Attenuation of Vascular Smooth Muscle Cell Proliferation by 1-Cyclohexyl-3-dodecyl Urea Is Independent of Soluble Epoxide Hydrolase Inhibition

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ABSTRACT

Epoxyeicosatrienoic acid(s) (EET) have variable hemodynamic, anti-inflammatory, and growth regulatory effects, and inhibitors of their regulatory enzyme, soluble epoxide hydrolase (sEH), can mimic these effects. For this reason, sEH inhibitors are being studied as potential pharmaceuticals for the treatment of hypertension, atherosclerosis, and inflammatory diseases. We now show that a highly selective urea-based sEH inhibitor 1-cyclohexyl-3-dodecyl urea (CDU) attenuates human aortic vascular smooth muscle (HVSM) cell proliferation independently of any effect on sEH. CDU also inhibits endothelial cells when stimulated with basic fibroblast growth factor or serum. In addition, we demonstrate that EET, as well as several newer generation sEH inhibitors and a urea-based weak sEH inhibitor, do not affect proliferation in HVSM cells. Structure-activity relationships demonstrate that the addition of an acid group to the dodecyl carbon chain, changing the cyclohexyl group to an adamantyl group, and shortening the carbon chain to two carbons all abolish the antiproliferative effect. Our finding that a highly selective urea-based inhibitor of sEH can alter biology independently of its putative target enzyme suggests that there may be other useful properties of this class of compounds unrelated to their influence on epoxyeicosanoids. In addition, our results show that caution should be used when attempting to infer conclusions of EET biology based solely on the effects these inhibitors in tissue culture models, especially when used at micromolar concentrations.
The development of highly selective and systemically available urea-based inhibitors of sEH has greatly advanced the study of EET and sEH biology. In various studies, including many from our laboratories, these sEH inhibitors have been associated with attenuation of human vascular smooth muscle (HVSM) and endothelial cell proliferation in cultured cells (Davis et al., 2002), reduction of blood pressure in spontaneously hypertensive animal models (Yu et al., 2000; Imig et al., 2002; Zhao et al., 2004), and anti-inflammatory effects (Schmelzer et al., 2005; Smith et al., 2005). Although these compounds seem to act directly through attenuation of sEH activity in some cell systems (Fang et al., 2001), in other situations, such as those described in this study, the biological response of the compounds at low micromolar concentrations is independent of inhibition of sEH.

We now show that CDU, which attenuates HVSM cell proliferation, does so independently of sEH inhibition and does not function through increased EET levels. Whereas we have yet to ascertain the precise mechanism of this growth-inhibitory effect, we report herein the first description of an sEH-independent effect by a compound in this class of selective and specific urea-based sEH inhibitors. Our finding that a compound in this class of sEH inhibitors can alter biology independently of its putative target enzyme suggests that there may be other useful properties of this class of compounds unrelated to their influence on epoxyeicosanoids. In addition, our data indicate that caution should be used when attempting to infer conclusions of EET biology based solely on the effects these inhibitors, especially when they are used at micromolar concentrations in tissue culture models.

Materials and Methods

Materials. Human recombinant platelet-derived growth factor (PDGF)-BB and FGF-2 were obtained from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal β-actin was obtained from Sigma-Aldrich (St. Louis, MO) and used at a 1:4000 dilution. Polyclonal anti-sEH antibody against human sEH was raised from rabbits and has been described previously (Wixtrom et al., 1988) and was used at 1:2000 dilution. Horseradish peroxidase-conjugated anti-rabbit IgG and horseradish peroxidase-conjugated anti-mouse IgG were obtained from Bio-Rad and used at a 1:5000 dilution. Methyl-[3H]thymidine was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). All other reagents were from Sigma-Aldrich.

Cell Culture. Human aortic smooth muscle cells and human aortic endothelial cells were obtained from Cambrex Bio Science Walkersville (Walkersville, MD) at passage 3. Cells were maintained in smooth muscle growth medium-2 or endothelial growth medium-2 medium and from Cambrex Bio Science Walkersville. Medium was changed every two to three days. Cells were growth-arrested by placing them in serum-free smooth muscle growth medium-2 or endothelial growth medium-2 medium supplemented with 20 mM Hepes (pH 7.4), 5 mg/ml transferrin, 0.5 mg/ml bovine serum albumin, 50 units/ml penicillin, 50 units/ml streptomycin, and 2.5 μg/ml amphotericin B.

Proliferation Assays. All cells were used at passages four to eight. Before each experiment, the cells were harvested, plated onto 24-well plates, grown to 80% confluence, and then serum-starved for 24 h. To stimulate proliferation, 30 ng/ml PDGF or 100 ng/ml FGF-2 was added to cells along with DMSO (vehicle), sEH inhibitor, or EET for 24 h. Cells were incubated with [3H]thymidine for the final 18 h of the assay. [3H]Thymidine incorporation was assayed by trichloroacetic acid precipitation and subsequent scintillation counting as previously described and reported as counts per minute (Weiss and Nucetelli, 1992).

Western Blots. Six-centimeter plates of HVSM and Huh-7 cells were lysed, and protein concentrations were determined by the Lowry method. Equal protein quantities were electrophoresed and Western-blotted as described previously (Weiss et al., 1998). Nitrocellulose membranes were probed with anti-sEH antibody. To confirm equal protein loading, blots were either reprobed with β-actin or equal amounts of lysates were loaded in duplicate lanes in the same gel and separated after transfer to be probed for β-actin separately.

sEH Activity. sEH activity was determined using racemic [3H]-trans-1,3-diphenylpropene oxide (tDPPO) as the substrate (Borhan et al., 1995). In brief, 1 μl of a 5 mM solution of tDPPO in N,N-dimethylformamide was added to 100 μl of homogenate in sodium phosphate buffer (0.1 M, pH 7.4) containing 0.1 mg/ml bovine serum albumin ([S] final = 50 μM). The mixtures were incubated for 30 min at 30°C, and the reaction was quenched by the addition of 60 μl of methanol and 200 μl of iso-octane. Incubations were vortexed vigorously to extract tDPPO into the iso-octane, leaving the diol metabolites in the aqueous phase. Radioactive diol metabolites were quantified by liquid scintillation counting. Assays were performed in triplicate.

Lipid Extraction and Analysis. HVSM or Huh-7 cells were plated onto 6-cm tissue culture plates and grown to 70 to 80% confluence and treated with 500 nM of EET for 24 h with and without the sEH inhibitor CDU. An equal number of cells were plated for each treatment condition. Media were removed, and cells were washed with phosphate-buffered saline three times and then they were collected with a scraper in 500 μl of phosphate-buffered saline and stored at −80°C until the lipid extraction was performed. Cellular lipids were extracted by chloroform/methanol extraction after the addition of analytical surrogates and then were subsequently subjected to base hydrolysis. EET and DHET levels in the lipid extractions were then measured using a liquid chromatography tandem mass spectrometry (LC-MS) method (Newman et al., 2002).

Results

Attenuation of Human VSM Cell Proliferation Is Independent of sEH Inhibitor Potency. We have previously shown that incubation of HVSM or endothelial cells with CDU causes dose-dependent attenuation of proliferation, as assessed by [3H]thymidine incorporation, with no apparent toxicity (Davis et al., 2002). To extend this observation, we tested for HVSM antiproliferative effects of four novel sEH inhibitors that have similar or higher inhibition potency than CDU, as well as a urea-based compound with minimal sEH inhibitory activity (Table 1) (Morisseau et al., 2002; Jones et al., 2005; Schmelzer et al., 2005). Two of these compounds were used to test whether changes to the cyclohexyl moiety or the dodecyl moiety alter the antiproliferative effects of these compounds. 1-Adamantyl-3-dodecyl urea (ADU) differs from CDU only in the replacement of the cyclohexyl group with an adamantyl group, and 12-(3-cyclohexylureido)-dodecanoic acid (CUDA) only differs in the addition of an acid group to the dodecyl tail (Table 1). The other two compounds were chosen because they were orally available sEH inhibitors that are excellent candidates for pharmaceutical use. Serum-starved HVSM cells were stimulated with PDGF-BB in the presence or absence of the each specific sEH inhibitor for
24 h, and [3H]thymidine incorporation was assessed. As reported previously (Davis et al., 2002), CDU at 5 μM reduces cell proliferation by approximately 50% (Fig. 1A). Surprisingly, despite their potency with respect to sEH inhibition, the other sEH inhibitors, CUDA, ADU, 12-(3-adamantylureido)-dodecanoic acid butyl ester (AUDA-BE), and 1-adamantyl-3-(5-(2-(2-ethoxyethoxy)ethoxy)pentyl)urea (950), do not inhibit HVSM cell proliferation at concentrations as high as 10 μM, suggesting that the action of CDU is independent of sEH inhibition (Fig. 1A).

To further examine the antiproliferative effect of CDU, we tested whether the hydrocarbon chain was required for this effect. We assessed the antiproliferative effect of the relatively poor sEH inhibitor CEU, which differs from CDU in that an ethyl group is substituted for a dodecyl tail (see Table 1). Using this compound, we found that the antiproliferative effects of CDU are abolished by the shortening the hydrocarbon chain to two carbons (CEU; Fig. 1B). Thus, the addition of a carboxylic acid group to the dodecyl tail (CUDA; Fig. 1A), replacing the cyclohexyl group with an ADU group (Fig. 1A), and shortening the hydrocarbon chain (CEU; Fig. 1B) all abolished the HVSM cell antiproliferative effects of CDU.

To determine whether the CDU inhibitory effect is a general phenomenon and is not restricted to PDGF-BB and HVSM cells, we examined the antiproliferative effect of CDU in human aortic endothelial cells (EC) stimulated with either bFGF (FGF-2) or serum. CDU caused attenuation of EC proliferation after stimulation with either bFGF (Fig. 1C) or 10% serum (Fig. 1D), showing results similar to those obtained with PDGF-BB in HVSM cells. We have previously shown that CDU inhibits proliferation of PDGF-BB-stimulated foreskin fibroblasts (Davis et al., 2002). Thus, the antiproliferative effect of CDU is restricted to neither HVSM cells nor PDGF-BB.

### EET Do Not Inhibit HVSM Cell Proliferation

We previously hypothesized that the antiproliferative effect of CDU was a result of stabilization of EET, such that the latter compound was the cellular agent inhibiting the proliferation of the cells (Davis et al., 2002). This hypothesis is supported by our previous findings that a mixture of three EET regioisomers inhibited HVSM cell proliferation, and when added in conjunction with CDU, the regioisomer mixture reduced proliferation in these cells further (Davis et al., 2002). Given the results obtained with novel sEH inhibitors described above, we reinvestigated this hypothesis.

We first retested the preparation of EET used in the earlier study and found that this preparation still inhibited HVSM proliferation (data not shown). However, LC-MS analysis showed that over time 99% of these EET had been converted to DHETs, suggesting that the epoxides were not the factor responsible for the antiproliferative effect. To confirm this hypothesis, we used a mixture of EET regioisomers recently produced in our laboratory as well as individual EET regioisomers purchased commercially (Cayman Chemical). The newly synthesized 1:4:5 mixture of 8,9–11,12–14,15-EET from our laboratory did not significantly alter proliferation in HVSM cells at concentrations ranging from 8 to 1 μM (Fig. 2A). Likewise, incubation of the cells with as much as 5 μM of either 11,12-EET or 14,15-EET from Cayman Chemical did not alter proliferation in these cells (Fig. 2B). Taken together, these data indicate that EET are not the agent(s) directly responsible for inhibiting the proliferation of cultured vascular cells in response to sEH inhibition. Furthermore, the antiproliferative effect seen with the earlier EET

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mixture is not due to DHET, because we did not find a correlation between the DHET content of the initial EET preparation and its activity. These data are consistent with observation described above that the action of CDU is independent of sEH inhibition.

Because both our newly synthesized EET and two commercially purchased EET regioisomers did not inhibit proliferation, we decided to test the hypothesis that our initial EET preparation contains an impurity that is responsible for its antiproliferative properties. The purity of these mixtures had been previously assessed by both HPLC/MS and $^1$H NMR, and the mixtures were found to be $\geq 98\%$ pure. However, during the initial synthesis, toluene had been used to azeotrope residual acetic acid in the HPLC-purified EET isomer fractions. More recently, pentane extraction has been used to remove the EET from the water/methanol-based HPLC solvent and considered as the single procedural difference in the EET synthesis within our laboratory. Therefore, using gas chromatography/MS methods, we directly evaluated the residual toluene level in the original EET preparation and assessed the possibility that this trace impurity could be responsible for the reported EET effect on vascular proliferation. From this analysis, the 10 mM stock solution of EET that had been stored in ampules at $30^\circ C$ since synthesis were found to have 0.6 ± 0.1% toluene by volume. This translates to a concentration of <0.0005% toluene being in-
introduced into the VSM proliferation assays, and this concentration did not inhibit proliferation (Fig. 3). Therefore, the source of the antiproliferative effect reported in our initial study, while not toluene, is unknown.

**Cultured HVSM Cells Lack Functional sEH.** The differential effects of various sEH inhibitors on HVSM cell proliferation and the lack of a growth inhibitory effect by the sEH substrates (the EET) led us to investigate whether HVSM cells in culture conditions possess functional sEH. The presence of sEH in HVSM cells was evaluated using three independent assays.

Cell lysate was obtained from both continuously growing HVSM cells, as well as a known sEH-containing human hepatoma cell line HuH7 (as a positive control). Immunoblotting of both of these lysates, using a monoclonal antibody to human sEH with recombinant human sEH as positive control, showed the absence of an sEH band at the appropriate molecular mass of 58 kDa in HVSM cells (Fig. 4). To confirm these data, the HVSM cell lysate was also immunoblotted using a rabbit anti-mouse sEH antibody (data not shown). This immunoblot showed a different nonspecific pattern of bands and did not detect the 65-kDa band, confirming that this band does not represent an alternate form of sEH.

Because the intracellular concentration of sEH necessary to regulate intracellular EET level is not known, it is possible that sEH could be undetectable in HVSM cells by immunoblot and yet could still be catalyzing the conversion of EET regioisomers to DHET regioisomers. To address this possibility, we measured sEH specific activity in HVSM cells and HuH7 cells using racemic tDPPO as the substrate (Borhan et al., 1995). sEH specific activity in HVSM cells was found to be less than 0.06 \pm 0.02 nmol/min \cdot mg, which is the detection limit of the assay, in marked contrast to the control sEH-positive HuH7 cell line, which has an sEH activity of 0.16 \pm 0.05 nmol/min \cdot mg. These data confirm the immunoblotting data above.

To further substantiate the lack of sEH enzyme in these HVSM cells, we used a liquid chromatography tandem mass spectrometry method to monitor changes in sEH metabolites in the presence of an sEH inhibitor. The positive conversion of EET to DHET (as indicated by an increased ratio of EET to DHET) would be evidence of the presence of sEH. HVSM and HuH-7 cells were treated with 500 nM mixed EET with and without the CDU (1 \mu M) overnight after which cellular lipids were extracted and analyzed. For this assay, a nongrowth inhibitory dose of CDU (Davis et al., 2002) was used to separate the antiproliferative effect from sEH inhibition. The addition of the sEH inhibitor CDU to these HVSM cells treated with EET did not increase the ratio of EET to DHET in cellular lipids (Fig. 5A), indicating that EET are not being converted to DHET by sEH in these HVSM cells and further supporting the likelihood that sEH is not expressed in these cells. As expected, the ratio of EET to DHET in the sEH-positive HuH7 cells was higher in cells treated with EET and CDU than it was in cells treated with EET alone (Fig. 5B). This increase in EET to DHET ratio in CDU treated HuH7 cells demonstrates that CDU inhibits sEH activity in these cells at 1 \mu M. To our knowledge, this is the lowest concentration of an sEH inhibitor that has been used to inhibit its target enzyme in cell culture.

**Discussion**

In this study, we reproduce the inhibition of HVSM cell proliferation by CDU as we have reported previously (Davis et al., 2002). However, although we have yet to ascertain the precise mechanism of its vascular antiproliferative effects, we show that CDU action is not mediated by sEH inhibition or stabilization of EET in this system. Our finding that a substituted urea compound has substantial and important biological properties, independent of its effect on sEH, suggests that there may exist other uses of this class of compounds that are unrelated to their effects on the epoxyeicosanoids.

The effects of both EET and the inhibition of sEH on proliferation in cultured mammalian cells are variable. EET increase proliferation in human umbilical vein endothelial cells (Potente et al., 2003), pig VSM cells (Fang et al., 1998), rat mesangial cells (Harris et al., 1990), rat cerebral capillary
endothelial cells (Zhang and Harder, 2002), and pig renal epithelial cells (Chen et al., 1998), all while in culture conditions. EET have no effect on proliferation in cultured bovine VSM cells (Sun et al., 2003) but we now show that EET do not effect proliferation in human aortic VSM cells. Inhibition of sEH with 4-phenylchalcone oxide potentiated EET-increased proliferation in pig VSM cells (Fang et al., 1998), and the sEH inhibitor CDU inhibits proliferation in human VSM cells, aortic ECs, and foreskin fibroblasts (Davis et al., 2002). Our findings in this study demonstrate that, in marked contrast to CDU, the sEH inhibitors CUDA, ADU, ADUA-BE, and 950 do not affect proliferation in HVSM cells.

These cell- and species-specific variation in proliferative responses to EET and sEH inhibitors may be due to normal variation in sEH expression in these cell types or an artifact of cell culture. Data from our other and laboratories have revealed sEH expression or activity in intact kidney arterioles (Yu et al., 2004), human saphenous vein, and human aorta (Yu et al., 2004). Furthermore, in the kidney, sEH expression is localized to the smooth muscle cell layer of the afferent renal arteriole (Yu et al., 2004), a finding that may help explain the antihypertensive effects of sEH inhibitors. In light of the current study and another study showing that cultured saphenous vein endothelial and VSM cells lack sEH activity (Yu et al., 2004), it is possible that sEH expression is gradually abolished during the process of cell culture.

Our finding that the sEH inhibitor CDU attenuates proliferation in HVSM cells independently of sEH may lead to reinterpretation of some of the extant data from other such studies using sEH inhibitors in cell culture systems. It may be possible to avoid these sEH-independent effects by using sEH inhibitors at lower concentrations. In this study, we demonstrate that CDU inhibits sEH activity in cultured cells at 1 μM. To our knowledge, this is the lowest concentration of an sEH inhibitor that has been used to inhibit its target enzyme in cell culture. It is unlikely that the sEH-independent effects of CDU contributed to results of past in vivo studies from our laboratory (Imig et al., 2002; Zhao et al., 2004), because the plasma concentration of CDU in those studies is more than 50-fold lower than the growth-inhibitory dose we report in this study. Additionally, the major in vivo metabolite of CDU (CUDA) does not affect HVSM cell proliferation.

Two recent studies have demonstrated that some sEH inhibitors can activate PPARα independently of sEH (Fang et al., 2005) (C. Morisseau and B. D. Hammock, submitted for publication). In those studies, structure-activity relationships of sEH inhibitors demonstrate that compounds with an acid group (i.e., CUDA, and AUDA) are more potent PPARα agonists than CDU. Our findings that CUDA does not reduce proliferation in HVSM cells and the above-referenced study showing that CDU does not activate PPARα at concentrations that inhibit proliferation in HVSM cells (Fang et al., 2005) suggest that the mechanism by which CDU elicits its antiproliferative effect is unrelated to PPARα activation.

A possible explanation for the antiproliferative effect of CDU is the presence of a heretofore undetected impurity in CDU. Although we cannot rule out this possibility, evidence suggests that an impurity is not the cause of this antiproliferative effect. First, data from another study demonstrate that an inhibitor of sEH not tested for its inhibitory effects in this study (AUDA) inhibits HVSM cell proliferation, similar to our findings with CDU (C. Morisseau and B. D. Hammock, submitted for publication). Second, both inhibitors with and without antiproliferative effects were synthesized in our laboratory using similar methodology.

Correlating the different effects of various sEH inhibitors on HVSM proliferation with their structural differences might allow us to predict sEH-independent effects in the next generation sEH inhibitors, but as of yet, we have not been successful in deducing such a correlation. Although CDU inhibits proliferation, we observed in this study that the addition of an acid group to dodecyl carbon chain (CUDA) abolishes this effect. Likewise, changing the cyclohexyl group to an ADU group leaving the dodecyl tail alone also abolished the antiproliferative properties of CDU. Lastly, shortening the carbon chain to two carbons (CEU) abolishes the antiproliferative properties of CDU (Fig. 1B). Investigation of such structure-activity relationships may allow us to predict sEH-independent effects in the next generation sEH inhibitors, but as of yet, we have not been successful in deducing such a correlation.

Further research on urea-based inhibitors of sEH may uncover clinically useful properties of these compounds independent of their effects on sEH activity. In addition to hypertension and inflammation, VSM proliferation is a major contributor to the processes of atherosclerosis and angio-plasty restenosis. Because inhibition of sEH lowers blood pressure and reduces inflammation, compounds that attenuate VSM cell proliferation and inhibit sEH would theoreti-
CDU-Induced Growth Inhibition Is Independent of sEH


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