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Divergent roles of prokineticin receptors in the endothelial cells: angiogenesis and fenestration

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Guilini C, Urayama K, Turkeri G, Dedeoglu DB, Kurose H, Messaddeq N, Nebigil CG. Divergent roles of prokineticin receptors in the endothelial cells: angiogenesis and fenestration. *Am J Physiol Heart Circ Physiol* 298: H844–H852, 2010. First published December 18, 2009; doi:10.1152/ajpheart.00898.2009.—Prokineticins are secreted peptides that activate two G protein-coupled receptors: PKR1 and PKR2. Prokineticins induce angiogenesis and fenestration, but the cognate receptors involved in these functions are unknown. We hypothesized a role for prokineticin receptor signaling pathways and expression profiles in determining the selective effects of prokineticins on coronary endothelial cells (H5V). Activation of the PKR1/MAPK/Akt signaling pathway stimulates proliferation, migration, and angiogenesis in H5V cells, in which PKR1 predominates over PKR2. PKR1 was colocalized with $G\alpha_{11}$ and was internalized following the stimulation of these cells with prokineticin-2. Knock down of PKR1 or $G\alpha_{11}$ expression in H5V cells effectively inhibited prokineticin-2-induced vessel formation and MAPK/Akt activation, indicating a role for PKR1/$G\alpha_{11}$ in this process. However, in conditions in which PKR2 predominated over PKR1, these cells displayed a fenestrated endothelial cell phenotype. H5V cells overexpressing PKR2 displayed large numbers of multivesicular bodies and caveolar clusters and a disruption of the distribution of zonula occluden-1 tight junction protein. Prokineticin-2 induced the colocalization of PKR2 with $G\alpha_{12}$, and activated $G\alpha_{12}$, which bound to zonula occluden-1 to trigger the degradation of this protein in these cells. Prokineticin-2 induced the formation of vessel-like structures by human aortic endothelial cells expressing only PKR1, and disorganized the tight junctions in human hepatic sinusoidal endothelial cells expressing only PKR2, confirming the divergent roles of these receptors. Our findings show the functional characteristics of coronary endothelial cells depend on the expression of PKR1 and PKR2 levels and the divergent signaling pathways used by these receptors.

angiogenesis; signaling; G proteins

THE PROKINETICINS, prokineticin-1 (also called endocrine gland-VEGF) and prokineticin-2 (also called Bv8), belong to the AVIT family of secreted proteins (9). Prokineticins were first identified in the gastrointestinal tract as potent agents mediating muscle contraction (15) and have been isolated from cow's milk (20). They have been shown to be widely distributed in mammalian tissues (2). Prokineticins are potent angiogenic factors (13) also involved in the regulation of hematopoiesis (14), monocyte differentiation (6), macrophage activation (19) and olfactory bulb activation (21, 27), pain sensitization (8, 26), circadian rhythm (5, 16), and the coordination of circadian behavior and physiology (31). In addition to their role in angiogenesis, prokineticins seem to be potent survival/mitogenic factors in various cells, including endothelial cells (11), neuronal cells (28), lymphocytes, hematopoietic stem cells, and cardiomyocytes (35). Prokineticins exert their biological activities by stimulating two similar G protein-coupled receptors: prokineticin receptor 1 (PKR1) and 2 (PKR2) (17). Prokineticin-2 is the most potent agonist of both receptors (34). These receptors are ubiquitously expressed in mammalian tissues (20), including cardiac tissues (35). Their amino acid sequences are 85% identical, but they play very different roles in the heart. The interaction of prokineticin-2 with PKR1 protects cardiomyocytes against hypoxia-induced apoptosis (35). Transient expression of a PKR1 transgene decreases mortality and preserves left ventricular function, by promoting angiogenesis and cardiomyocyte survival, in a mouse model of myocardial infarction (35). Transgenic mice overexpressing PKR1 in cardiomyocytes display postnatal coronary angiogenesis and vasculogenesis under paracrine regulation (36). However, transgenic mice overexpressing PKR2 in cardiomyocytes display eccentric hypertrophy and vascular leakage under paracrine regulation (37). These data demonstrate the functional importance of prokineticin receptors and show that the PKR1 and PKR2 signaling pathways may have opposite effects in heart. PKR1 is strongly expressed in endothelial cells of arterioles and vessels, whereas PKR2 is strongly expressed in fenestrated endothelial cells (30). The cell-autonomous functions and signaling pathways of these receptors have not yet been studied in the endothelial cell system. To evaluate the physiological role of the different pathways activated by PKR1 and PKR2, we used coronary endothelial cells (H5V), which constitute an excellent model system for studying the role of prokineticin receptors, because they express both PKR1 and PKR2 (35–37). We confirmed the divergent role of these receptors in human aortic endothelial cells (HAEC), expressing only PKR1, and in human hepatic sinusoidal endothelial cells (HHSEC) expressing only PKR2.

The expression profiles of these receptors and the divergent roles of the signaling pathways they mediate may determine the cell-autonomous phenotypic heterogeneity of endothelial cells. An understanding of these roles may facilitate the development of new treatments targeting PKR1 and PKR2 in cardiovascular diseases.

MATERIALS AND METHODS

Reagents. Human recombinant prokineticin-2 was purchased from Peprotech (France). Growth factor-reduced Matrigel was obtained from BD Biosciences, and gelatin and collagen were obtained from

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Sigma (St. Louis, MO). Antibodies against Akt, phospho-Akt, MAPK, and phospho-MAPK were obtained from Cell Signaling Technology (Beverly, MA), and antibodies against $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{q/11}$, Ki67, phospho-Akt (Thr 308), GAPDH, and caveolin-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, San Diago, CA). Antibodies against zonula occluden-1 (ZO-1) (Invitrogen, San Diago, CA), PKR2 (Abcam, Cambridge, UK), and PKR1 (IGBMC, Illkirch, France) were also used. Specific inhibitors of phosphatidylinositol 3-kinase (PI3K) (LY294002) and of members of the mitogen-activated protein kinase (MAPK) family (PD98059) were purchased from Calbiochem (La Jolla, CA).

Cell culture. H5V endothelial cells derived from mouse heart were kindly provided by Dr. Annunciata Vecchi (Istituto Clinico Humanitas, Rozzano, Italy). HHSEC, provided by Innoprot, were isolated by ScienCell Research Laboratories from human liver. HAEC were purchased from Invitrogen. Cells were maintained as described in the supplemental data. (The online version of this article contains supplemental data.)

Proliferation assay. H5V cells (2×10^4/well) were used to seed 24-well plates containing Dulbecco's modified Eagle medium (DMEM) supplemented with 2% FCS, and the cells were then incubated with or without prokineticin-2 (1–15 nM) for 1 or 4 days. Cells were counted, in a hemocytometer, at time $t = 0$ and $t = 1–4$ (see supplemental data, Fig. I, *top*). The same experiment was also performed in H5V cells 48 h after transfection with small interfering RNA (siRNA) (for negative control, PKR1 or PKR2). In another set of experiments, the H5V cells were preincubated with neutralizing PKR1-Ab (100 μl/ml) 30 min before prokineticin-2 was added.

Wound-healing migration assay. Cells were counted at seeding, and equal numbers of cells (7 or 8×10^5 cells per well) were incubated in a six-well plate containing DMEM supplemented with 10% FCS for 24 h (18). Confluent H5V cells were kept in DMEM supplemented with 2% FCS for 24 h and were then wounded with a pipette tip and subjected to prokineticin-2 stimulation. Cells at the edge of the wound migrated into the wound, gradually closing it. The wound was photographed within 4 h. The cells were cultured for an additional 20 h and then photographed again. To distinguish between enhanced restitution by migration and proliferation, H5V cells were treated by 0.5 μm/ml mitomycin C for 2 h before wounding assays were performed and during the 24-h incubation with test substances. Preliminary experiments showed that incubation of H5V cells with 0.5 μg/ml mitomycin C for 24 h completely inhibited proliferation without affecting cell viability (86% over control).

Assessment of the formation of capillary-like structures by endothelial cells. H5V cells or H5V cells transfected with siRNA (for the negative control), or PKR1 and $G\alpha_{11}$ were used to seed 24-well culture plates coated with Matrigel. The cells were then incubated in the presence or absence of prokineticin-2 for 24 h (BD Biosciences, Bedford, MA), according to the manufacturer's instructions, and as previously described (35) (see supplemental data).

Transfection. H5V cells were transfected with a control plasmid (pcDNA3.1), or with plasmids encoding $G\alpha_{12}$, $G\alpha_{12QL}$, or PKR2, in the presence of Lipofectamine 2000 (Invitrogen). Cells were stimulated, 48 h after transfection, with or without 5 nM prokineticin-2, at 37°C, under an atmosphere containing 5% CO_2, for the times indicated. The reaction was stopped by washing twice with cold PBS on ice. The washed cells were used for Western blot or immunofluorescence analyses.

siRNA transfection. H5V cells were transfected in siPORT Amine Transfection Reagent (Ambion, Austin, TX), with 100 nM siGENOME siRNA for mouse $G\alpha_{11}$ (Dharmacon, Lafayette, CO), 100 nM siRNA for the negative control (Ambion siRNA no. AM4611), 100 nM siRNA for mouse PKR1 (Ambion siRNA no. 181827; UAUCCAGAUAGUUAACAGCTT), or mouse PKR2 (Ambion siRNA ID no. 152628, CAUCCGUGGUUUCAAAGGGTG), according to the manufacturer's instructions (Ambion) (35) (see supplemental data, Fig. III). Cells transfected with negative control siRNA were used as a control.

Western blot analysis. H5V cells were lysed in lysis buffer. Total protein was separated by SDS-PAGE in 7 or 12% acrylamide gels and blotted onto polyvinylidene difluoride membranes. The membrane was blocked and incubated with primary antibodies. It was then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary immunoglobulins. The signal was then detected by

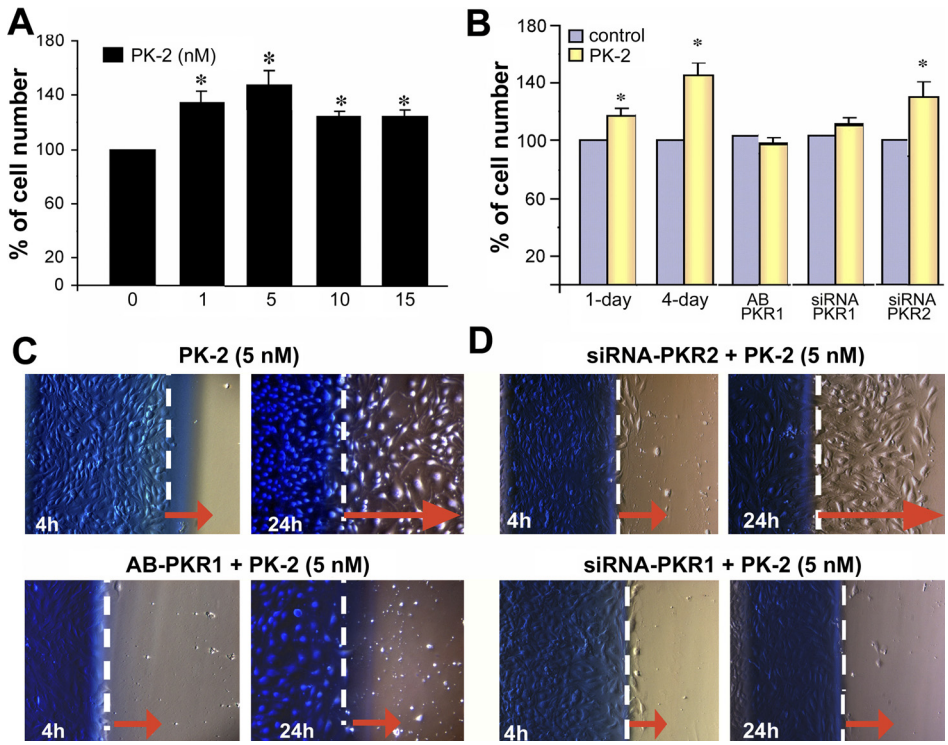

Fig. 1. *A*: proliferation rate of coronary endothelial cells (H5V) 4 days after treatment with prokineticin-2 (PK-2) at various concentrations ($n = 3$). *$P < 0.05$. *B*: treatment with 5 nM PK-2 resulted in the largest increase in cell number on *days 1* and *4*. PK-2-mediated cell proliferation (4 days) was completely abolished in H5V cells treated with the neutralizing anti-prokineticin receptor 1 (PKR1) antibody (AB) or in which PKR1 expression was knocked down, but not in cells in which PKR2 expression was knocked down. *$P < 0.05$. *C*: migration assays 4 and 24 h after the PK-2 (5 nM) treatment of H5V cells. The discontinuous line (limit of the blue marker) shows area from which cells were scraped at *time 0*. *Top*: PK-2 induces cell migration within 24 h. *Bottom*: prior treatment of the cells with neutralizing AB against PKR1 completely inhibits PK-2-mediated cell migration ($n = 3$). *D*: PK-2 induced migration in small interfering RNA (siRNA)-PKR2-transfected cells (*top*), but had no effect in siRNA-PKR1-transfected cells (*bottom*). Red arrows show the migrating cell distances between the edges of the wound cells (discontinuous line) at the wound edge (*time 0*) migrated into the wound (time 4 h), closing it over time (24 h after PK-2 treatment).

enhanced chemiluminescence (Pierce, Rockford, IL), according to the manufacturer's protocol (see supplemental data).

Immunoprecipitation with anti-Gα_{12} antibody. H5V cells growing in a 10-cm dish were starved of serum (only 2% serum in the medium) overnight and stimulated with prokineticin-2 (5 nM) at 37°C for the time indicated. The reaction in stimulated H5V cells was stopped by washing with cold PBS on ice, and the cells were then lysed in lysis buffer. The lysates were cleared by centrifugation at 15,000 *g* for 20 min. Total protein concentration was adjusted and, 32 h later, the lysates were precleared on Protein A/G PLUS Agarose (Santa Cruz Biotechnology) for 30 min at 4°C. Gα_{12} proteins were immunoprecipitated from the precleared lysates by incubation with 2 μg/ml anti-Gα_{12} or anti-ZO-1 antibody, followed by 20 μl of protein A/G agarose. The agarose beads were washed three times with lysis buffer, boiled in SDS-PAGE loading buffer, and subjected to SDS-PAGE and immunobloting (24).

Measurement of Gα_{12} activity. We investigated Gα_{12} activity in H5V cells cotransfected with constructs encoding constitutively active Gα_{12QL} or Gα_{12} and PKR2, using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The transfected cells were stimulated by incubation with 5 nM prokineticin-2 for the times indicated, and the supernatant was incubated with 5 μg of glutathione *S*-transferase-tetratricopeptide repeat and glutathione-Sepharose beads overnight at 4°C. The beads were washed with ice-cold lysis buffer, and the bound proteins were eluted in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting with anti-Gα_{12} antibody (39). Densitometric analyses were performed with NIH Image software (see supplemental data).

Immunofluorescence and microscopy. Immunofluorescence analysis was performed on H5V cells, as previously described (36). Fluorescent signals were visualized and photographed with a Leica microscope equipped with a digital camera. In some of the confocal microscopy analyses, signal intensity was quantified on digitized images, as the product of averaged pixel intensity per unit area. Densitometric analysis was carried out with LEICA software or Scion Image. The laser was selected according to the sample and was used to compare pixel intensity and pixel distribution. We analyzed these data, using pixel data from which background intensity had been subtracted (35).

Statistical analysis. Data are expressed as means ± SE. Multigroup comparisons were performed by one-way ANOVA with post hoc correction (in Figs. 1*A* and 5*B*). Comparisons between pairs of groups were made with the unpaired Student's *t*-test. For all analyses, $P < 0.05$ was considered significant.

RESULTS

Prokineticin-2 enhances endothelial cell proliferation and migration. We investigated the possible mechanisms underlying the direct angiogenic effects of prokineticin-2 on endothelial cells, by analyzing the effects of prokineticin-2 on the major steps of angiogenesis in vitro: endothelial cell proliferation and migration. We investigated the possible effects of prokineticin-2 on the rate of proliferation of H5V cells, by counting cells 4 days after treatment with various concentrations of prokineticin-2 (Fig. 1*A*). Cell numbers were maximal at a prokineticin-2 concentration of 5 nM, for cell counts on both *days 1* and *4* ($P < 0.05$, Fig. 1*B*). Proliferation rate as an elevation of DNA incorporation was also assessed by Ki67 immunostaining of H5V cells 24 h after treatment with prokineticin, verifying mitogenic effect of prokineticin-2 in these cells (supplemental data, Fig. I, *bottom*). No mitogenic effect of prokineticin-2 was seen in cells previously treated with a neutralizing antibody against PKR1 (35) (Fig. 1*B*). Prokineticin-2-mediated stimulation of endothelial cell proliferation was completely abolished by knocking down PKR1 expression in H5V cells (siRNA-PKR1) (Fig. 1*B*). However, knocking down PKR2 expression in H5V cells (siRNA-PKR2) had no effect on prokineticin-2-mediated proliferation (Fig. 1*B*), consistent with the involvement of PKR1, but not of PKR2.

We investigated the involvement of prokineticin-2 in inducing endothelial cell migration, by carrying out a wound-healing assay. Prokineticin-2 treatment significantly increased the level of cell migration, within 24 h (Fig. 1*C*, *top*). In the same experiment, the proliferation of H5V cells was inhibited with 0.5 μg/ml of mitomycin C for 2 h before wounding assays were performed and during the 24-h incubation with prokineticin-2, to distinguish between enhanced restitution by migration and proliferation (supplemental data, Fig. II). The motogenic (inducing motility) effect of prokineticin-2 was not seen in cells previously treated with the neutralizing antibody against PKR1 (Fig. 1*C*, *bottom*), or in siRNA-PKR1 cells (Fig. 1*D*, *bottom*), consistent with the involvement of PKR1. However, prokineticin-2-mediated motogenic effect was not affected by knocking down the expression of PKR2 in siRNA-PKR2 cells (Fig. 1*D*, *top*).

Prokineticin-2/PKR1 signaling promotes vessel-like formation: involvement of kinases. Our laboratory has previously shown that the activation or overexpression of PKR1 induces

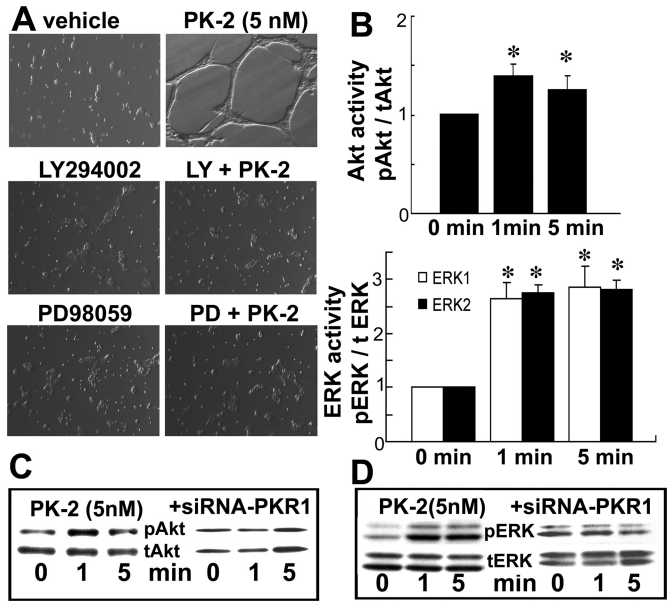

Fig. 2. *A*: representative demonstration of the formation of vessel-like structures by H5V cells. We observed the formation of vessel-like structures 24 h after treatment of the H5V cells with PK-2 (5 nM), whereas no such structures were induced in untreated cells (vehicle; $n = 4$, $P < 0.001$; *top*). Original magnification = ×10. The PK-2-induced formation of vessel-like structures was completely abolished by inhibitors of phosphatidylinositol 3-kinase (LY294002, *middle*) and MAPK (PD98059, *bottom*). *B*: relative intensity of phosphorylated (p) vs. total (t) Akt and MAPK protein signals in the presence of PK-2 (5 nM). Western blot analysis revealed that the PK-2 treatment of H5V cells increased the amounts of the phosphorylated forms of Akt (56 kDa, *top*) and MAPK (42/44 kDa *bottom*) without altering tAkt and tMAPK protein levels ($n = 3$). *$P < 0.05$. *C*: representative PK-2-mediated Akt activation detected by an AB recognizing pAkt within 1 min and completely abolished by knocking down PKR1 expression in siRNA-PKR1 cells. *D*: representative PK-2-mediated MAPK activation detected by an AB recognizing pERK1 (42 kDa) and pERK2 (44k Da) within 5 min. This activation was completely abolished by knocking down PKR1 expression in H5V cells (siRNA-PKR1).

vessel-like formation on Matrigel, used as an in vitro model of angiogenesis (35). We investigated the downstream signaling pathway for prokineticin-2/PKR1-induced vessel-like formation by H5V cells, by initially treating cells with specific inhibitors of various kinases, along with prokineticin-2. Inhibitors of PI3K (LY294002) and MAPK kinase-1 (PD98059) significantly inhibited the formation of vessel-like structures in response to prokineticin on Matrigel (Fig. 2*A*). In light of these findings, suggesting that the PI3K/Akt and MAPK signaling pathways play a critical role in the prokineticin-2-induced formation of vessel-like structures by H5V cells, we further investigated the activation of these pathways. Prokineticin-2 induced Akt phosphorylation at serine 473, within 1 min ($P < 0.05$, Fig. 2*B*, *top*). Akt phosphorylation at threonine 308 was also observed 1 and 5 min after prokineticin-2 treatment by 10 and 40%, respectively (see supplemental data, Fig. VI). Prokineticin-2 also induced phosphorylation of the ERK1 and ERK2 kinases within 1 min ($P < 0.05$, Fig. 2*B*, *bottom*). We investigated the role of PKR1, by carrying out similar experiments with the siRNA-PKR1 cells. Knocking down PKR1 expression in H5V cells abolished the effects of prokineticin-2 on Akt and MAPK phosphorylation, indicating PKR1 involvement (Fig. 2, *C* and *D*).

PKR1 binds $G\alpha_{q/11}$ to promote angiogenesis in H5V cells. We then investigated whether the classic $G\alpha_{q/11}$ signaling axis mediated the endothelial cell activity. We assessed the colocalization of $G\alpha_{q/11}$ (red) and PKR1 (green) in H5V cells by confocal microscopy. In resting permeabilized cells, both PKR1 and $G\alpha_{q/11}$ were diffusely distributed in the plasma membrane and cytosol. After PKR1 activation, some colocalization of PKR1 and $G\alpha_{q/11}$ was observed at the tips and bases of membrane protrusions (Fig. 3*A*, *middle*). Confocal analyses

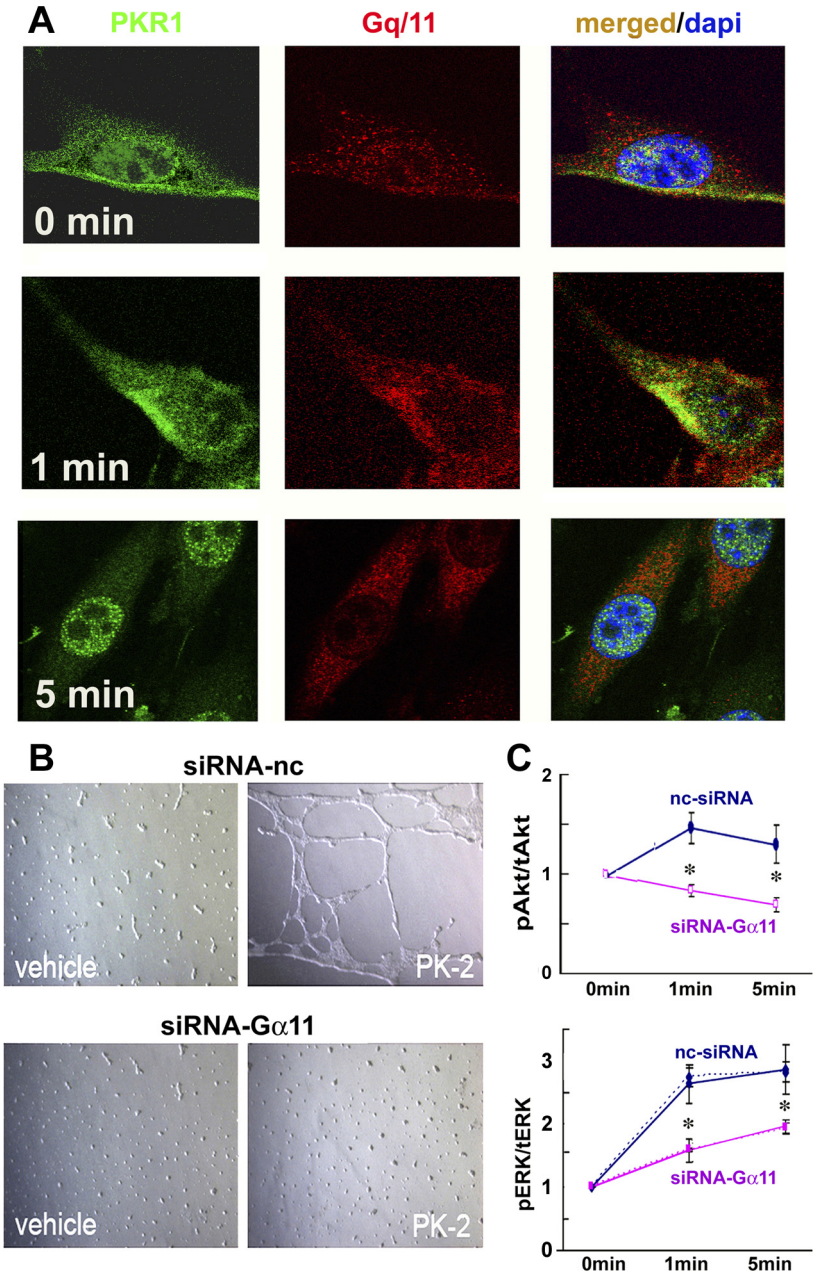

Fig. 3. *A*: localization of PKR1 (green) and $G\alpha_{q/11}$ (red) in the H5V cells before and after PK-2 treatment at the indicated times. The cell nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI; blue; $n = 3$). PKR1 and $G\alpha_{q/11}$ staining overlapped 1 min after the PK-2 treatment of the cells (yellow). *B*: representative demonstration of the formation of vessel-like structures in response to PK-2 in H5V cells transfected with the siRNA negative control (nc) or siRNA-$G\alpha_{11}$. The formation of vessel-like structures (*top*) was observed 24 h after the treatment of siRNA-nc-transformed H5V cells with PK-2 (5 nM). Knocking down $G\alpha_{11}$ expression in H5V cells completely abolished the formation of vessel-like structures induced by PK-2 (*bottom*). Original magnification $= \times 2.5$. *C*: quantitative analyses of PK-2-mediated Akt (*top*) and MAPK (*bottom*) activation, based on detection with an AB recognizing phosphorylated forms of the proteins in the siRNA nc (blue lines) and in the siRNA-$G\alpha_{11}$ cells (pink lines, $n = 3$). $^*P < 0.001$.

revealed that staining for $G\alpha_{q/11}$ and PKR1 overlapped within 1 min of the prokineticin-2 treatment of H5V cells. PKR1 was completely internalized within 5 min of PKR1 activation (Fig. 3*A*, *bottom*). We also verified ligand-mediated PKR1 internalization in human embryonic kidney-293 cells stably expressing green fluorescent protein-PKR1 by time-lapse analyses (supplemental data, Fig. VII A). In these cells, prokineticin treatment induces calcium mobilization that is consistent with coupling to $G\alpha_{q/11}$ activation (supplemental data, Fig. VII B).

We addressed the involvement of $G\alpha_{q/11}$ signaling in PKR1-mediated angiogenesis, by knocking down $G\alpha_{11}$ expression in H5V cells (supplemental data, Fig. IV). Surprisingly, knocking down $G\alpha_{11}$ expression in H5V cells abolished the prokineticin-2-mediated formation of vessel-like structures (Fig. 3*B*). It also significantly inhibited prokineticin-2-mediated MAPK and Akt activation, consistent with the binding of PKR1 specifically to $G\alpha_{11}$ to activate MAPK and Akt (Fig. 3*C*).

PKR2 signaling does not induce angiogenesis in vitro. We then altered the ratio of PKR2 to PKR1 in H5V cells, by overexpressing PKR2. We investigated whether PKR2 overexpression in H5V (H5V-PKR2) cells could induce angiogenesis in vitro. In H5V-PKR2 cells, PKR2 transcript and protein levels were increased three and approximately two times higher (supplemental data, Fig. V), respectively, than those in H5V cells ($P < 0.05$). H5V-PKR2 cells were placed on Matrigel for 24 h, and the formation of vessel-like structures was quantified. The overexpression of PKR2 or stimulation with prokineticin-2 did not result in any vessel-like structures similar to those seen in cells transfected with the positive control vector (H5V-control; Fig. 4*A*). Thus PKR2 overexpression, unlike PKR1 overexpression, does not trigger angiogenic signaling in H5V cells.

PKR2 signaling increases the formation of caveolin clusters and vesicle trafficking in the endothelial cells. H5V-PKR2 cells had a morphology different from that of H5V-control cells (Fig. 4*B*). H5V-PKR2 cells were rounder, with visible vacuoles in the cytoplasm. PKR1 overexpression did not induce fenestration of the endothelial cells, ruling out the possibility of a transfection- or overexpression-dependent effect of PKR2 in H5V-PKR2 cells. We then investigated whether PKR2 signaling in endothelial cells impaired function and led to the pathological fenestration of endothelial cells. Electron microscopy showed that H5V-PKR2 cells had larger numbers of lysosomes, multivesicular bodies, phagolysosomes, and clathrin-coated endocytotic vesicles, and vesiculo-vacuolar permeability organelles typical of fenestrated endothelial cells (1) (Fig. 4, *C* and *D*). H5V-PKR2 cells displayed prominent caveolin-l-positive aggregates in their cytoplasm, particularly around the nucleus (Fig. 4*E*). Western blot analyses confirmed the presence of larger amounts of caveolin-1 in the H5V-PKR2 cells ($P < 0.05$, Fig. 4*F*). These studies suggest that PKR2 overexpression in H5V endothelial cells resulted in a phenotype more like that of fenestrated endothelial cells.

PKR2 binds $G\alpha_{12}$ in H5V cells. As PKR1 and PKR2 are 85% identical but have different effects in endothelial cells, we investigated the possibility of binding to different G proteins for PKR2. In resting cells, both PKR2 (red) and $G\alpha_{12}$ (green) were diffusely distributed throughout the cytosol. One min after the treatment of H5V-PKR2 cells with prokineticin-2, confocal analyses demonstrated the colocalization of $G\alpha_{12}$ with PKR2 (Fig. 5*A*). PKR2 was fully internalized after 1 min of prokineticin-2 treatment. Similar results were obtained following the activation of endogenous PKR2 by prokineticin-2.

Glutathione *S*-transferase-tetratricopeptide repeat pulldown assays of activated $G\alpha_{12}$ showed that prokineticin-2 activated $G\alpha_{12}$ within 1 min, with this activation lasting from 5 to 10 min in H5V cells (Fig. 5*B*, *left*). As expected, we detected a robust activation of $G\alpha_{12}$ by prokineticin-2 treatment in H5V-PKR2 cells similar to that observed with the constitutively active form of $G\alpha_{12}$, $G\alpha_{12QL}$ expressing cells (Fig. 5*B*, *right*).

Prokineticin treatment leads to the association of $G\alpha_{12}$ with ZO-1, altering the distribution of ZO-1 in H5V cells. $G\alpha_{12}$ has been shown to associate with the ZO-1 junctional protein to regulate the cell permeability (23). We, therefore, investigated whether prokineticin-2 induced the association of $G\alpha_{12}$ with ZO-1 and regulated the distribution or expression of ZO-1. ZO-1 was coimmunoprecipitated with $G\alpha_{12}$, 5 min after prokineticin-2 treatment (Fig. 6*A*). Moreover, the ZO-1 protein

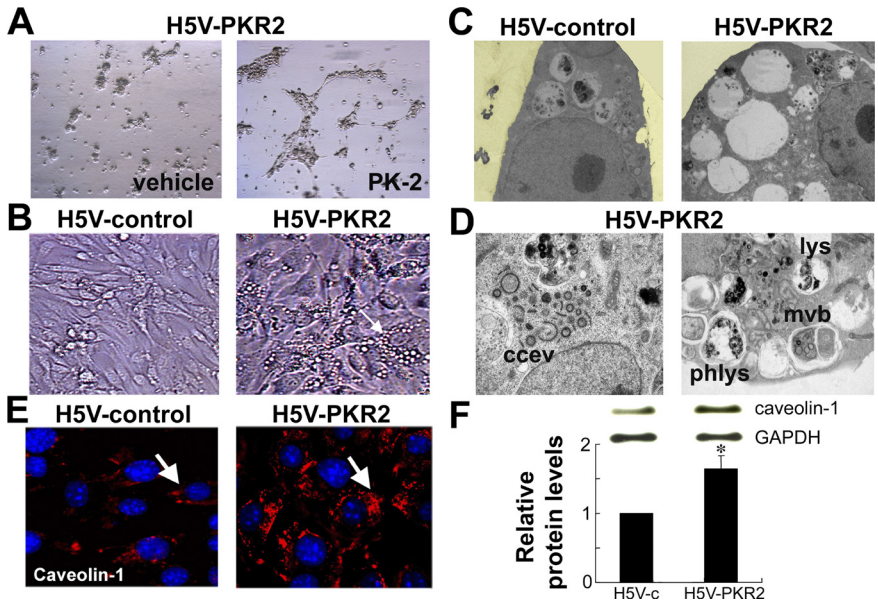

Fig. 4. *A*: formation of vessel-like structures in Matrigel by H5V cells transfected with control vector (H5V-control) alone or vector carrying PKR2 cDNA (H5V-PKR2). No formation of vessel-like structures was observed in H5V-PKR2 cells upon treatment with PK-2. Original magnification = ×10. *B*: H5V-PKR2 cells were morphologically different from H5V-control cells. Original magnification = ×20. *C*: electron microscopy of H5V cells transfected with plasmid-control (H5V-control) or plasmid carrying PKR2 cDNA (H5V-PKR2). *D*: H5V-PKR2 cells contained larger numbers of lysosomes (lys), multivesicular bodies (mvb), phagolysosomes (phlys), and clathrin-coated endocytotic vesicles (ccev). *E*: H5V-PKR2 cells have larger numbers of caveolin-1 clusters in the cytoplasm, as detected by immunostaining with caveolin-1-specific AB. *F*: Western blot analyses showed that cells overexpressing PKR2 contained significantly larger amounts of caveolin-1 (histogram, $n = 3$). H5V-c, H5V-control. *$P < 0.05$.

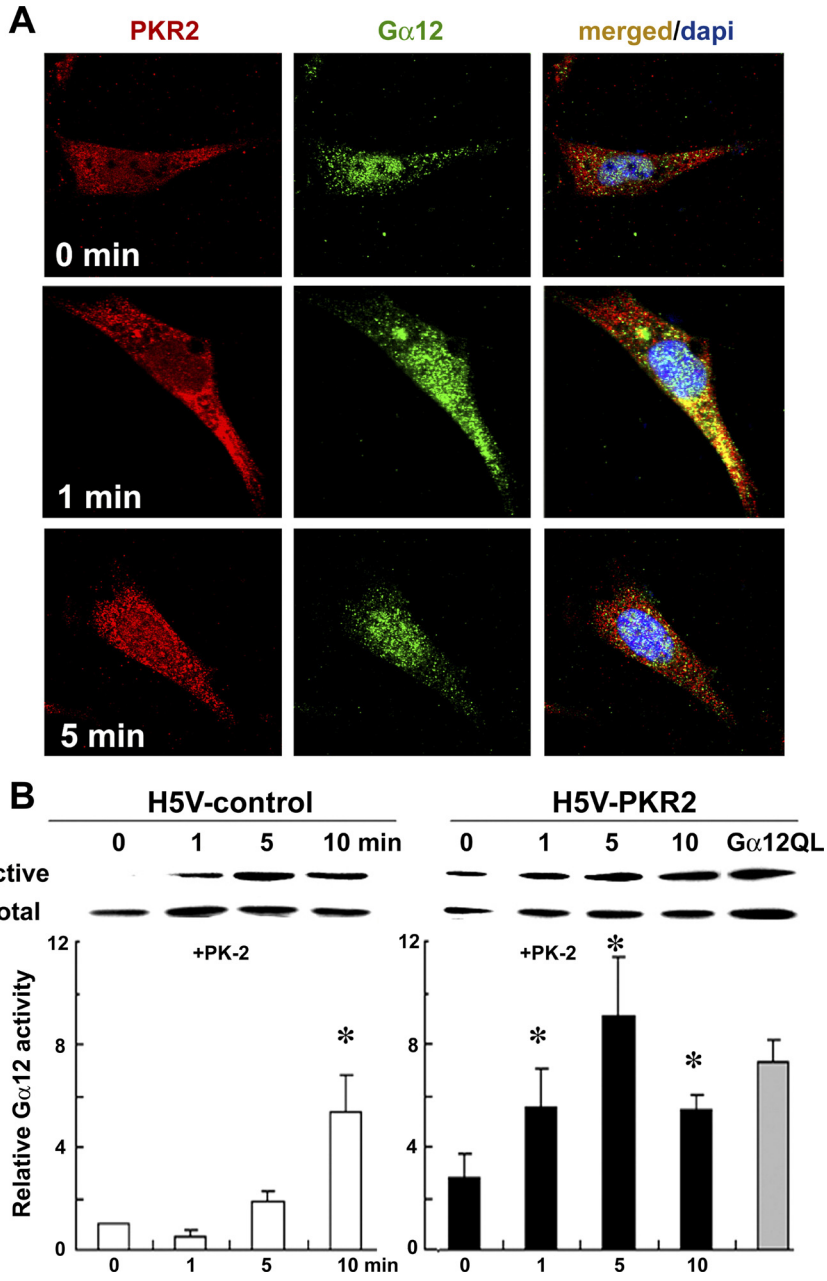

Fig. 5. *A*: localization of PKR2 (red) and $G\alpha_{12}$ (green) in H5V-PKR2 cells before and after PK-2 treatment at the indicated times. Cell nuclei are stained with DAPI (blue; $n = 3$). PKR2 and $G\alpha_{12}$ staining overlapped, with internalization occurring within 1 min of the PK-2 treatment of H5V-PKR2 cells. *B*: relative $G\alpha_{12}$ activity, detected by glutathione *S*-transferase-tetratricopeptide repeat pull-down assay in cells cotransfected with H5V plasmid (*left*) or H5V PKR2 (*right*) and constructs encoding $G\alpha_{12}$ or its constitutively active form, $G\alpha_{12QL}$, after PK-2 treatment at the indicated times. $^*P < 0.05$.

was internalized within 10 min of prokineticin-2 treatment of the H5V-PKR2 cells (Fig. 6*B*). Western blot analyses on cell lysates from H5V-PKR2 cells confirmed that H5V-PKR2 cells had lower levels of ZO-1 than controls within 24 h (Fig. 6*C*).

Divergent roles of PKR1 and PKR2 receptors in human endothelial cells. HAEC express only PKR1, whereas HHSEC express only PKR2. Both receptors are present in human explanted heart samples (Fig. 7*A*). HAEC stimulation with prokineticin-2 induced the formation of vessel-like structures on Matrigel (Fig. 7*B*). The prokineticin-2 treatment of HHSEC led to the internalization of ZO-1 protein within 60 min and the disruption of cell organization, resulting in a clear punctate pattern of staining for ZO-1 (Fig. 7*C*). These data clearly highlight the divergent roles of the two prokineticin receptors in human endothelial cells as a function of their expression profiles.

DISCUSSION

There is evidence to suggest that there is structural and functional heterogeneity in the endothelial cells of the vascular and microvascular beds of various organs that may be altered by pathological conditions (1). Current knowledge about the regulation of coronary endothelial cell structure and functional integrity is still fragmentary. Here we showed that a balance between PKR1 and PKR2 expression, and their coupling to different G protein signaling pathways, could be particularly important for regulation of the coronary endothelial structure and function. The prokineticin receptors have been shown to be coupled to different G proteins. Ca^{2+} mobilization and phosphoinositide turnover by prokineticins may be consistent with coupling to $G\alpha_{q/11}$. Prokineticins have also been shown to induce cAMP accumulation in cells overexpressing prokineti-

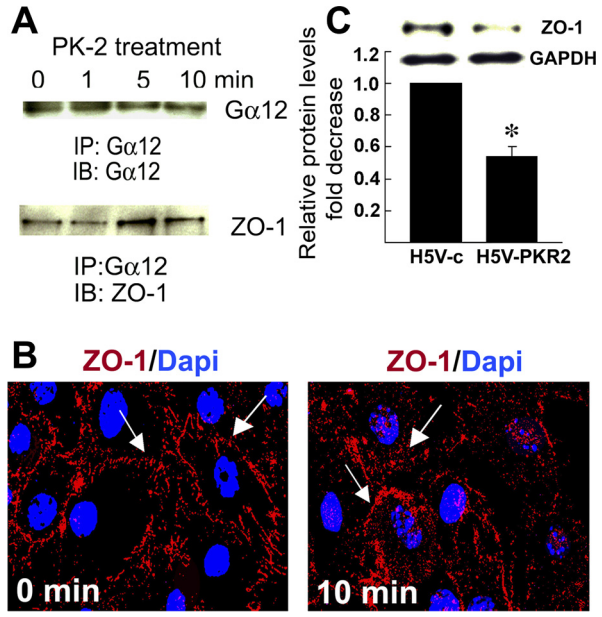

Fig. 6. *A*: coimmunoprecipitation of $G\alpha_{12}$ and zonula occluden-1 (ZO-1). The H5V-control and H5V-PKR2 cells were stimulated, as indicated, with PK-2 (5 nM) and subjected to immunoprecipitation (IP) with the anti-$G\alpha_{12}$ AB. The immunoprecipitated $G\alpha_{12}$ was immunoblotted (IB) with antibodies against $G\alpha_{12}$ or ZO-1. *B*: representative demonstration of the internalization of ZO-1 protein in H5V-PKR2 cells 10 min after treatment with PK-2. *C*: quantitative analyses of ZO-1 expression in H5V-control and H5V-PKR2 cells, shown as a histogram ($n = 4$). $^*P < 0.05$.

cin receptors (L17). The involvement of $G\alpha_{i/o}$ proteins in prokineticin signaling has also been suggested (4). The functional significance of the interactions between prokineticin receptors and their cognate G proteins remains unclear in the endothelial cells.

We report here the divergent roles of PKR1 and PKR2, possibly due to their coupling to different G proteins, defining the functional heterogeneity of endothelial cells. We found that the activation of endogenously high levels of PKR1 in H5V coronary endothelial cells or the overexpression of PKR1 in these cells promoted the formation of vessel-like structures. However, in conditions in which PKR2 predominated over PKR1, these cells displayed a fenestrated endothelial cell phenotype. Prokineticin-2-mediated angiogenesis resulted principally from the stimulation of endothelial cell proliferation and migration, both of which were completely abolished by prior treatment of the cells with a neutralizing antibody against PKR1 or by knocking down PKR1 expression in H5V cells. Our laboratory previously showed that the overexpression of PKR1 in H5V cells in the absence of the ligand of this receptor promoted angiogenesis in vitro (35), consistent with a role for PKR1 in this process. Our findings indicate that MAPK and PI3K are involved in the prokineticin-mediated angiogenesis signaling pathway, because prokineticin-2 activated MAPK and Akt kinase, and prokineticin-2/PKR1-mediated formation of vessel-like structures was inhibited by pharmacological inhibitors of these kinases. Our findings are consistent with those of previous studies showing that prokineticin activates MAPK in steroidogenic adrenocortical cells (10) and enteric neural crest cells (28) and induces the PI3K pathway to promote the survival of neuroblastoma cells (29). In this study, we found that $G\alpha_{11}$ colocalized with PKR1 and that the knocking down of $G\alpha_{11}$ expression abolished PKR1-mediated angiogenesis and the activity of MAPK and Akt in H5V cells.

Heterotrimeric G proteins regulate fundamental endothelial cell properties, interacting with structural proteins. $G\alpha_{q/11}$ proteins have been shown to be involved in endothelial cell proliferation rather than fenestration, via the tyrosine phosphorylation of VEGFR-2 in HUVEC cells (40). A number of $G\alpha_{q/11}$-coupled receptors have been shown to play a role in angiogenesis (32). For example, the activation of thrombin (22) or angiotensin II receptors (7) regulates microcapillary endothelial cell activation to induce angiogenesis. By interacting with $G\alpha_{q/11}$-coupled receptors, oxytocin stimulates the phosphorylation of endothelial nitric oxide synthase via the PI3K/AKT pathway, thereby promoting migration and angiogenesis in HUVEC cells (3). We provide here the first demonstration of a direct role of $G\alpha_{11}$ signaling in PKR1-mediated angiogenesis.

It has recently been shown that PKR2 is strongly expressed in fenestrated endothelial cells, such as those found in the endocrine glands (12), corpus luteum, kidney, and liver (25), indicating the possible involvement of PKR2 in endothelial cell fenestration (30). We provide here the first evidence that the overexpression of PKR2 in H5V cells, conferring a phenotype more like that of fenestrated endothelial cells, increases endothelial subcellular injury levels, thereby increasing cell permeability. The overexpression of PKR2, unlike that of PKR1, did not induce angiogenesis in vitro in H5V cells. PKR2 overexpression increased the formation of vesiculo-vacuolar permeability organelles and caveolae, small invaginations of the

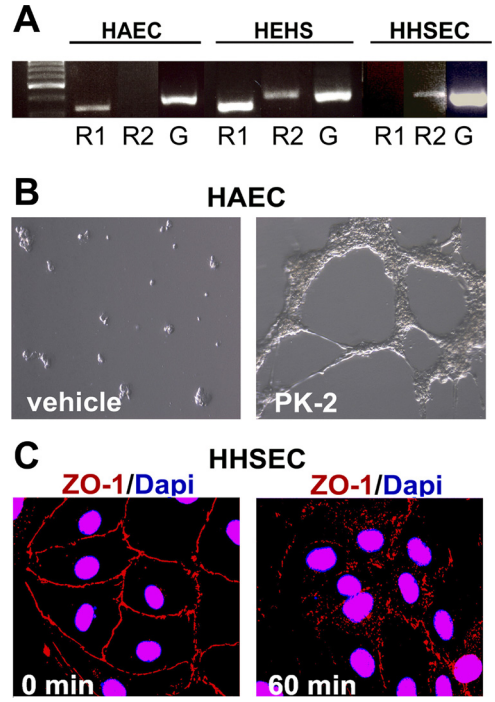

Fig. 7. *A*: RT-PCR analyses of PKR1, PKR2, and GAPDH (G) expression on human aortic endothelial cells (HAEC), human explanted heart samples (HEHS), and human hepatic sinusoidal endothelial cells (HHSEC). *B*: PK-2 induces the formation of vessel-like structures by HAEC expressing PKR1 in Matrigel. *C*: however, in PKR2-expressing HHSEC, treatment with PK-2 induces ZO-1 internalization and cellular disorganization within 60 min.

plasma membrane facilitating the vesicular transport of small proteins through the cytoplasm of an individual endothelial cell, thereby increasing the transcellular permeability of H5V cells. PKR2 signaling leads to an abnormal organization of endothelial cells and the disconnection of cells, through effects on the cell-cell adhesion molecule ZO-1 at tight junctions. Through $G\alpha_{12}$-ZO-1 protein interactions, PKR2 may regulate the paracellular permeability between cells (38). An additional role has also recently been described for $G\alpha_{12}$ signaling, in increasing permeability and tight junctional disassembly via interaction with ZO-1 in Madin-Darby canine kidney cells (33). We provide here the first demonstration that PKR2 signaling is involved in the activation of $G\alpha_{12}$, which then interacts with ZO-1, thereby downregulating ZO-1-mediated cell-cell adhesion.

The divergent roles of prokineticin-2 action via these two receptors were evident in two different types of cell, each expressing only one of the receptors. In HAEC, which express only PKR1, prokineticin-2 induced angiogenesis. In HHSEC, which express only PKR2, prokineticin-2 promoted cellular disorganization through changes in the distribution of ZO-1.

In conclusion, our data provide in vitro evidence that two highly similar G protein-coupled receptors have divergent endothelial cell functions due to their coupling to two different G proteins for signaling. It is unclear how representative H5V cells are of the coronary endothelium in vivo, but our findings indicate that prokineticin receptor expression profile may determine the selective effects of prokineticins on coronary endothelial cells, favoring endothelial cell barrier dysfunction or compensatory angiogenesis during myocardial infarction. In samples from humans with end-stage heart failure, a specific decrease in PKR1 levels has been observed (35). PKR1/PKR2 levels in cardiovascular diseases remain to be investigated. Our study should facilitate the discovery of specific agonists and antagonists targeting PKR1 and PKR2 for possible use in the treatment of cardiovascular diseases.

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DISCLOSURES

I am not aware of financial conflict(s) with the subject matter or materials discussed in this manuscript with any of the authors, or any of the authors' academic institutions or employers.

REFERENCES

1. **Aird WC.** Phenotypic heterogeneity of the endothelium. II. Representative vascular beds. *Circ Res* 100: 174–190, 2007.
2. **Bullock CM, Li JD, Zhou QY.** Structural determinants required for the bioactivities of prokineticins, and identification of prokineticin receptor antagonists. *Mol Pharmacol* 65: 582–588, 2003.
3. **Cattaneo MG, Chini B, Vicentini LM.** Oxytocin stimulates migration and invasion in human endothelial cells. *Br J Pharmacol* 153: 728–736, 2008.
4. **Chen J, Kuei C, Sutton S, Wilson S, Yu J, Kamme F, Mazur C, Lovenberg T, Liu C.** Identification and pharmacological characterization of prokineticin 2 beta as a selective ligand for prokineticin receptor 1. *Mol Pharmacol* 67: 2070–2076, 2005.
5. **Cheng MY, Bullock CM, Li C, Lee AG, Bermak JC, Belluzzi J, Weaver DR, Leslie FM, Zhou QY.** Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature* 417: 405–410, 2002.
6. **Dorsch M, Qiu Y, Soler D, Frank N, Duong T, Goodearl A, O'Neil S, Lora J, Fraser .** PK1/EGVEGF CC induces monocyte differentiation and activation. *J Leukoc Biol* 78: 426–434, 2005.
7. **Fujiyama SMH, Nozawa Y, Maruyama K, Mori Y, Tsutsumi Y, Masaki H, Uchiyama Y, Koyama Y, Nose A, Iba O, Tateishi E, Ogata N, Jyo N, Higashiyama S, Iwasaka T.** Angiotensin AT(1) AT(2) receptors differentially regulate angiopoietin-2 and vascular endothelial growth factor expression and angiogenesis by modulating heparin binding-epidermal growth factor (EGF) mediated EGF receptor transactivation. *Circ Res* 88: 22–29, 2001.
8. **Hu WP, Zhang C, Li JD, Luo ZD, Amadesi S, Bunnett N, Zhou QY.** Impaired pain sensation in mice lacking prokineticin 2. *Mol Pain* 2: 35–39, 2006.
9. **Kaser A, Winklmayr M, Lepperdinger G, Kreil G.** The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions. *EMBO Rep* 4: 469–473, 2003.
10. **Keramidas M, Faudot C, Cibiel A, Feige JJ, Thomas M.** Mitogenic functions of endocrine gland-derived vascular endothelial growth factor and Bombina variegata 8 on steroidogenic adrenocortical cells. *J Endocrinol* 196: 473–482, 2008.
11. **Kisliouk T, Podlovni H, Spanel-Borowski K, Ovadia O, Zhou QY, Meidan R.** Prokineticins (endocrine gland-derived vascular endothelial growth factor and BV8) in the bovine ovary: expression and role as mitogens and survival factors for corpus luteum-derived endothelial cells. *Endocrinology* 146: 3950–3958, 2005.
12. **LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller GA, Peale F, Gurney A, Hillan KJ, Ferrara N.** Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature* 412: 877–884, 2001.
13. **LeCouter J, Lin R, Tejada M, Frantz G, Peale F, Hillan KJ, Ferrara N.** The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: localization of Bv8 receptors to endothelial cells. *Proc Natl Acad Sci USA* 100: 2685–2690, 2003.
14. **Le Couter J, Zlot C, Tejada M, Peale F, Ferrara N.** Bv8 and endocrine gland-derived vascular endothelial growth factor stimulate hematopoiesis and hematopoietic cell mobilization. *Proc Natl Acad Sci USA* 101: 16813–16818, 2004.
15. **Li M, Bullock CM, Knauer DJ, Ehlert FJ, Zhou QY.** Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle. *Mol Pharmacol* 59: 692–698, 2001.
16. **Li JD, Hu WP, Boehmer L, Cheng MY, Lee AG, Jilek A, Siegel JM, Zhou QY.** Attenuated circadian rhythms in mice lacking the prokineticin 2 gene. *J Neurosci* 26: 11615–11623, 2006.
17. **Lin DC, Bullock CM, Ehlert FJ, Chen JL, Tian H, Zhou QY.** Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. *J Biol Chem* 277: 19276–19280, 2002.
18. **Mahadev K, Wu X, Donnelly S, Ouedraogo R, Eckhart AD, Goldstein BJ.** Adiponectin inhibits vascular endothelial growth factor-induced migration of human coronary artery endothelial cells. *Cardiovasc Res* 78: 376–384, 2008.
19. **Martucci C, Franchi S, Giannini E, Tian H, Melchiorri P, Negri L, Sacerdote P.** Bv8, the amphibian homologue of the mammalian prokineticins, induces a proinflammatory phenotype of mouse macrophages. *Br J Pharmacol* 147: 225–234, 2006.
20. **Masuda Y, Takatsu Y, Terao Y, Kumano S, Ishibashi Y, Suenaga M, Abe M, Fukusumi S, Watanabe T, Shintani Y, Yamada T, Hinuma S, Inatomi N, Ohtaki T, Onda H, Fujino M.** Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochem Biophys Res Commun* 293: 396–402, 2002.
21. **Matsumoto S, Yamazaki C, Masumoto KH, Nagano M, Naito M, Soga T, Hiyama H, Matsumoto M, Takasaki J, Kamohara M, Matsuo A, Ishii H, Kobori M, Katoh M, Matsushime H, Furuichi K, Shigeyoshi**

Y. Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2. *Proc Natl Acad Sci USA* 103: 4140–4145, 2006.

22. **McLaughlin JN, Mazzoni MR, Cleator JH, Earls L, Perdigoto AL, Brooks JD, Muldowney JA 3rd, Vaughan DE, Hamm HE.** Thrombin modulates the expression of a set of genes including thrombospondin-1 in human microvascular endothelial cells. *J Biol Chem* 280: 22172–22180, 2005.
23. **Meigs TE, Fields TA, McKee DD, Casey PJ.** Interaction of G alpha 12 and G alpha 13 with the cytoplasmic domain of cadherin provides a mechanism for beta-catenin release. *Proc Natl Acad Sci USA* 98: 519–524, 2001.
24. **Meyer TN, Schwesinger C, Denker BM.** Zonula occludens-1 is a scaffolding protein for signaling molecules. G alpha(12) directly binds to the Src homology 3 domain and regulates paracellular permeability in epithelial cells. *J Biol Chem* 277: 24855–24858, 2002.
25. **Monnier J, Piquet-Pellorce C, Feige JJ, Musso O, Clement B, Turlin B, Theret N, Samson M.** Prokineticin 2/Bv8 is expressed in Kupffer cells in liver and is down regulated in human hepatocellular carcinoma. *World J Gastroenterol* 214: 1182–1191, 2008.
26. **Negri L, Lattanzi R, Giannini E, Colucci M, Margheriti F, Melchiorri P, Vellani V, Tian H, De Felice M, Porreca F.** Impaired nociception and inflammatory pain sensation in mice lacking the prokineticin receptor PKR1: focus on interaction between PKR1 and the capsaicin receptor TRPV1 in pain behavior. *J Neurosci* 26: 6716–6727, 2006.
27. **Ng KL, Li JD, Cheng MY, Leslie FM, Lee AG, Zhou QY.** Dependence of olfactory bulb neurogenesis on prokineticin 2 signaling. *Science* 308: 1923–1927, 2005.
28. **Ngan ES, Lee KY, Sit FY, Poon HC, Chan JK, Sham MH, Lui VC, Tam PK.** Prokineticin-1 modulates proliferation and differentiation of enteric neural crest cells. *Biochim Biophys Acta* 1773: 536–545, 2007.
29. **Ngan ES, Sit FY, Lee K, Miao X, Yuan Z, Wang W, Nicholls JM, Wong KK, Garcia-Barcelo M, Lui VC, Tam PK.** Implications of endocrine gland-derived vascular endothelial growth factor/prokineticin-1 signaling in human neuroblastoma progression. *Clin Cancer Res* 13: 868–875, 2007.
30. **Podlovni H, Ovadia O, Kisliouk T, Klipper E, Zhou QY, Friedman A, Alfaidy N, Meidan R.** Differential expression of prokineticin receptors by endothelial cells derived from different vascular beds: a physiological basis for distinct endothelial function. *Cell Physiol Biochem* 18: 315–326, 2006.
31. **Prosser HM, Bradley A, Chesham JE, Ebling FJ, Hastings MH, Maywood ES.** Prokineticin receptor 2 (Prokr2) is essential for the regulation of circadian behavior by the suprachiasmatic nuclei. *Proc Natl Acad Sci USA* 104: 648–653, 2007.
32. **Richard DE, Vouret-Craviari V, Pouysségur J.** Angiogenesis and G protein coupled receptors: signals that bridge the gap. *Oncogene* 20: 1556–1562, 2001.
33. **Sabath E, Negoro H, Beaudry S, Paniagua M, Angelow S, Shah J, Grammatikakis N, Yu AS, Denker BM.** G alpha 12 regulates protein interactions within the MDCK cell tight junction and inhibits tight-junction assembly. *J Cell Sci* 121: 814–824, 2008.
34. **Soga T, Matsumoto S, Oda T, Saito T, Hiyama H, Takasaki J, Kamohara M, Ohishi T, Matsushime H, Furuichi K.** Molecular cloning and characterization of prokineticin receptors. *Biochim Biophys Acta* 1579: 173–179, 2002.
35. **Urayama K, Guilini C, Messaddeq N, Hu K, Steenman M, Kurose H, Ert G, Nebigil CG.** The prokineticin receptor-1 (GPR73) promotes cardiomyocyte survival and angiogenesis. *FASEB J* 21: 2980–2993, 2007.
36. **Urayama K, Guilini C, Turkeri G, Takir S, Kurose H, Messaddeq N, Dierich A, Nebigil CG.** Prokineticin receptor-1 induces neovascularization and epicardial-derived progenitor cell differentiation. *Arterioscler Thromb Vasc Biol* 28: 841–849, 2008.
37. **Urayama K, Dedeoglu DB, Guilini C, Frantz S, Ertl G, Messaddeq N, Nebigil CG.** Transgenic myocardial overexpression of prokineticin receptor-2 (GPR73b) induces hypertrophy and capillary vessel leakage. *Cardiovasc Res* 81: 28–37, 2009.
38. **Weis SM, Cheresh DA.** Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* 437: 497–504, 2005.
39. **Yamaguchi Y, Katoh H, Mori K, Negishi M.** G alpha(12) and G alpha(13) interact with Ser/Thr protein phosphatase type 5 and stimulate its phosphatase activity. *Curr Biol* 12: 1353–1358, 2002.
40. **Zeng H, Zhao D, Mukhopadhyay D.** KDR stimulates endothelial cell migration through heterotrimeric G protein Gq/11-mediated activation of a small GTPase RhoA. *J Biol Chem* 277: 46791–46798, 2002.