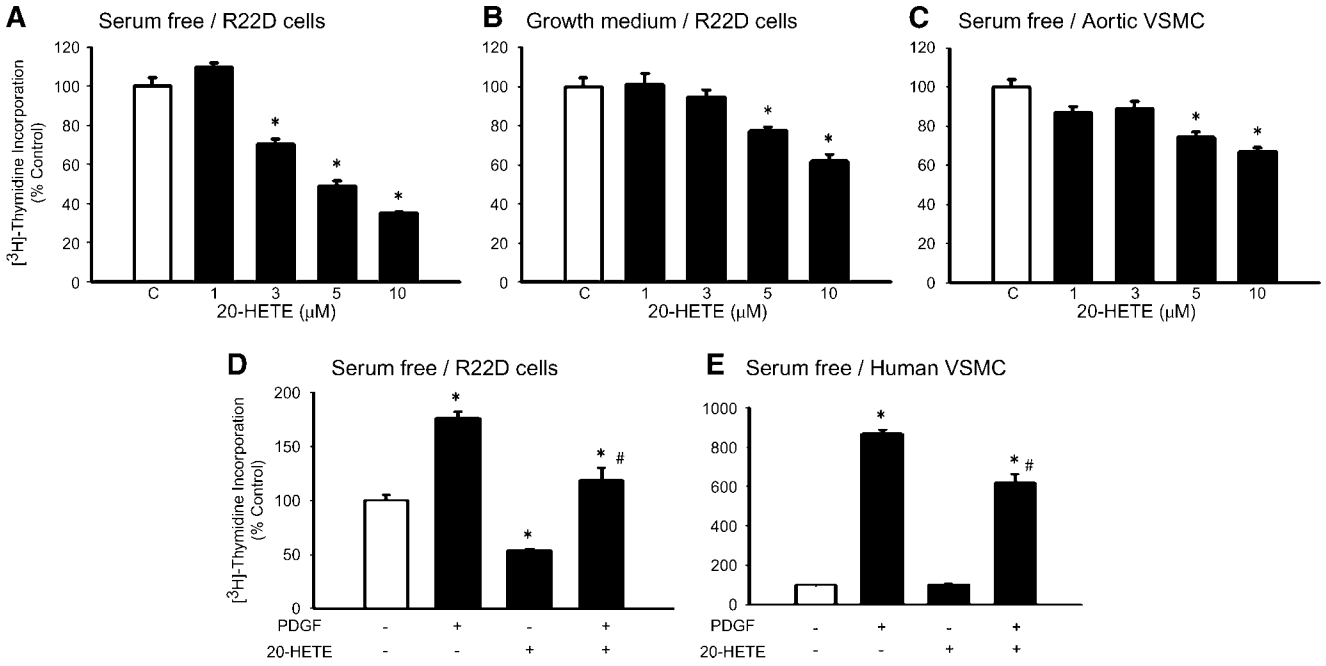

Fig. 1. 20-Hydroxyeicosatetraenoic acid (20-HETE) attenuated DNA synthesis in vascular smooth muscle cells (VSMCs) from neonatal rat and human. R22D cells (A, B, D), aortic VSMCs (C), or human VSMCs (E) were cultured in growth medium (B) or in serum-free medium for 24 h (A, C) before treatment with 20-HETE. In some experiments, quiescent R22D cells (D) and human VSMCs (E) were incubated with platelet-derived growth factor (PDGF; 5 ng/ml) in the presence or absence of 20-HETE (5 μM). After 24 h, [^{3}H]thymidine incorporation was determined (n = 4). Values are means ± SEM. * $P < 0.05$ compared with control values; # $P < 0.05$ compared with the PDGF group without 20-HETE. The results are representative of three experiments.

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 $MgCl_2$ (15 mM). The reaction was conducted for 90 min at 37°C. Quantitative real-time PCR was carried out with a sequence detection system (ABI PRISM 7000; Applied Biosystems) in reaction mixture containing 1× Smart Quant Green Master Mix (Protech Technology, Enterprise), RNA (50 ng), and the following primers: sense (5′-GCCCTGGATACCAACTACTGCT-3′) and antisense (5′-AGGCT-CCAAATGTAGGGGCAGG-3′) for TGF-β_1 and sense (5′-GTAA-CCCGTTGAACCCCATT-3′) and antisense (5′-CCATCCAATCG-GTAGTAGCG-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, and 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 FBS (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 (1:2,000), total ERK 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.

[^{3}H]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 RII antibody, or nonspecific IgG. After incubation for 18 h, 1 μCi/ml [^{3}H]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 [^{3}H]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_1 (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 H_2O_2 (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 [^{3}H]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

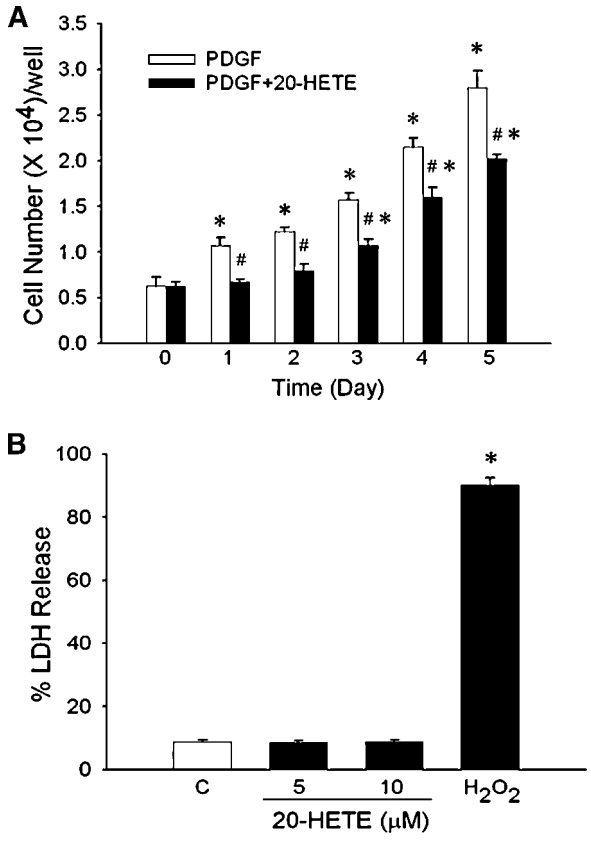

Fig. 2. 20-HETE inhibited the PDGF-induced proliferation of R22D cells without causing lactate dehydrogenase (LDH) release. A: R22D cells were exposed to serum-free medium for 24 h before the addition of PDGF (5 ng/ml) in the presence or absence of 20-HETE (5 μM; n = 4). The results shown are representative of three experiments. B: Quiescent R22D cells were exposed to 20-HETE (5 or 10 μM) or H_2O_2 (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.

[^{3}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]thymidine incorporation was also observed in aortic VSMCs (Fig. 1C); 20-HETE (5 μM) significantly inhibited [^{3}H]thymidine incorporation by 26 ± 2% (n = 4; $P < 0.05$). In addition, coadministration of 20-HETE (5 μM) with PDGF (5 ng/ml)

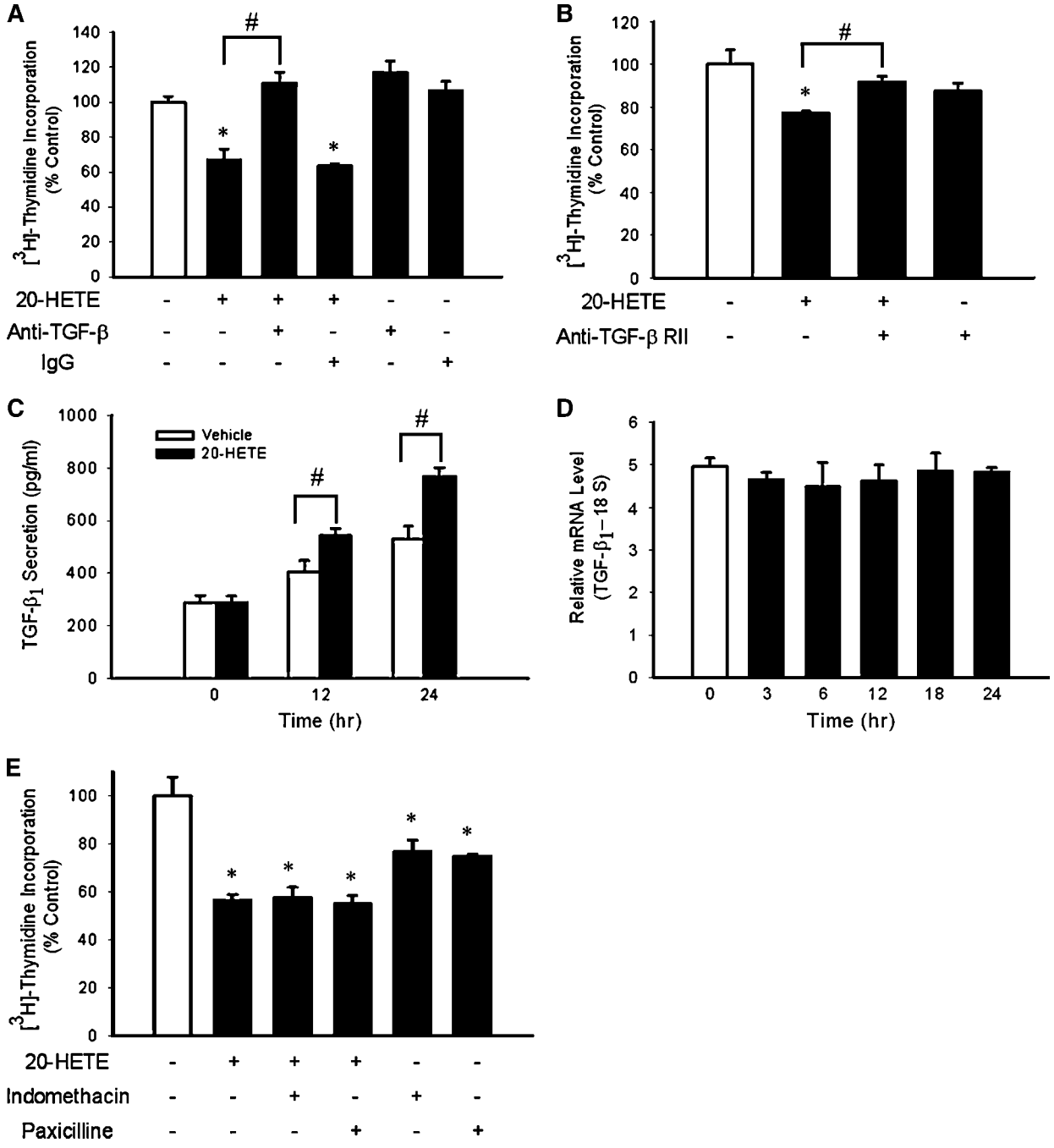

Fig. 3. Anti-transforming growth factor-β (TGF-β) antibody reversed the inhibitory effects of 20-HETE on DNA synthesis in R22D cells. A: Quiescent cells were treated with anti-TGF-β or nonspecific IgG (10 μg/ml) in the presence or absence of 20-HETE (5 μM) for 24 h before the determination of incorporated [^{3}H]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]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-β_1 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-β_1 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]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 [^{3}H]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 [^{3}H]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 H_2O_2 (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 [^{3}H]thymidine incorporation in R22D cells; however, it completely reversed the inhibitory effect of 20-HETE on [^{3}H]thymidine incorporation. Nonspecific IgG exhibited no effect on either basal [^{3}H]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 [^{3}H]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-β_1 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-β_1 secretion was 45% higher than that of the vehicle group, which corresponds to 237 ± 26 pg/ml more secreted TGF-β_1 in the medium. However, the mRNA expression of TGF-β_1 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 Ca^{2+}-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-β_1 may exert an inhibitory effect on DNA synthesis in R22D

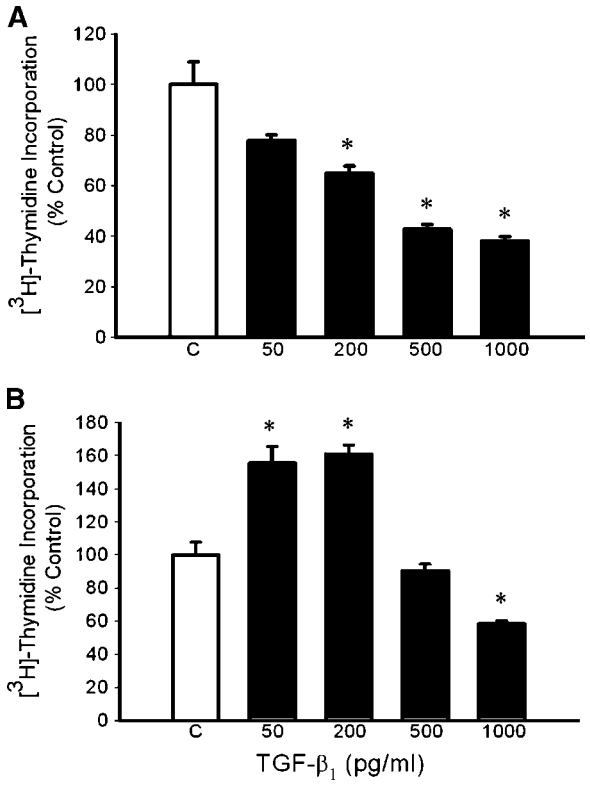

Fig. 4. Effects of the exogenous addition of TGF-β_1 on DNA synthesis in VSMCs from neonatal rats. Quiescent R22D cells (A) or aortic VSMCs (B) were treated with the indicated concentrations of TGF-β_1 for 24 h before the determination of [^{3}H]thymidine incorporation (n = 4). The radioactivity of incorporated [^{3}H]thymidine was determined by liquid scintillation counting. Values are means ± SEM. * $P < 0.05$ compared with the corresponding control values. The results shown are representative of three experiments.

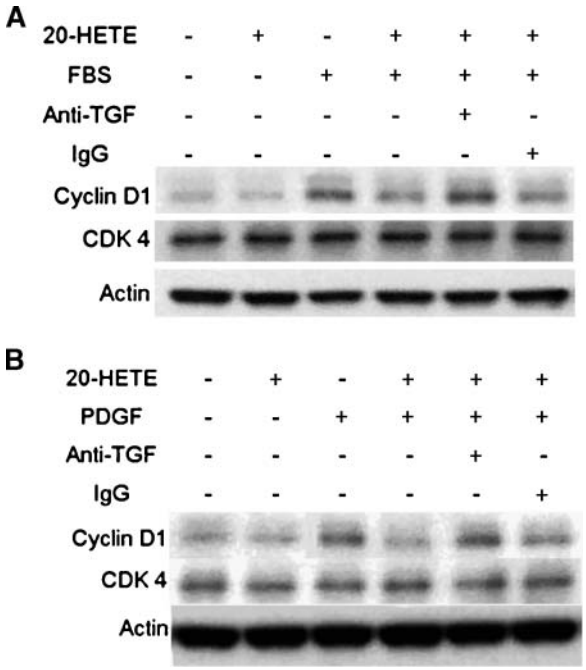

Fig. 5. Anti-TGF-β antibody reversed the inhibitory effects of 20-HETE on cyclin D1 expression in R22D cells. Quiescent cells were incubated with a mixture of 20-HETE (5 μM) and FBS (10%; A) or PDGF (5 ng/ml; B) in the presence or absence of anti-TGF-β antibody for 12 h. The expression of cyclin D1 and cyclin-dependent kinase 4 (CDK4) was determined using Western blot. The results shown are representative of four experiments.

cells, the effect of TGF-β_1 on [^{3}H]thymidine incorporation was studied in these cells. As illustrated in **Fig. 4A**, TGF-β_1 inhibited [^{3}H]thymidine incorporation of R22D cells in a concentration-dependent manner, with significant inhibition first seen at 200 pg/ml. At 200 pg/ml, TGF-β_1 significantly inhibited [^{3}H]thymidine incorporation by 36 ± 3% (n = 4; $P < 0.05$). As illustrated in Fig. 4B, TGF-β_1 exhibited a different pattern on [^{3}H]thymidine incorporation in aortic VSMCs. Low concentrations of TGF-β_1 (≤200 pg/ml) stimulated [^{3}H]thymidine incorporation, whereas high concentrations (>500 pg/ml) inhibited [^{3}H]thymidine incorporation in aortic VSMCs.

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

It has been established that TGF-β inhibits [^{3}H]thymidine incorporation via 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-

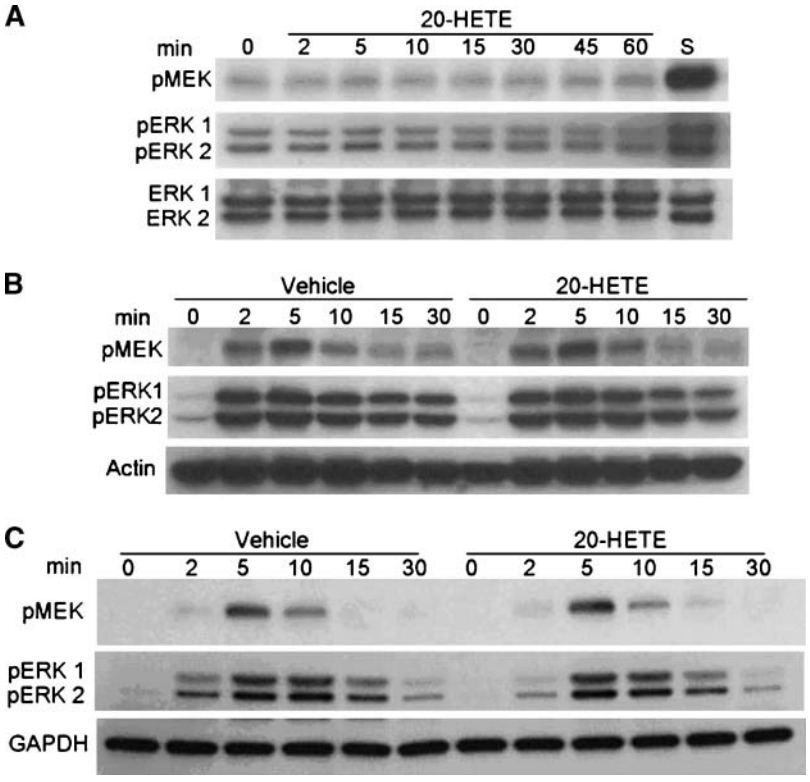

Fig. 6. 20-HETE did not affect phosphorylation of MAPK/ERK kinase (MEK) or extracellular regulated protein kinase 1/2 (ERK 1/2) in R22D cells. A: Quiescent cells were incubated with 20-HETE (5 μM) for the indicated time periods, with FBS (10%; S)-induced increase in MEK and ERK 1/2 phosphorylation as 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 RII 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-β_1 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-β_1.

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-β_1 stimulated 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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