Sunitinib inhibits catecholamine synthesis and secretion in pheochromocytoma tumor cells by blocking VEGF receptor 2 via PLC-γ-related pathways

Yuichi Aita,1 Kiyo-aki Ishii,2 Yuria Saito,1 Tatsuhiko Ikeda,3 Yasushi Kawakami,1 Hitoshi Shimano,2 Hisato Hara,3 and Kazuhiro Takekoshi1

1Department of Molecular Laboratory Medicine, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan; 2Department of Internal Medicine (Endocrinology and Metabolism), Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan; and 3Department of Breast-Thyroid-Endocrine Surgery, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan

Submitted 27 March 2012; accepted in final form 15 August 2012

SUNITINIB IS A RATIONALLY designed, oral, small molecule multitargeted receptor tyrosine kinase (RTK) inhibitor with antiangiogenic and antitumor activity that targets platelet-derived growth factor receptors (PDGFs), vascular endothelial growth factor receptors (VEGFRs), stem cell factor receptor (c-Kit), colony stimulating factor 1 receptor (CSF-1R), and fms-related tyrosine kinase 3 (Flt-3) (1, 2, 9, 13, 19, 21, 32, 43). It has been well established that sunitinib suppresses tumor angiogenesis mainly through VEGFR-2 (KDR/Flk-1), which is expressed on endothelial cells and is known to play a critical role in angiogenesis (9, 13, 19). In addition, sunitinib is reported to have direct antiproliferative and/or apoptotic effects on tumor cells, which express target RTKs (22, 33, 39).

It was previously shown that, unlike other representative RTKs, VEGFR-2 utilizes the phospholipase C (PLC)-γ/protein kinase C (PKC)/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase pathway (ERK) as the major signaling pathway. In contrast, Ras is poorly activated in VEGFR-2-related signaling pathways (40, 41).

Advanced-stage malignant pheochromocytomas are resistant to conventional therapy, such as radiation therapy and chemotherapy. However, based on three very recent reports, sunitinib appears to be useful for the treatment of malignant pheochromocytomas (15, 25, 26). Pheochromocytomas often display hypervascularity (5, 14, 27, 35). Therefore, it is reasonable to speculate that inhibition of angiogenesis could be a rational therapeutic target in these tumors, probably through a logical mechanism of action similar to that in other hypoxia-inducible tumors, such as renal cell carcinoma. However, the antitumor effects of sunitinib in malignant pheochromocytomas have not been clearly established. In particular, it is unclear whether sunitinib acts primarily through only antiangiogenic mechanisms or also directly targets tumor cells. Recent studies revealed that sunitinib markedly reduced plasma catecholamine levels in patients with pheochromocytomas (15, 25, 26). This inhibitory effect of sunitinib on catecholamine strongly suggests the possibility that sunitinib may have direct inhibitory effects on catecholamine synthesis and secretion. To support this, we recently demonstrated that sunitinib directly induced apoptosis through inhibition of the protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase 1 (pS6 kinase 1 (S6K1) pathway in PC-12 cells. Additionally, it is of note that VEGFR-2 silencing attenuated these sunitinib-induced effects, indicating that sunitinib exerts its effects through VEGFR-2 (36).

Tyrosine hydroxylase (TH) is a rate-limiting enzyme in the biosynthesis of catecholamine. TH activity is regulated by both short- and long-term mechanisms. Short-term regulation occurs at the posttranslational level. Central to this regulation is

Address for reprint requests and other correspondence: K. Takekoshi, Department of Molecular Laboratory Medicine, Graduate School of Comprehensive Human Sciences, Univ. of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan (e-mail: K-takek@md.tsukuba.ac.jp).
the phosphorylation of TH, which results in enzyme activation (4, 8, 10, 16, 17, 45). Indeed, TH is phosphorylated and activated by a variety of protein kinases, although phosphorylation of only three serine residues (Ser19, Ser31, and Ser40) is regulated in vivo. The most likely physiological candidates for the phosphorylation of TH at Ser19, Ser31, and Ser40 are Ca2+/calmodulin-dependent protein kinase II, ERK, and protein kinase A/PKC, respectively. However, only phosphorylation at Ser40 has a major effect on TH activity. In addition to the short-term regulation of TH activity, long-term control occurs at the transcriptional level (6, 20, 29). Mobilization of the short-term regulation of TH activity, long-term control of TH activity, and intracellular storage sites mediated by inositol 1,4,5-trisphosphate (IP3) formation following PLC-γ activation (12, 18).

The aim of the present study was to determine whether sunitinib directly inhibits catecholamine synthesis and/or secretion in undifferentiated PC-12 cells, which are a well-established experimental model of pheochromocytoma. However, it is known that PC-12 cells do not accurately reflect the pathogenesis of malignant cells. Recently, certain neural crest-derived tumors, such as pheochromocytoma and neuroblastoma, were reported to have common genetic background (37, 38). Therefore, we confirmed the effects of sunitinib in a human neuroblastoma cell line, SK-N-SH. An additional objective of this study was to clarify the mechanisms underlying sunitinib-mediated inhibition of catecholamine synthesis and/or secretion in these cells.

MATERIALS AND METHODS

Reagents. Unless otherwise noted, all reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sunitinib was a gift from Pfizer.

Cell culture. The PC-12 cell line (RCB0009) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). The cells were grown in DMEM (Invitrogen) containing 10% horse serum (Invitrogen) and 10% FBS (Invitrogen) containing 10% horse serum (Invitrogen) and 10% FBS. DMEM was obtained from the RIKEN Cell Bank. The cells were maintained in minimal essential medium-/H9251/ (Invitrogen) in a humidified atmosphere of 5% CO2 and 95% O2 at 37°C. DMEM was derived from Wako Pure Chemical Industries (Osaka, Japan). Sunitinib was a gift from Pfizer.

The PC-12 cell line (RCB0009) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). The cells were grown in DMEM (Invitrogen) containing 10% horse serum (Invitrogen) and 10% FBS (Invitrogen) in a humidified atmosphere of 5% CO2 and 95% O2 at 37°C. The human neuroblastoma cell line SK-N-SH (RCB0426) was obtained from the RIKEN Cell Bank. The cells were maintained in minimal essential medium-α (Invitrogen) containing 10% FBS.

TH activity. TH activity was measured using the method previously reported (3). Cells were incubated with either dimethyl sulfoxide (DMSO) (control) or sunitinib at 37°C for 12 h. The cells were then harvested, homogenized, and incubated for 30 min with 100 μl reaction buffer solution containing a pseudosubstrate and various phospholipids from a commercially available kit (Pep Taq-Non-radioactive PKC activity kit; Promega). The reaction was stopped by heating, and then the reaction mixture was separated into phosphorylated and nonphosphorylated substrates on a 0.9% agarose gel. The band intensities were measured by densitometry.

Determination of catecholamine secretion. The level of catecholamine in medium was determined as follows. Briefly, cells were grown in culture medium, which was then replaced with medium containing either DMSO (control) or sunitinib. Next, the cells were incubated at 37°C for 12 h. An aliquot of incubation medium was analyzed for catecholamine.

Determination of intracellular catecholamine contents. Catecholamine contents in cells were determined as follows. Briefly, cells were treated with either DMSO (control) or sunitinib at 37°C for 12 h. The cells were harvested and homogenized, and then the homogenates were diluted and analyzed for catecholamine. Catecholamine contents were corrected for protein concentration.

Immunoblotting. Immunoblotting was carried out as previously described (23, 36). Antibody to phospho-specific TH at Ser40 and Ser19/Ser31 were from New England Biolabs and Calbiochem, respectively. The other antibodies were purchased from Cell Signaling Technology. The band intensities were measured by densitometry.

IP3 production. Measurement of IP3 production was carried out using the fluorescence polarization detection kit [HitHunter Inositol (1,4,5)-Triphosphate Assay; DiscoveryRx]. Briefly, cells were incubated with either DMSO (control) or sunitinib at 37°C for 12 h. The cells were then harvested and titrated in a 96-well microtiter plate. Assay ran on Infinite F200 PRO (Tecan Group, Mannedorf, Switzerland). In this assay kit, the polarized signal is inversely proportional to the amount of IP3 in the cell lysate. All assays were according to the manufacturer’s instructions.

Statistical analysis. The results are expressed as means ± SE. The significance of differences in the data was determined by an analysis of the paired Student’s t-test or Dunnett’s test. P values <0.05 were considered significant. All data were analyzed using the software SPSS version 19.0 (SPSS).
also examined the effect of sunitinib on the phosphorylation of TH at Ser\(^{19}\), Ser\(^{31}\), and Ser\(^{40}\), which functions in the short-term regulation of enzyme activity. Sunitinib significantly decreased TH phosphorylation at Ser\(^{31}\) and Ser\(^{40}\) in a dose- and time-dependent manner (Fig. 2). In contrast, sunitinib did not affect phosphorylation at Ser\(^{19}\) (data not shown).

Effect of sunitinib on TH protein levels in PC-12 cells. We also examined the effect of sunitinib on the long-term regulation of TH activity. Cells were treated with sunitinib (1,000 nM), and TH protein level was examined by immunoblotting. As shown in Fig. 3, sunitinib significantly decreased the level of TH protein, to ~60% of the basal value.
Effect of sunitinib on catecholamine release and intracellular catecholamine in PC-12 cells.

As shown in Fig. 4A, sunitinib (1,000 nM) significantly inhibited catecholamine secretion to ~40% of the basal level. It is known that the intracellular catecholamine level is correlated with catecholamine synthesis. Therefore, to confirm that sunitinib inhibits catecholamine synthesis, we examined the effect of sunitinib on intracellular catecholamine. As shown in Fig. 4C, sunitinib (1,000 nM) significantly reduced intracellular catecholamine to ~60% of the basal level.

Effect of VEGFR-2 knockdown on sunitinib-mediated inhibition of catecholamine synthesis and secretion in PC-12 cells.

To further confirm whether sunitinib exerts its effects through VEGFR-2, we examined the effect of VEGFR-2 knockdown on catecholamine synthesis and secretion. VEGFR-2 knockdown by small-interfering RNA (siRNA) was previously confirmed by immunoblotting (36). As shown in Figs. 1A, 4A, and 4C, VEGFR-2 silencing attenuated sunitinib-induced effects, such as the reduction of TH activity, catecholamine secretion, and intracellular catecholamine, compared with that of the negative control siRNA. These results indicate that these sunitinib-mediated inhibitory effects could be mediated by VEGFR-2.

It is known that sunitinib exerts its effects via multiple RTKs. Indeed, Manley et al. (30) reported that the half-maximal inhibitory concentration (IC50) values of sunitinib for PDGFR-α and PDGFR-β are 55 and 35 nM, whereas those for VEGFR-1, -2, and -3 are 15, 38, and 30 nM, respectively. Moreover, sunitinib is also a potent inhibitor of CSF-1R, Flt-3, and c-Kit (IC50 values of 35, 21, and 10 nM, respectively) (30). Consistent with this notion, multitargeted therapies, such as sunitinib, are likely to have more antitumor activities than those targeting a single pathway. Therefore, we determined whether multiple RTKs, such as PDGFR-α, PDGFR-β, c-Kit, Flt-3, and CSF-1R, were involved in sunitinib-mediated inhibition of catecholamine synthesis and secretion. As shown in Fig. 1B, mRNA expression was determined by quantitative real-time PCR, confirming the knockdown of target genes by siRNA. In contrast to
what was observed with knockdown of VEGFR-2, knockdown of these other RTKs did not affect the sunitinib-mediated inhibition of either catecholamine synthesis or secretion (Figs. 1C and 4B).

**Effect of sunitinib on PLC-γ and PKC in PC-12 cells.** It is known that VEGFR-2 utilizes the PLC-γ/PKC/Raf/MEK/ERK pathway (40, 41). To clarify the mechanisms underlying sunitinib-mediated inhibition of TH activity, we examined the effect of sunitinib on PLC-γ phosphorylation. As shown in Fig. 5A, sunitinib at 100 and 1,000 nM significantly decreased PLC-γ phosphorylation to ~50% of the basal value. Phosphorylation of TH at Ser40 has a major effect on activity and is known to be regulated by PKC. Combining these findings with the inhibition of TH activity and Ser40 phosphorylation by sunitinib, we examined the effect of sunitinib on PKC activity. As shown in Fig. 5B, sunitinib at 100 and 1,000 nM significantly decreased PKC activity to ~70 and 60% of the basal value, respectively. Therefore, sunitinib may inhibit TH phosphorylation at Ser40 via inhibition of the VEGFR-2/PLC-γ/PKC pathway.

**Effect of sunitinib on mitogen-activated protein kinase activity in PC-12 cells.** Mitogen-activated protein kinases (MAPK) pathways are known to be among the most significant cellular signaling sequences (11). Therefore, we determined whether sunitinib affects the activity of MAPKs, including p44 and p42 MAPK (ERK), p46 and p54 c-Jun NH2-terminal kinase, and p38 MAPK (p38-α, -β, -γ, and -δ), in PC-12 cells. When cells were treated with sunitinib for 12 h, ERK phosphorylation decreased significantly with 100 nM sunitinib, and was ~50% at 1,000 nM (Fig. 6A). Inhibition was evident 12 h after onset of treatment and lasted for at least 48 h (at ~50% of the basal value) (Fig. 6B). In contrast to the sunitinib-mediated inhibition of ERK phosphorylation, sunitinib did not affect the other MAPKs tested (data not shown).

**Effect of sunitinib on IP3 production in PC-12 cells.** Because sunitinib was shown to inhibit PLC-γ phosphorylation (Fig. 5A), we tested whether sunitinib inhibited the production of catecholamine production in PC-12 cells. Cells were treated with either DMSO (control) or sunitinib (1,000 nM) for 12 h. In other experiments, cells were transfected with either siVEGFR-2 or siCON, and, 48 h later, the cells were treated with sunitinib (1,000 nM) for 12 h. The media were examined as described in MATERIALS AND METHODS. B: the sunitinib-mediated inhibition of catecholamine secretion is not affected by the silencing of PDGFR-α, PDGFR-β, c-Kit, Flt-3, or CSF-1R in PC-12 cells. Cells were treated with either DMSO (control) or sunitinib (1,000 nM) for 12 h. In other experiments, cells were transfected with either siCON or siRNA targeting PDGFR-α, PDGFR-β, c-Kit, Flt-3, and CSF-1R, and, 48 h later, the cells were treated with sunitinib (1,000 nM) for 12 h. The medium were examined as described in MATERIALS AND METHODS. C: sunitinib inhibits intracellular catecholamine content, and this inhibition was abolished by VEGFR-2 silencing in PC-12 cells. Cells were treated with either DMSO (control) or sunitinib (1,000 nM) for 12 h. In other experiments, cells were transfected with either siCON or siRNA targeting PDGFR-α, PDGFR-β, c-Kit, Flt-3, and CSF-1R, and, 48 h later, the cells were treated with sunitinib (1,000 nM) for 12 h. Cell lysates were examined as described in MATERIALS AND METHODS. The values shown represent the means ± SE (n = 4–6). Because the major catecholamine synthesized in PC-12 cells is dopamine, and the production of norepinephrine and epinephrine was negligible, dopamine levels are presented. *P < 0.05 vs. the basal value. #P < 0.05 vs. the value in siCON-transfected cells treated with sunitinib (1,000 nM).
IP₃, a messenger for the mobilization of Ca²⁺ from intracellular storage sites. As shown in Fig. 7, sunitinib at 100 and 1,000 nM significantly increased the polarized signal, representing the reduction of IP₃ production in cells.

Effect of sunitinib on TH activity in a human neuroblastoma cell line. To confirm the key findings of this study, we examined the effect of sunitinib on TH activity in a human neuroblastoma cell line, SK-N-SH (37, 38). Similar to what was observed in PC-12 cells, TH activity in SK-N-SH cells decreased significantly, to ~60% of the basal value (Fig. 8). In addition, this inhibition was abolished by VEGFR-2 silencing in SK-N-SH cells.

DISCUSSION

In this study, we demonstrated that sunitinib directly inhibits catecholamine synthesis and secretion in pheochromocytoma PC-12 cells, indicating that the sunitinib-mediated inhibition of catecholamine secretion observed in a previous paper may be explained, at least in part, through the direct inhibition of tumor cells rather than the inhibition of angiogenesis. Indeed, we demonstrated that sunitinib (10 nM) significantly decreased TH activity (Fig. 1A). Because sunitinib exhibits a similar inhibi-
Sunitinib inhibits TH activity, and this inhibition is abolished by VEGFR-2 silencing in a human neuroblastoma cell line. SK-N-SH cells were incubated for 12 h with either DMSO (control) or sunitinib (1,000 nM). In other experiments, SK-N-SH cells were transfected with either siVEGFR-2 or siCON, and, 48 h later, the cells were treated with sunitinib (1,000 nM) for 12 h. TH activity was measured as described in MATERIALS AND METHODS. The data shown represent the means ± SE of 3 independent experiments (1 experiment was performed with 2 samples). *P < 0.05 vs. the basal value. **P < 0.05 vs. the value in siCON-transfected cells treated with sunitinib (1,000 nM).

To reinforce these findings, sunitinib significantly decreased VEGFR-2 phosphorylation at two sites, Ser31 and Ser40, known to correlate with TH activity (Fig. 2). Specifically, sunitinib at 1,000 nM significantly decreased TH protein (Fig. 3). Therefore, it is likely that sunitinib directly reduces TH activity by controlling short- and long-term regulation in PC-12 cells. In agreement with the reduction in TH activity, intracellular catecholamine was significantly decreased (Fig. 4C).

To further confirm that VEGFR-2 was involved in sunitinib-mediated inhibition, we silenced VEGFR-2 with siRNA. Indeed, VEGFR-2 knockdown attenuated the sunitinib-induced effects, including the reduction of TH activity, catecholamine secretion, and intracellular catecholamine, confirming that these sunitinib-induced effects could be mediated by VEGFR-2 (Figs. 1A, 4A, and 4C). It is known that sunitinib exerts its effects via multiple RTKs (30). To exclude the possibility that other RTKs were involved in the sunitinib-induced effects we observed, we knocked down multiple RTKs, such as PDGFR-α, PDGFR-β, c-Kit, Flt-3, and CSF-1R (Fig. 1B). However, silencing of other RTKs did not affect the inhibitory effect of sunitinib on either catecholamine synthesis or secretion (Figs. 1C and 4B). Although we did not examine all of the RTKs reported, our results support the idea that the inhibitory effects obtained were mainly due to inhibition of VEGFR-2 and not due to the inhibition of the other RTKs, in agreement with the fact that silencing of VEGFR-2 alone markedly attenuated the sunitinib-induced effects.

To reinforce these findings, sunitinib significantly decreased PLC-γ phosphorylation and PKC activity in PC-12 cells (Fig. 5). Because TH phosphorylation at Ser40 is positively regulated by PKC, it is reasonable to speculate that sunitinib inhibits TH phosphorylation at Ser40 through the inhibition of VEGFR-2, followed by the PLC-γ/PKC pathway, thus resulting in a significant reduction of TH activity in PC-12 cells.

The physiological relevance of TH phosphorylation at Ser31 is unclear. However, it was reported that at Ser31 phosphorylation might be required for full TH activation (7). In our experimental system, we demonstrated that sunitinib decreased ERK phosphorylation (Fig. 6). Although the physiological relevance of the differences between MAPKs observed in the present study remains unclear, it suggests that sunitinib may reduce TH phosphorylation at Ser31 through inhibition of the VEGFR-2/PLC-γ/PKC/Raf/MEK/ERK pathway.

Another important point made in this study was that sunitinib inhibited catecholamine secretion from PC-12 cells. Additionally, we demonstrated that sunitinib significantly inhibited IP3 production (Fig. 7). It is well established that [Ca2+]i, is regulated by Ca2+ release from intracellular storage sites mediated by IP3 formation following PLC-γ activation (12). Therefore, we can suggest that sunitinib inhibits PLC-γ phosphorylation and leads to reduced IP3 production, which mediates [Ca2+]i, and is consistent with the notion that sunitinib directly inhibits catecholamine secretion. The precise relationship between VEGFR-2 and Ca2+ mobilization remains obscure. However, it has been previously shown that PLC-γ activation via VEGFR stimulation, followed by IP3 production and increased PKC activity, leads to increased [Ca2+]i, in primary endothelial cells (31). The pathophysiological role of RTKs, including VEGFR, in regulating catecholamine secretion in chromaffin cells also remains uncertain (28). Further studies are needed to clarify these points.

It is known that pheochromocytoma PC-12 cells, which are well established and extensively used, do not accurately reflect the pathogenesis of malignant tumor cells. Pheochromocytomas and neuroblastomas are derived from the sympathetic nervous system. Despite their embryologic relationship, a review of the literature suggests the lack of an association
between them. However, regarding the molecular pathogenesis, these neural crest-derived tumors reportedly share a common genetic background, such as SDHB and KIF1Bβ (37, 38). Therefore, we confirmed that sunitinib exerted the same effects on catecholamine synthesis in a human neuroblastoma cell line (Fig. 8).

In the clinical setting, sunitinib is reported to significantly inhibit catecholamine secretion (15, 25, 26). This, along with our present findings, suggests that sunitinib directly inhibits catecholamine synthesis and secretion during treatment of malignant pheochromocytomas. In addition, it is of note that TH is strongly activated in pheochromocytomas compared with normal adrenal medullas (24). Therefore, inhibition of TH activity by sunitinib seems to be the rationale for treatment of malignant pheochromocytomas.

Growing evidence suggests that the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR/S6K1 pathways may play an important role in the pathogenesis of pheochromocytomas. TMEM127, a new tumor suppressor gene reported to be associated with pheochromocytoma development, encodes a protein that suppresses mTOR activation. Indeed, the authors found that S6K1 phosphorylation increased in TMEM127 mutant pheochromocytomas compared with normal adrenal medullas (34, 42, 44). Therefore, activation of the PI3K/Akt/mTOR/S6K1 pathways may also be involved in the pathogenesis of pheochromocytomas.

We recently demonstrated that sunitinib directly induces apoptosis in PC-12 cells by inhibiting the VEGFR-2/Akt/mTOR/S6K1 pathways through modulation of Bcl-2 and Bad (36). Based on this finding along with our present results, it would appear that sunitinib exerts its antitumor effects, at least in part, through direct inhibition of catecholamine synthesis and secretion as well as apoptosis rather than by inhibiting angiogenesis (Fig. 9).

GRANTS
This study was supported in part by a Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, No. 21591168 (to K. Takekoshi) and No. 23591889 (to H. Hara).

DISCLOSURES
The authors have nothing to declare.

AUTHOR CONTRIBUTIONS

REFERENCES
SUNITINIB INHIBITS CATECHOLAMINE PRODUCTION IN PC-12 CELLS


