Overexpression of G Protein–Coupled Receptor Kinase-2 in Smooth Muscle Cells Attenuates Mitogenic Signaling via G Protein–Coupled and Platelet-Derived Growth Factor Receptors

Karsten Peppel, PhD; Anne Jacobson, BA; Xuewei Huang, BS; John P. Murray, BS; Martin Oppermann, MD; Neil J. Freedman, MD

Background—Neointimal hyperplasia involves activation of smooth muscle cells (SMCs) by several G protein–coupled receptor (GPCR) agonists, including endothelin-1, angiotensin II, thrombin, and thromboxane A₂. Signaling of many GPCRs is diminished by GPCR kinase-2 (GRK2). We therefore tested whether overexpression of GRK2 in SMCs could diminish mitogenic signaling elicited by agonists implicated in the pathogenesis of neointimal hyperplasia.

Methods and Results—Overexpression of GRK2 was achieved in primary rabbit aortic SMCs with a recombinant adenovirus. Control SMCs were infected with an empty vector adenovirus. Inositol phosphate responses to endothelin-1, angiotensin II, thrombin agonist peptide, and platelet-derived growth factor (PDGF) were attenuated by 37% to 72% in GRK2-overexpressing cells (P<0.01), but the response to the thromboxane A₂ analogue U46619 was unaffected. GRK2 also inhibited SMC [³H]thymidine incorporation stimulated not only by these agonists (by 30% to 60%, P<0.01) but also by 10% FBS (by 35%, P<0.05). However, GRK2 overexpression had no effect on epidermal growth factor–induced [³H]thymidine incorporation. Agonist-induced tyrosine phosphorylation of the PDGF-β receptor, but not the epidermal growth factor receptor, was reduced in GRK2-overexpressing SMCs. GRK2 overexpression also reduced SMC proliferation in response to endothelin-1, PDGF, and 10% FBS by 62%, 51%, and 29%, respectively (P<0.01), without any effect on SMC apoptosis.

Conclusions—GRK2 overexpression diminishes SMC mitogenic signaling and proliferation stimulated by PDGF or agonists for several GPCRs. Gene transfer of GRK2 may therefore be therapeutically useful for neointimal hyperplasia.

Key Words: muscle, smooth ■ cells ■ signal transduction ■ receptors

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From the Department of Medicine (Cardiology), Duke University Medical Center, Durham, NC, and the Department of Immunology, Universitätskliniken, Göttingen, Germany (M.O.).

Correspondence to Neil J. Freedman, MD, Box 3187, Duke University Medical Center, Durham, NC 27710. E-mail neil.freedman@duke.edu

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system, we assessed SMC responsiveness to the G\textsubscript{x}-coupled receptor agonists ET-1, thrombin, Ang II, and TXA\textsubscript{2}, and also—because of its apparent role in NH—platelet-derived growth factor (PDGF).

**Methods**

**Primary SMC Isolation and Culture**

Thoracic aortas were harvested from euthanized male New Zealand White rabbits, and primary SMCs were obtained by the explant outgrowth technique\textsuperscript{11} in the presence of mycoplasma removal agent (50 \mu g/mL, ICN Pharmaceuticals, Inc). Immunofluorescence for SMC \(\alpha\)-actin confirmed SMC identity.\textsuperscript{11} SMCs were passaged in DMEM/10% FBS with antibiotics,\textsuperscript{8} split 1:4, and discarded after passage 7.

**Adenovirus Production and Infection**

The bovine GRK2 cDNA\textsuperscript{12} was inserted into the plasmid pSKAC, as described previously.\textsuperscript{13} The GRK2ct adenovirus has been described.\textsuperscript{13}

Subconfluent SMCs were infected in batches of 150-mm plates. A single plate was trypsinized and counted; subsequently, plates for infection were washed and exposed (37°C, 30 minutes) to 5 mL of infection medium (DMEM/2% FBS/25 mmol/L HEPES; pH 7.4) with or without virus (multiplicity of infection\textsuperscript{=}100), with gentle agitation. Virus-containing medium was removed, and plates were incubated in fresh infection medium for 24 hours. Cells were then trypsinized and divided into aliquots in various dishes for assays, at either 2.6 \times 10^4 cells/cm\textsuperscript{2} (phosphoinositide hydrolysis and \([\text{3H}]\)thymidine \([\text{3H}]\text{TdR}\) incorporation) or 1.3 \times 10^5 cells/cm\textsuperscript{2} (proliferation, cell cycle, and immunoblotting assays).

**Phosphoinositide Hydrolysis**

SMCs were rendered quiescent by a 72-hour incubation in low-mitogen medium (DMEM supplemented with 20 mmol/L HEPES [pH 7.4], fatty acid–free BSA [1 mg/mL], 0.2% FBS, insulin [1.7 \mu g/mL], transferrin [5.5 \mu g/mL]), sodium selenite [6.7 ng/mL], and antibiotics). SMCs were labeled in this medium containing 2 \mu Ci/mL of \([\text{3H}]\)inositol for 12 to 18 hours, and then assayed for agonist-stimulated phosphoinositide (PI) hydrolysis, as described previously.\textsuperscript{8}

\([\text{3H}]\text{TdR}\) Incorporation

SMCs were plated in 24-well plates, rendered quiescent as described above, and treated on day 1 with the indicated stimulus diluted in low-mitogen medium. On day 6, SMCs were re-treated as on day 1. On day 12, SMCs were washed, incubated with Hoechst 33258 (5 \mu g/mL) for 30 minutes (25°C), washed with PBS, and visualized with a Chroma Blue GEP filter on a Leica fluorescence microscope. From each slide chamber, 4 images (at \(\times 10\) magnification) were captured with Adobe Photoshop. Nuclei were counted with the UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio and available from the Internet by anonymous FTP from maxrad6.uthscsa.edu). At least 200 cells per well of each chamber slide were counted.

**Cell Cycle Analysis**

SMCs in low-mitogen medium were assayed flow-cytometrically for DNA content on days 6 and 12 of the proliferation assays.\textsuperscript{15} Analyses on days 6 and 12 were equivalent, so only those from day 12 are presented. To ensure that apoptotic or necrotic SMCs could be identified,\textsuperscript{15} we harvested and pelleted debris or floating SMCs in the medium and pooled this pellet with that from SMCs removed from the plate by trypsinization. SMCs were stained with Hoechst 33258, and 10\(^3\) cells per cell line were analyzed. The fraction of apoptotic cells increased 4- to 6-fold with a 4-hour treatment with 1 \mu mol/L staurosporine (Sigma) (data not shown).

**Statistical Analysis**

To facilitate pooling of results across several independent experiments, results within each experiment were normalized to values obtained from SMCs infected with the empty vector adenovirus. With Prism software (GraphPad), data from uninfected, vector-infected, and GRK2- or GRK2ct-virus–infected SMCs were analyzed by repeated-measures (ie, paired within individual experiments) 1-way ANOVA. Tukey’s multiple comparison test was used to compare the SMC groups with one another. Data are presented in the text as \pm SD, and probability values are 2-tailed.

**Results**

**Overexpression of GRK2 in SMCs**

Cells migrating out from de-endothelialized aortic explants were identified as SMCs by immunofluorescent staining for SMC \(\alpha\)-actin\textsuperscript{14} (Figure 1A). The \(\approx 90\%\) prevalence of cell staining for SMC \(\alpha\)-actin was assessed by comparison of immunofluorescence for SMC \(\alpha\)-actin with fluorescent nuclear staining of all cells (data not shown).

To achieve overexpression of GRK2 in these primary SMCs, we infected them with a recombinant adenovirus that effected GRK2 overexpression in nearly 100% of the SMCs (compare Figure 1C and 1D), with a predominantly cytoplas-
The distribution of the GRK2 (Figure 1B). This GRK2 overexpression was $40\times$ above endogenous levels, as assessed by immunoblotting (Figure 2). GRK2 overexpression persisted for 12 days in SMCs maintained in low-mitogen medium. Immunoblotting of solubilized SMC protein along with a purified GRK2 standard demonstrated that these GRK2 adenovirus–infected SMCs expressed 12 to 25 pmol GRK2/mg total cell protein. Uninfected and empty vector adenovirus–infected SMCs were indistinguishable with regard to GRK2 expression, and GRK2 overexpression did not alter SMC $\alpha$-actin expression (data not shown).

**Agonist-Induced SMC PI Hydrolysis and $[^3H]$TdR Incorporation: Effects of GRK2 Overexpression**

To assess SMC second-messenger signaling in response to agonists implicated in NH, we studied PI hydrolysis mediated by phospholipase C isoforms $\beta$ (stimulated by $G_{q}$-coupled, heptahedral receptors) and $\gamma$ (stimulated by receptor tyrosine kinases, like the PDGFR) (Figure 3A). ET-1, Ang II, thrombin agonist peptide, and PDGF-BB each promoted a time-dependent accumulation of inositol phosphates, ranging from 1.5±0.3- to 9±4-fold over basal at 15 minutes.
Agonist-promoted PI hydrolysis was reduced by 37% to 72% in SMCs overexpressing GRK2 (Figure 3B). Interestingly, signaling inhibition by GRK2 appeared to be receptor-specific, because PI hydrolysis induced by the TXA2 analogue U46619 (3.8-fold over basal) was unaffected by GRK2 overexpression. Thus, GRK2 overexpression attenuated second-messenger signaling promoted not only by several GPCR-coupled receptors but also, surprisingly, by the PDGF-BB receptor in SMCs, which either did or did not overexpress GRK2 (Figure 6). GRK2 overexpression reduced agonist-induced tyrosine phosphorylation of the SMC EGFR (Figure 5). The GRK2ct contains the pleckstrin homology (PI3K-binding) and G protein βγ-binding domains of GRK2 but lacks the central catalytic and amino-terminal targeting domains of GRK2. Because we performed these experiments with equivalent levels of GRK2 and GRK2ct expression (Figure 5A), differences between the effects of GRK2 and the GRK2ct could be ascribed to differences in protein activity, rather than to differences in intracellular protein concentration.

PDGF-stimulated PI hydrolysis was inhibited by 38% in SMCs overexpressing GRK2 (Figure 5B). This degree of inhibition was more than twice that (P < 0.05) effected by equivalent expression of the GRK2ct. Despite these discordant findings in PI hydrolysis, GRK2 overexpression inhibited PDGF-induced [3H]Tdr incorporation only insignificantly more than GRK2ct, however, the more robust [3H]Tdr incorporation promoted by the combination of PDGF and ET-1 (Figure 4) was reduced (by 32 ± 3%, v0.01) only in SMCs overexpressing GRK2, but not in those overexpressing GRK2ct (data not shown).

These observations suggest that overexpressed GRK2 may reduce PDGF-promoted signaling, in part, by binding to the ligands for its pleckstrin homology/PI3K-binding carboxyl-terminal domain, as the GRK2ct does. However, the larger part of GRK2-mediated inhibition of PDGF-promoted signaling appears to operate through either the central catalytic domain, the amino-terminal targeting domain, or perhaps both of these domains.

If GRK2 interacted via its N-terminal or catalytic domain with the PDGFR, GRK2 might interfere with agonist-promoted tyrosine phosphorylation of the PDGFR. To test this possibility, we assessed the phosphotyrosine content of PDGFRβs immunoprecipitated from PDGF-challenged SMCs, which either did or did not overexpress GRK2 (Figure 6). GRK2 overexpression reduced agonist-induced tyrosine phosphorylation of the PDGFRβs by 37 ± 20% (P < 0.01). However, GRK2 overexpression had no effect on agonist-promoted tyrosine phosphorylation of the SMC EGFR (Figure 6). Thus, the ability of GRK2 to attenuate agonist-promoted receptor tyrosine kinase activity appears to be receptor-specific. Moreover, the specificity of GRK2-mediated receptor tyrosine kinase inhibition is congruent at any receptor tyrosine kinase. We therefore found it surprising that overexpression of GRK2 attenuated both PI hydrolysis (Figure 3) and [3H]Tdr incorporation (Figure 4) stimulated by the PDGFR(s) in primary SMCs. One potential explanation for these phenomena could lie in the ability of the GRK2 carboxyl-terminal domain to bind phosphatidylinositol-4,5-bisphosphate (PIP2), the preferred substrate for 2 important PDGFR-stimulated effector enzymes: phospholipase C-γ and phosphatidylinositol 3'-kinase. Sequestration of PIP2 by overexpressed GRK2 might therefore inhibit PDGF-stimulated PI hydrolysis as well as downstream events, such as DNA synthesis.

Could attenuation of PDGF-promoted signaling by overexpressed GRK2 involve more than competition for binding of PIP2? To address this question, we compared PDGF-promoted signaling in SMCs overexpressing either GRK2 or a polypeptide encompassing just the carboxyl-terminal 195 amino acids of GRK2 (GRK2ct) (Figure 5). The GRK2ct contains the pleckstrin homology (PI3K-binding) and G protein βγ-binding domains of GRK2 but lacks the central catalytic and amino-terminal targeting domains of GRK2. Because we performed these experiments with equivalent levels of GRK2 and GRK2ct expression (Figure 5A), differences between the effects of GRK2 and the GRK2ct could be ascribed to differences in protein activity, rather than to differences in intracellular protein concentration.

Figure 4. Effects of GRK2 overexpression on stimulus-induced SMC [3H]thymidine incorporation. SMCs were infected as in Figure 3B and exposed to low-mitogen medium without (basal) or with stimuli, as in Figure 3, Additional stimuli: 10% FBS, 1.7 nmol/L human EGF, and 0.4 nmol/L PDGF-BB with 100 nmol/L ET-1 (PDGF/ET). Stimulus-induced [3H]Tdr incorporation is plotted (mean ± SEM) from 4–8 experiments performed in triplicate, as dpm above basal: [(stimulated cells) – (unstimulated cells)]. Incorporation of [3H]Tdr by unstimulated cells (dpm × 10–6) was 4–2, 9–5, and 14–8 for uninfected, GRK2-infected, and vector-infected cells, respectively. Uninfected and vector-infected SMCs showed indistinguishable stimulus-induced [3H]Tdr incorporation. * P < 0.01, ** P < 0.05 vs control SMCs.

Effects of GRK2 on PDGFR Signaling

Although GRK2 can phosphorylate a wide array of heptahelical GPCRs, it has yet to be implicated in the regulation of
GRK2 Overexpression Reduces SMC Proliferation

Agonist-stimulated [3H]TdR incorporation may not indicate either an increase in DNA synthesis or ongoing cellular proliferation. We therefore sought to determine whether the GRK2 overexpression that inhibited agonist-induced [3H]TdR incorporation would also inhibit SMC proliferation. Accordingly, aliquots of the same SMCs as those subjected to assays for [3H]TdR incorporation were subjected to proliferation studies, presented in Figure 7. In response to either ET-1, PDGF-BB, or a combination of these agonists, the number of control SMCs increased 1.4±0.3-, 1.8±0.4-, or 2.0±0.8-fold in 12 days. The number of SMCs increased 2.7±0.7-fold over 4 days in response to 10% FBS. Compared with control cells, the proliferation of GRK2-overexpressing cells was reduced by 50% to 60% (P<0.01) in response to either ET-1, PDGF-BB, or the 2 agonists together (Figure 6). Likewise, proliferation of GRK2-overexpressing cells was reduced by 29% (P<0.01) in response to 10% FBS (Figure 7). This reduction in SMC proliferation by GRK2 overexpression was achieved without any evidence of GRK2 adenovirus-induced apoptosis in SMCs maintained in low-mitogen medium (Table). Thus, GRK2 overexpression in SMCs attenuates not only agonist-stimulated [3H]TdR incorporation but also agonist-stimulated SMC proliferation in vitro.

Discussion

In primary cultures of vascular SMCs, we have shown that overexpression of GRK2 diminishes signaling elicited via several receptors believed to be important in the pathogenesis of NH, including the PDGFR. GRK2 overexpression suppressed SMC signaling assessed either at the level of the second messenger or considerably downstream from the second messenger, at the level of SMC [3H]TdR incorporation. Moreover, GRK2 overexpression attenuated SMC proliferation without potentiating SMC apoptosis. The possibility that GRK2 overexpression may attenuate NH is suggested by the ability of GRK2 specifically binds and phosphorylates agonist-bound, or activated, heptahelical GPCRs. Expressed at physiological levels, GRK2 appears to initiate desensitization of GPCRs in a manner dependent on GRK2-mediated phosphorylation of the heptahelical receptor. When expressed at physiological levels, however, GRK2-mediated inhibition of receptor signaling involves primarily agonist-promoted association of the receptor with the GRK (a process that is both receptor- and GRK-specific). Overexpression of just the amino-terminal domain of GRK2 can...
attenuate G<sub>i</sub>-coupled receptor signaling. This phenomenon may result from binding to either the heptahelical receptor or the G protein itself, because the GRK2 amino-terminal domain possesses homology with regulator of G-protein signaling (RGS) proteins.

Surprisingly, GRK2 overexpression inhibited signaling not only via SMC GPCRs but also via the SMC PDGFR<sub>β</sub>.<sup>16</sup> By what mechanisms could overexpressed GRK2 suppress PDGFR-mediated signaling in SMCs? PIP<sub>2</sub> sequestration has been discussed above. In addition, overexpressed GRK2 may interfere with PDGFR signaling by sequestering G<sub>βγ</sub> subunits. The heterotrimeric protein G<sub>i</sub> has been implicated in PDGFR-mediated stimulation of p42/p44 mitogen-activated protein kinases in airway SMCs.<sup>21</sup> G<sub>i</sub> can also mediate mitogen-activated protein kinase activation elicited through heptahedral receptors, and this process is inhibited by GRK2ct, presumably by sequestration of G<sub>βγ</sub> subunits.<sup>22</sup> Thus, to the extent that PDGFRs signal via a G<sub>βγ</sub>-related mechanism in our vascular SMCs, GRK2ct and GRK2 itself should be expected to inhibit the signaling. Inhibition of PDGF-evoked mitogenesis through sequestration of G<sub>βγ</sub>, and PIP<sub>2</sub>, may, in part, underlie the efficacy of GRK2ct in attenuating NH in rabbit jugular vein bypass grafts.<sup>23</sup>

If the cytoplasmic tail of the PDGFR can mimic heptahedral receptor cytoplasmic domains by activating G<sub>i</sub>,<sup>21</sup> perhaps it can also mimic heptahedral receptor cytoplasmic domains by activating GRK2, as the wasp venom peptide mastoparan can.<sup>24</sup> If the PDGFR cytoplasmic domain can activate GRK2, it may also be phosphorylated by GRK2, with a consequent impairment of downstream signaling. Indeed, our observation that overexpressed GRK2 reduces PDGFR<sub>β</sub> tyrosine phosphorylation is consistent with the possibility of GRK2-mediated PDGFR<sub>β</sub> phosphorylation. Recently, casein kinase Iγ<sub>2</sub> has been shown to phosphorylate the PDGFR<sub>β</sub> on serine(s) and consequently to reduce PDGF-promoted receptor tyrosine phosphorylation.<sup>25</sup> Intriguingly, sites phosphorylated by casein kinase Iγ<sub>2</sub> are strikingly similar to those that can be phosphorylated by GRK2.<sup>2,25</sup> Whether GRK2 directly phosphorylates the PDGFR<sub>β</sub> remains to be determined.

Conceptually, inhibiting mitogenic signaling at the level of the receptor seems to be strategically advantageous in treating NH. Receptor-mediated signaling involves catalytic cascades that amplify signals as they propagate toward the cell nucleus. Such signal amplification may at least partly explain the incomplete effectiveness in treating NH observed with molecular strategies targeting transcriptional or other cell cycle-regulatory proteins.<sup>26</sup> With GRK2 overexpression in SMCs, multiple signaling pathways are attenuated simultaneously at the level of cell surface receptors signaling through G<sub>i</sub>-coupled and tyrosine kinase pathways. Our data regarding GRK2 overexpression therefore suggest that GRK2 overexpression may be therapeutically useful as a novel, plasma membrane–targeted treatment for NH.

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