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

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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/Flik-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 (ERK)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase 1 (S6K1) pathway in PC-12 cells. Additionally, it is of note that VEGFR-2 knockdown attenuated these effects, indicating inclusion of TH activity and catecholamine secretion, suggesting that they were mediated by VEGFR-2. Sunitinib significantly decreased phospholipase C (PLC)-γ phosphorylation and subsequent protein kinase C (PKC) activity. Because Ser40 phosphorylation significantly affects TH activity and is known to be regulated by PKC, sunitinib may inhibit Ser40 phosphorylation via the VEGFR-2/PLC-γ/PKC pathway. Additionally, sunitinib markedly decreased the activity of extracellular signal-regulated kinase (ERK), but not c-Jun NH2-terminal kinase or p38 mitogen-activated protein kinase. Therefore, sunitinib may reduce TH Ser41 phosphorylation through inhibition of the VEGFR-2/PLC-γ/PKC/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase/ERK pathway. Sunitinib also significantly reduced inositol 1,4,5-trisphosphate production. However, because PC-12 cells do not precisely reflect the pathogenesis of malignant cells, we confirmed the key findings in a human neuroblastoma cell line, SK-N-SH. In conclusion, sunitinib directly inhibits catecholamine synthesis and secretion in pheochromocytoma PC-12 cells.
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

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) 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 homogenized in 0.25 M sucrose (50 volumes) using a glass tissue grinder. The standard incubation medium consisted of the following components in a total volume of 250 μl: 100 μl cell homogenate, 40 μl of 1 M sodium acetate buffer (pH 6.0), 40 μl of 1 mM L-tyrosine or D-tyrosine, 20 μl of 1 M 6-methyl-5,6,7,8-tetrahydropterine in 1 M 2-mercaptoethanol, 20 μl of 20 mg/ml catalase, and 30 μl water. The medium was incubated at 37°C for 30 min, and the reaction was stopped by addition of 1 M perchloric acid containing dihydroxybenzylamine as an internal standard, and 0.2 M EDTA, and by storage at ice temperature. Next, 1 M potassium carbonate and 0.2 M Tris-HCl (pH 8.5) containing 2% EDTA were added. The 3,4-dihydroxyphenylalanine (DOPA) was extracted using the aluminum oxide method. Two hundred forty microliters of extracted medium were mixed with 0.1 N NaOH and Amberlite CG-50 (PM Biomedicals, Ilkirch, France) and analyzed by HPLC. The mobile phase consisted of the following components: 50 mM sodium acetate, 20 mM citric acid, 20 mM sodium octyl sulfate, 1 mM di-n-butylamine, and 0.134 mM EDTA. All separations were performed isocratically at a flow rate of 0.6 ml/min at 29°C. The enzyme activity was calculated as the amount of DOPA formed from tyrosine per milligram protein in 30 min.

Small-interfering RNA transfection. RNA interference was performed as previously described (36). Briefly, cells were grown in culture medium, and, 24 h later, the cells were transfected with Silencer Select siRNA (Ambion) using RNAiMAX (Invitrogen) according to the manufacturer’s instructions. At the same time, nontargeting Silencer Select siRNA (Ambion) was transfected as a negative control. Quantitative real-time PCR was carried out as described previously (36).

PKC activity. PKC activity was measured as previously described (3). Cells were incubated with either 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.

Effect of sunitinib on TH activity in PC-12 cells. Because TH is the initial and rate-limiting enzyme in catecholamine biosynthesis, we tested the effect of sunitinib on TH activity. Nicotine is known to stimulate catecholamine synthesis in chromaffin cells. Initially, we confirmed that nicotine (10 μM) increased TH activity ~1.5-fold compared with the basal value in PC-12 cells; thus, this was used as a convenient positive control in this study. As shown in Fig. 1A, sunitinib at 10, 100, and 1,000 nM significantly decreased TH activity to 79, 74, and 68% of the basal value, respectively.

Effect of sunitinib on TH phosphorylation in PC-12 cells. To reinforce the sunitinib-mediated inhibition of TH activity, we...
also examined the effect of sunitinib on the phosphorylation of TH at Ser\textsuperscript{19}, Ser\textsuperscript{31}, and Ser\textsuperscript{40}, which functions in the short-term regulation of enzyme activity. Sunitinib significantly decreased TH phosphorylation at Ser\textsuperscript{31} and Ser\textsuperscript{40} in a dose- and time-dependent manner (Fig. 2). In contrast, sunitinib did not affect phosphorylation at Ser\textsuperscript{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 \sim 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 (IC_{50}) 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 (IC_{50} 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

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**Fig. 3.** Effect of sunitinib on TH protein level in PC-12 cells. Cells were incubated with sunitinib (1,000 nM) for the indicated times, and then TH protein was measured by immunoblotting as described in MATERIALS AND METHODS. Representative data are shown, and these data were reproducible in 3 independent experiments. The value in the histogram represents the mean ± SE of the densitometric measurements of the indicated parameter. Under basal conditions, this ratio was set to 100%. *P < 0.05 vs. the basal value.

**Fig. 4.** A: sunitinib inhibits catecholamine secretion, and this inhibition is 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 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 media 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).
SUNITINIB INHIBITS CATECHOLAMINE PRODUCTION IN PC-12 CELLS

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-

**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-

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**Fig. 5.** Sunitinib inhibits phospholipase C (PLC)-γ phosphorylation and protein kinase C (PKC) activity in PC-12 cells. Cells were treated with either DMSO (control) or various concentrations of sunitinib (10, 100, and 1,000 nM) for 12 h. Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-phospho-specific PLC-γ (Try783) or anti-PLC-γ antibody (A). PKC activity was assayed as described in MATERIALS AND METHODS (B). Representative data are shown, and these data were reproducible in 3 independent experiments. The value in the histogram represents the mean ± SEM of the densitometric measurements of the indicated parameter. Under basal conditions, this ratio was set to 100%. *P < 0.05 vs. the basal value.

**Fig. 6.** Effect of sunitinib on extracellular signal-regulated kinase (ERK) activity in PC-12 cells. Cells were treated with sunitinib at the indicated concentrations for 12 h (A) or at 1,000 nM for the indicated times (B). Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-phospho-p44 and p42 mitogen-activated protein kinase (MAPK) antibody or anti-p44 and p42 MAPK antibody. Details are described in MATERIALS AND METHODS. Representative data are shown, and these data were reproducible in 3 independent experiments. The value in the histogram represents the mean ± SEM of the densitometric measurements of the indicated parameter. Under basal conditions, this ratio was set to 100%. *P < 0.05 vs. the basal value.

**Fig. 7.** Effect of sunitinib on inositol 1,4,5-trisphosphate (IP₃) production in PC-12 cells. Cells were treated with sunitinib at the indicated concentrations for 12 h, and IP₃ levels were measured as described in MATERIALS AND METHODS. The polarized signal is inversely proportional to the amount of IP₃. The data shown represent the mean ± SE of 3 independent experiments (1 experiment was performed with 2 samples). *P < 0.05 vs. the basal value.
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).

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...
SUNITINIB INHIBITS CATECHOLAMINE PRODUCTION IN PC-12 CELLS

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 pheochromocytomas, 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).

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DISCLOSURES

The authors have nothing to declare.

AUTHOR CONTRIBUTIONS


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