Antiangiogenic Effect of KR-31372 by Apoptosis via Mediation of Mitochondrial KATP Channel Opening and the Phosphatase and Tensin Homolog Deleted from Chromosome 10 Phosphorylation

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ABSTRACT
Antiangiogenic action of (2R,3R,4S)-N'-cyano-N-(6-nitro-3,4-dihydroxy-2-methyl-2-dimethoxymethyl-2H-1-benzopyran-4-yl)-N'-benzyl guanidine (KR-31372) was examined with its proapoptotic action in human umbilical vein endothelial cells (HUVECs) compared with diazoxide. KR-31372 as well as diazoxide significantly suppressed the neovascularization in mice induced by the Matrigel-containing recombinant human vascular endothelial growth factor (VEGF)165 in vivo and the basal tube formation of HUVECs in vitro with suppression of proliferation of HUVECs stimulated by VEGF165. KR-31372 and diazoxide enhanced DNA fragmentation associated with increase in phosphatase and tensin homolog deleted from chromosome 10 (PTEN) and decrease in serine/threonine kinase phosphorylation, which were accompanied by augmented Bax and cytochrome c release, and suppressed Bcl-2 in HUVECs. In the U87-MG cells, when transfected with expression vectors for sense PTEN, KR-31372 enhanced DNA fragmentation, but not in naive U87-MG cells. The suppression by KR-31372 and diazoxide of these variables was significantly antagonized by 5-hydroxydecanoic acid, a mitochondrial KATP channel blocker. Taken together, KR-31372 strongly inhibited angiogenesis in HUVECs by proapoptotic mechanism via mediation of 5-hydroxydecanoic acid-inhibitable mitochondrial KATP channel opening and PTEN phosphorylation.

Angiogenesis is a process that involves enzymatic degradation and remodeling of the extracellular matrix, migration, and proliferation of capillary endothelial cells (Cao et al., 1996). Vascular endothelial growth factor (VEGF), as an endothelial cell-specific mitogen, promotes angiogenesis by stimulation of endothelial cell proliferation and inhibition of endothelial cell apoptosis (Kuzuya et al., 1999). Apoptosis, a programmed cell death, is critical during embryonal development and in tissue renewal (Cohen, 1993). Induction of endothelial cell apoptosis has recently attracted considerable interest as a potential target for antitumor therapy (Sgouros et al., 1998).

Phosphatase and tensin homolog deleted from chromosome 10 (PTEN) has dual specificity protein phosphatase (toward phospho-serine/threonine and phospho-tyrosine) and phosphoinositide 3-phosphatase activities (Myers et al., 1997; Maehama and Dixon, 1998) and antagonizes the phosphatidylinositol 3-kinase (PI3-K) pathway by catalyzing degradation of the phosphatidylinositol (3,4,5)-triphosphate [PI(3,4,5)P3], which is generated by PI3-K, thereby leading to a control role in converting PI(3,4,5)P3 to phosphatidylinositol (3,4)-diphosphate, an inactive state (Stambolic et al., 1998; Cantley and Neel, 1999).

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ABBREVIATIONS: VEGF, vascular endothelial growth factor; PTEN, phosphatase and tensin homolog deleted from chromosome 10; Akt, serine/threonine kinase; PI3-K, phosphatidylinositol 3-kinase; PI(3,4,5)P3, phosphatidylinositol (3,4,5)-triphosphate; mitoKATP, mitochondrial ATP-sensitive potassium channel; KATP, ATP-sensitive potassium channel; 5-HD, 5-hydroxydecanoic acid; HUVEC, human umbilical vein endothelial cell; MEM, minimal essential medium; bp, base pair(s); sPTEN, sense phosphatase and tensin homolog deleted from chromosome 10.
higher sensitivity to opening by diazoxide, which increases the sensitivity of sarcoplasmic calcium channels by 2000-fold (Garlid et al., 1996). Glitazolamide, a potent and nonselective K<sub>ATP</sub> channel blocker has been demonstrated to inhibit the mitochondria in the heart and brain (Jaburek et al., 1998; Bajgar et al., 2001). 5-Hydroxydecanoic acid (5-HD) blocks the mitochondria reconstituted in liposomes and isolated mitochondria, but it does not block cardiac type sarcoplasmic K<sub>ATP</sub> channels (Jaburek et al., 1998). There is, however, little information on the relationships between mitoK<sub>ATP</sub> activation and regulation of PTEN phosphorylation in the angiogenesis in relation with apoptosis.

In the preliminary study, KR-31372 showed very weak vasodilator action despite having a benzopyran moiety in its structure, which is a striking contrast to levocromakalim, and suggested a glitazolamide-inhibitable opening of K<sub>ATP</sub> channel in isolated rat ventricular myocytes, suggestive of the K<sub>ATP</sub> channel opener. Recently, KR-31372 exerted an inhibitory effect on the oxidized low-density lipoprotein-stimulated synthesis of [H]thymidine incorporation and migration of the cultured rat aortic smooth muscle cells (Kim et al., 2000). The antiangiogenic effect of KR-31372 was first demonstrated in rat sponge implant model, in that oral administration of KR-31372 (1 mg/kg for 7 days) significantly suppressed the antiangiogenic effect of KR-31372. The injected Matrigel rapidly formed a single, solid gel plug. After 5 days, mice were sacrificed, and Matrigel plug was recovered, fixed with 10% formaldehyde/phosphate-buffered saline (pH 7.4), and embedded in paraffin and examined with hematoxylin and eosin stain. To quantify the formation of new blood vessel, the amount of hemoglobin was measured using the total hemoglobin kit (Sigma-Aldrich, St. Louis, MO). Four mice were used for one group, and the experiment was repeated twice.

DNA Fragmentation Assays. Cells were lysed in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K). Digestion was continued for 1 to 3 h at 55°C, followed by addition of RNase A to 0.1 mg/ml and running dye (10 mM EDTA, 0.25% bromophenol blue, and 50% glycerol). Equivalent amounts of DNA (15–20 μg) were loaded into wells of 1.6% agarose gel and electrophoresed in 0.5× TAE buffer (40 mM Tris-acetate and 1 mM EDTA) for 2 h at 6 V/cm. DNA was visualized by ethidium bromide staining. Gel pictures were taken by UV transillumination with the Polaroid camera. Bands were quantified by the Molecular Analyst software using the Bio-Rad’s Image Analysis System (Bio-Rad).

Mitochondrial cytochrome c was prepared via following procedures. After washing cells (12 × 10<sup>6</sup>) with ice-cold phosphate-buffered saline, cell pellets were suspended in buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM Na-EDTA, 1 mM Na-EGTA, 1 mM dithiothreitol, and 0.1% (v/v) polyethyleneimine sulfonate) containing 250 mM sucrose. The cells were homogenized and then centrifuged twice at 750g for 10 min at 4°C. The harvested supernatants were centrifuged at 10,000g for 10 min at 4°C, and the resulting mitochondrial pellets were dissolved in 1× SDS sample buffer. Western blots were performed with the antibody of cytochrome c (Santa Cruz Biotechnology, Inc.). The immunoreactive bands were visualized using chemiluminescent reagent of the SuperSignal west dura extended duration substrate kit (Pierce Chemical, Rockford, IL). The signals of the bands were quantified using the calibrated imaging densitometer (GS-T10; Bio-Rad). The protein con-
centration of the lysate was determined using the Bio-Rad DC assay kit (Bio-Rad).

**Plasmid Construction.** The expression of plasmid encoding the human PTEN protein was cloned by reverse transcription-polymerase chain reaction using the total RNA of SK-N-SH cells. Sequence analysis was performed to confirm the nucleotide sequences. The following sequences of oligodeoxynucleotides were used as primers containing linker recognizable by XhoI as underlined: 5′-GGGCTCGAGATCAGCCTAAG-3′. Amplified 1264-bp fragments containing the human PTEN coding region were ligated into the XhoI site of pcDNA3.1 HisC (Invitrogen, Carlsbad, CA). pcDNA3.1-sPTEN is transcribed sense nucleotides.

**DNA Transfection.** U87-MG cells were seeded for 24 h before transfection in tissue culture dishes. At 50 to 70% confluence, the dishes were washed twice with Opti-MEM medium to remove the fetal bovine serum and a transfection cocktail containing 10 μg DNA and 10 μl of LipofectAMINE reagent (Invitrogen) per 100-mm dish was added. The medium was removed and then 7 ml of MEM medium containing 10% fetal bovine serum and 10 ml of LipofectAMINE reagent (Invitrogen) per 100-mm dish was added. The medium was removed and then 7 ml of MEM medium containing 10% fetal bovine serum was added to each dish.

**Drugs.** VEGF₁₆₅ was purchased from the R & D Systems (Minneapolis, MN). (2R,3R,4S)-N′-Cyano-N-(6-nitro-3,4-dihydro-hydroxy-2-methyl-2-dimethoxymethyl-2H-1-benzopyran-4yl)-N′-benzyl guanidine (KR-31372) was donated from The Korea Research Institute of Chemical Technology (Daejon, Korea). 5-Hydroxydecanoic acid (sodium salt), endothelial cell growth supplement, and total hemoglobin kit were purchased from the Sigma-Aldrich (St. Louis, MO). 5-Bromo-2′-deoxyuridine kit was from the Roche Diagnostics (Mannheim, Germany). Matrigel was from the BD Biosciences Discovery Labware (Bedford, MA). KR-31372 and diazoxide were dissolved in dimethyl sulfoxide as a 10⁻¹ M stock solution. 5-Hydroxydecanoic acid was dissolved in distilled water as a 10⁻¹ M stock solution.

**Statistics.** The results are expressed as means ± S.E.M. Two-way repeated measures analysis of variance was used for the comparison of concentration-dependent changes in cell proliferation in response to agonists between inhibitor-treated and untreated groups. Statistical differences between groups were determined by paired or unpaired Student’s t test or analysis of variance. P < 0.05 was considered significant.

**Results**

**Tubular Formation.** HUVECs plated on the Matrigel elongated and migrated to form a tubular network structure as shown in the morphological changes. When quantitatively estimated by measuring the length covered by the tubular network area using the image analysis program, both KR-31372 (10⁻⁶ M) and diazoxide (10⁻⁵ M) markedly suppressed the basal tubular formation to 53.5 ± 9.4% (P < 0.01) and 59.3 ± 8.7% (P < 0.01), respectively (Fig. 1, A and B). 5-HD (10⁻⁵ M) applied 30 min before KR-31372 or diazoxide treatment significantly reversed the suppressed basal tubular formation induced by either KR-31372 or diazoxide. In the basal tubular formation, 5-HD (10⁻⁵ M) alone showed little effect.

**Matrigel Plug Assay in Mice.** Five days after implantation, histological examination and hemoglobin content were

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Fig. 1. A, inhibitory effect of KR-31372 (10⁻⁶ M) and diazoxide (10⁻⁵ M) on the basal tube formation of HUVECs on Matrigel in the absence and presence of 5-HD (10⁻⁵ M). Photographs were taken after 18 h in culture (40×). B, area covered by the tube network was determined using the GS-710 calibrated imaging densitometer. Results are expressed as means ± S.E.M. of three different preparations with duplicate experiments. C, inhibitory effect of KR-31372 (10⁻⁶ M) and diazoxide (10⁻⁵ M) on the VEGF₁₆₅-induced angiogenesis in vivo by using Matrigel plug assay. C57BL/6 mice subcutaneously received Matrigel (0.5 ml) containing VEGF₁₆₅ (5 ng/ml) with KR-31372 (10⁻⁶ M) or diazoxide (10⁻⁵ M) in the absence or presence of 5-HD (10⁻⁵ M) (200×). Arrows indicate the active neomicrovessels containing red blood cells. D, analysis of hemoglobin contents within the Matrigel plug. Four mice were used as a group, and the experiment was repeated twice. ###, P < 0.001 versus none; *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus vehicle (Veh); †, P < 0.05 versus KR-31372 or diazoxide alone.
estimated. As shown in Fig. 1, C and D, the neomicrovessel formation significantly increased in the Matrigel containing VEGF165 (5 ng/ml), whereas it was not evident in the Matrigel without VEGF165. Matrigel containing either KR-31372 (10⁻⁶ M) or diazoxide (10⁻⁵ M) significantly reduced the formation of VEGF165-stimulated neomicrovessels, which was antagonized by 5-HD (10⁻⁵ M). The neovascular formation was further confirmed by measurement of the hemoglobin content in the Matrigel. The hemoglobin content was elevated in the Matrigel containing 5 ng/ml VEGF165 to 5.2 ± 0.6 g/dl (P < 0.001), which was markedly suppressed by treatment with 10⁻⁶ M KR-31372 (1.6 ± 0.3 g/dl, P < 0.001) and 10⁻⁵ M diazoxide (2.2 ± 0.4 g/dl, P < 0.05). Pretreatment with 5-HD (10⁻⁵ M) significantly reversed both KR-31372- and diazoxide-induced reduction in hemoglobin content (Fig. 1D), indicating that KR-31372 as well as diazoxide elicit antiangiogenic activities in the in vivo experiment.

**Cell Proliferation.** VEGF165 (1–20 ng/ml) concentration dependently increased DNA synthesis. After 48-h incubation, 10 ng/ml VEGF165 increased DNA synthesis to 185.3 ± 7.7% of control cells (Fig. 2, inset). Cell proliferation was concentration dependently suppressed by simultaneous incubation with either KR-31372 or diazoxide (10⁻⁸–10⁻⁴ M, each), respectively, 5-HD (10⁻⁵ M), when applied 30 min before KR-31372 or diazoxide treatment, significantly reversed the suppressed DNA synthesis induced by either KR-31372 or diazoxide (Fig. 2). After application of 5-HD (10⁻⁵ M) in the absence of VEGF165, the cells showed little effect on the proliferation of HUVECs (102.9 ± 3.7%).

**Apoptotic Effect.** Effect of KR-31372 was compared with diazoxide on the laddered feature of DNA fragmentation, which was pretreated with 10 ng/ml VEGF165 in the HUVECs. Exposure of HUVECs to KR-31372 (10⁻⁶ and 10⁻⁴ M) and diazoxide (10⁻⁵ M) induced prominent oligonucleosomal DNA fragmentation. Pretreatment with 5-HD (10⁻⁵ M) strongly suppressed the DNA laddering induced by KR-31372 (10⁻⁵ M) and diazoxide (10⁻⁵ M), respectively (Fig. 3).

On the other hand, the effect of KR-31372 on the DNA fragmentation was identified in the U-373MG cells, naive U87-MG cells, and U87-MG cells of sPTEN. The cells were exposed to KR-31372 (10⁻⁶–10⁻⁴ M) for 3 h in the presence of VEGF165 (10 ng/ml). KR-31372 (10⁻⁶–10⁻⁴ M) induced prominent oligonucleosomal DNA fragmentation in the U-373MG and U87-MG cells of sPTEN, but did not show DNA fragmentation in U87-MG lacking a wild-type PTEN (Fig. 4).

**PTEN and Akt Phosphorylation.** PTEN phosphorylation was concentration dependently increased by KR-31372 (1.82 ± 0.14-fold (P < 0.05) by 10⁻⁶ M and 2.78 ± 0.31-fold (P < 0.01) by 10⁻⁵ M KR-31372) when applied 3 h before VEGF165 (10 ng/ml) in the HUVECs. Pretreatment with 5-HD (10⁻⁵ M, P < 0.01) significantly suppressed the increased PTEN phosphorylation induced by KR-31372 (10⁻⁵ M) to 0.88 ± 0.09-fold (P < 0.01). Diazoxide (10⁻⁵ M) and 5-HD (10⁻⁵ M) showed similar interactions as shown with KR-31372 and 5-HD (Fig. 5A). The similar results were also evident in the U-373MG cells, human brain glioblastoma cells (Fig. 5B). However, the PTEN protein expression was not altered by these agents.

Otherwise, PTEN protein was expressed in the HUVECs and U-373MG cells, but not in the naive U87-MG cells, whereas the Akt expression was well identified in the three cells. Introduction of PTEN cDNA (sense oligodeoxynucleotide) into the U87-MG cells, lacking a wild-type PTEN, caused a large increase in PTEN expression (Fig. 6A). In the U87-MG cells of sPTEN, both KR-31372 and diazoxide significantly increased the PTEN phosphorylation as shown in the HUVECs and U-373MG cells, and the phosphorylated Akt levels were, in contrast, significantly decreased by these agonists (Fig. 6B). The alterations induced by KR-31372 and diazoxide were well antagonized by 10⁻⁶ M of 5-HD (Fig. 6, C and D). The levels of Akt and Akt phosphorylation were not altered by KR-31372 (10⁻⁶–10⁻⁴ M) and diazoxide (10⁻⁵ M) in the U87-MG cells lacking a wild-type PTEN (data not shown).

**Western Blot for Bcl-2, Bax, and Cytochrome c.** Under treatment with VEGF165 (10 ng/ml for 24 h), the Bcl-2 protein expression was markedly increased to 2.60 ± 0.54 relative density, which was strongly suppressed by KR-31372 (10⁻⁶–10⁻⁴ M) in a concentration-dependent manner (Fig. 7A). Diazoxide (10⁻⁵ M) also significantly decreased the Bcl-2 protein level. In the presence of 5-HD (10⁻⁵ M), both KR-31372- and diazoxide-induced inhibitions of Bcl-2 protein levels were significantly reversed (Fig. 7B).

In the presence of VEGF165 (10 ng/ml), the basal levels of Bax protein and cytochrome c release from mitochondria were 0.77 ± 0.06 and 0.97 ± 0.08 relative densities, respectively. In contrast, both were largely elevated by pretreatment with KR-31372 (10⁻⁶, 10⁻⁵, and 10⁻⁴ M) and diazoxide (10⁻⁵ M). 5-HD (10⁻⁵ M) significantly suppressed the increased Bax protein and cytochrome c release induced by either KR-31372 or diazoxide (Fig. 8, A and B).
Discussion

In the present study, the major findings were that KR-31372 as well as diazoxide markedly suppressed the in vitro basal tube formation in HUVECs and in vivo Matrigel-induced neovascularization in mice in association with augmentation of oligonucleosomal DNA fragmentation. Increased PTEN phosphorylation stimulated by KR-31372 and diazoxide was accompanied by decreased Akt phosphorylation and suppression of Bcl-2 expression in accordance with enhanced Bax protein level and cytochrome c release from mitochondria. All these variables were significantly reversed by 5-HD, a mitoKATP blocker. No previous study has defined the implication of mitoKATP opening- and PTEN phosphorylation-linked apoptotic mechanism in the antiangiogenesis.

The angiogenesis requires the triad processes: endothelial cell proliferation, migration, and local protease activity such as plasminogen activator and matrix metalloproteinases.

Fig. 3. A, increased DNA laddering by KR-31372 and diazoxide determined by agarose gel electrophoresis and ethidium bromide staining. HUVECs were incubated in the medium containing 10 ng/ml VEGF165 under pretreatment with KR-31372 and diazoxide for 2 days; none represents absence of VEGF165, and M represents the 100-bp DNA ladder markers. B, densitometric analysis. Results are expressed as means ± S.E.M. of three different experiments. **, *P < 0.01 versus VEGF165 alone; †††, *P < 0.001 versus 10⁻⁴ M KR-31372 or 10⁻⁵ M diazoxide alone.

Fig. 4. Effect of KR-31372 on the DNA fragmentation in the U-373MG, naïve U87-MG, and U87-MG cells of sPTEN. Cells were incubated in the medium containing 10 ng/ml VEGF165 under treatment with KR-31372 for 2 days. M represents the 100-bp DNA ladder markers. KR-31372 (10⁻⁵–10⁻⁴ M) induced prominent oligonucleosomal DNA fragmentation in the U-373MG and U87-MG cells of sPTEN, but did not show DNA fragmentation in U87-MG lacking a wild-type PTEN.

Fig. 5. Representative immunoblotting for PTEN phosphorylation in HUVECs (A) and U-373MG cells (B). Concentration-dependent elevation of PTEN phosphorylation by KR-31372 (10⁻⁶–10⁻⁴ M) and diazoxide (10⁻⁵ M) using anti-phospho-specific PTEN antibody. Effect of 5-HD (10⁻⁵ M) on the elevated PTEN phosphorylation by KR-31372 and diazoxide. Results are expressed as means ± S.E.M. of three different experiments. *, *P < 0.05; **, *P < 0.01; †, †P < 0.001 versus VEGF165 alone; ††, ††P < 0.01 versus 10⁻⁴ M KR-31372 or 10⁻⁵ M diazoxide alone.
as matrix metalloproteinases (Hananan and Folkman, 1996). Overexpression of VEGF and its receptors is associated with chronic inflammation, tumor growth, and diabetic retinopathy (Williams, 1998). Of the various VEGF species, VEGF165 is well characterized (Neufeld et al., 1999), and the expression of cell surface receptors for VEGF165 was demonstrated in the endothelial cells (Millauer et al., 1993). Binding of VEGF to VEGF receptor-2 leads to the receptor phosphory-

**Fig. 6.** A, representative Western blotting for PTEN and Akt in the HUVECs, U-373MG, naive U87-MG, and U87-MG cells transfected with expression vectors for sense PTEN (sPTEN). B, effects of KR-31372 and diazoxide on the expression of phosphorylation of PTEN and Akt in the presence and absence of 5-HD (10⁻⁵ M). C and D, densitometric analyses are showing elevation of the phosphorylated PTEN and inhibition of phosphorylated Akt levels by KR-31372 (10⁻⁶–10⁻⁴ M) and diazoxide (10⁻⁵ M), which were reversed under treatment with 5-HD (10⁻⁵ M). Results are expressed as means ± S.E.M. of three different experiments. ###, P < 0.001 versus none; ***, P < 0.01; ****, P < 0.001 versus VEGF 165 alone; †, P < 0.05; ††, P < 0.01 versus 10⁻⁵ M KR-31372 or 10⁻⁵ M diazoxide alone.

**Fig. 7.** Representative Western blots for Bcl-2 protein expression and the corresponding densitometric analysis. A, VEGF₁₆₅-induced Bcl-2 level was wholly suppressed by KR-31372 in a concentration-dependent manner. B, effect of 5-HD (10⁻⁵ M) on the Bcl-2 inhibition by KR-31372 and diazoxide. Results are expressed as means ± S.E.M. of three different experiments. ###, P < 0.001 versus none; ***, P < 0.001 versus vehicle (Veh); †, P < 0.05; ††, P < 0.01 versus 10⁻⁵ M KR-31372 or 10⁻⁵ M diazoxide alone.

**Fig. 8.** Representative Western blots for Bax protein expression (A) and cytochrome c release from mitochondria (B), and the corresponding densitometric analyses. Concentration-dependent increases in Bax protein and cytochrome c release by KR-31372 (10⁻⁶–10⁻⁴ M) and diazoxide (10⁻⁵ M), and their reverses by 5-HD (10⁻⁵ M). Results are expressed as means ± S.E.M. of three different assays. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus VEGF₁₆₅ alone; †, P < 0.05; ††, P < 0.01 versus 10⁻⁵ M KR-31372 or 10⁻⁵ M diazoxide alone.
PTEN was originally identified as a tumor suppressor gene based on its high frequency of mutation in a variety of tumors (Li et al., 1997). 3-Phosphoinositides are important substrates for PTEN both in vitro (Maehama and Dixon, 1998) and in vivo experiments (Sun et al., 1999). PTEN potently modulates VEGF-mediated signaling and function. Most recently, Huang and Kontos (2002) have provided evidence that adenovirus-mediated overexpression of a dominant negative PTEN mutant enhances VEGF-mediated endothelial cell proliferation and migration, whereas overexpression of wild-type PTEN, in contrast, shows inhibition of cell proliferation and chemotactic effects of VEGF. Our data showed for the first time that both KR-31372 and diazoxide exerted concentration-dependent increase in PTEN phosphorylation in the HUVECs, which was suppressed by 5-HD (10^{-5} M, P < 0.01). PTEN has the property to restrain the PI3-K pathway by catalyzing degradation of the PI(3,4,5)P3, which is generated by PI3-K. They did not identify whether the action site of glibenclamide is closely linked to 5-HD-sensitive mitoKATP opening and the reduced release of cytochrome c. Overall, PTEN, they exerted increased PTEN protein expression.

In our results, when the U87-MG cells, a glioblastoma cell line that lacks expression of wild type PTEN (Haas-Kogan et al., 1998), were transfected with expression vectors for sense PTEN, they exerted increased PTEN protein expression. These transfected cells also showed high phosphorylated PTEN accompanied by decreased Akt phosphorylation under treatment with KR-31372 and diazoxide, which was fully reversed by mitoKATP blocker 5-HD. Based on these facts, it is likely that KR-31372 has a role for regulation of PTEN phosphorylation as a mitoKATP opener.

In the previous results, glibenclamide reversed the KR-31372-induced suppression of the VEGF 165-stimulated chemotactic motility of HUVECs (Kim et al., 2003). At that time, they did not identify whether the action site of glibenclamide is on the sarcomemal K_{ATP} channels or the mitoKATP channels. In the light of the present study, it seemed that the action of glibenclamide was ascribed to the inhibition of the mitoK_{ATP} activation (Jaburek et al., 1998; Bajgar et al., 2001). Tamura et al. (1998) have emphasized the importance of PTEN in the cell migration, in that overexpression of PTEN dephosphorylates FAK in vivo and in vitro, and reduces its tyrosine phosphorylation and inhibits integrin-mediated cell spreading, whereas antisense PTEN enhanced migration. Most recently, Kim et al. (2003) have demonstrated that KR-31372 significantly inhibited the KDR/Flk-1 tyrosine phosphorylation-linked extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, and p125^FAK tyrosine phosphorylation, which were inhibited by glibenclamide, a K_{ATP} channel blocker. Considering that KR-31372 significantly suppressed the VEGF-stimulated triad of angiogenesis (DNA synthesis, migration, and MMP-2 release; data not shown) in HUVECs, it is likely predictable that KR-31372 might suppress the tube formation in HUVECs and Matrigel-induced neovascularization in mice via mediation of the mitoK_{ATP} opening, thereby leading to the antiangiogenesis.

Accumulating reports have shown that the mitoK_{ATP} is the receptor for cardioprotection by K_{ATP} channel openers and for 5-HD blockade and that 5-HD inhibits the mitoK_{ATP}, but not the sarcomemal K_{ATP} channel (McCullough et al., 1991; Grover and Garland, 2000). Garland et al. (1996, 1997) showed that diazoxide was 1000 to 2000 times more potent in opening mitoK_{ATP} than in opening sarcomemal K_{ATP} channels. Thus, it is intriguing that the mitoK_{ATP} opens, including diazoxide and KR-31372, increased the expression of PTEN phosphorylation and induced 5-HD-inhibitable apoptosis in the HUVECs, despite a number of studies demonstrating a cardioprotection by diazoxide and cromakalim as openers of mitoK_{ATP} (Grover and Garland, 2000). Currently, it is not possible to reconcile the disparate results of diazoxide and KR-31372 between cardiac myocytes and HUVECs. In contrast to other cells, the endothelial cells possessed the unique properties. The electrochemical gradient or driving force for Ca^{2+} entry into endothelial cells is characteristically influenced by the membrane potential (Lückhoff and Busse, 1990), such that depolarization decreases, whereas hyperpolarization increases, Ca^{2+} entry. Resting membrane potentials in endothelial cells are thought to be controlled primarily by K^+ channels; in particular, inwardly rectifying K^+ channels (Nilius et al., 1997).

Although the data are not shown, mitochondrial membrane potential and intramitochondrial calcium levels were decreased by both KR-31372 and diazoxide in a concentration-dependent manner. These values were significantly reversed by treatment with 5-HD. Moreover, these reductions were accompanied by increase in Bax protein and increased cytochrome c release, indicating that KR-31372 and diazoxide augmented the apoptotic action via mitoK_{ATP} opening. Presently, it remains to be clarified how the mitoK_{ATP} opening triggers to increase the PTEN phosphorylation.

Apoptosis of endothelial cells occurs during the vascular regression in the process of scar formation (Desmouliere et al., 1995), atherosclerosis (Dimmel et al., 1998), and progressive glomerulonephritis (Shimizu et al., 1997). The expression of Bcl-2 protein in the mitochondrial outer membrane prevents the association of the proapoptotic Bax protein with permeability transition pore and its pore forming activity, and then acts to inhibit cytochrome c release from mitochondria to cytosol ( Kluck et al., 1997; Shimizu and Tsujimoto, 2000). The consequence of mitoK_{ATP} activation in the isolated rat heart mitochondria was emphasized by Holmuhamedov et al. (1998), in that K_{ATP} channel openers (pinacidil, cromakalim, and levocromakalim) induced mitochondrial membrane depolarization with an increase in the rate of mitochondrial respiration and consequently a decrease in ATP synthesis, these cascades resulting in enhanced release of cytochrome c from mitochondria.

Together, these findings provide a strong evidence to support that the proapoptotic effect of KR-31372 in the HUVECs is closely linked to 5-HD-sensitive mitoK_{ATP} opening and the up-regulation of PTEN phosphorylation and down-regulation of Akt phosphorylation and Bcl-2 protein expression, thereby leading to antiangiogenesis in the HUVECs.
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References


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