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Footnotes

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Figure Legends

Fig. 1. Effect of PG on HIF-1α protein levels in cells

A and B, HEK293 cells (*A*, *left panel*), Hep3B cells (*A*, *right panel*), HUVECs (*B, left panel*), and HASMCs (*B, right panel*) were exposed to vehicle (lane 1), 100 μM PG (lane 2), or 100 μM DFX (lane 3) for 4 h and whole cell lysates were analyzed for HIF-1α or HIF-1β protein expression by immunoblot assay. *C*, HEK293 cells were exposed to the indicated dose of PG or DFX for 4 h (left panel) or were exposed to 100 μM PG or 100 μM DFX for the indicated time periods (right panel). Whole cell lysates were subject to immunoblot assay for HIF-1α protein expression.

Fig. 2. Effect of PG on HIF-1-mediated transcriptional activity

A, HEK293 cells were treated with vehicle (-), 10-100 μM PG, or 100 μM DFX for 24 h and total RNA was isolated. Expression levels of VEGF, EPO, and HIF-1α mRNA, and 18S rRNA were analyzed by RT-PCR. *B-E*, HEK293 cells were transfected with pSV40-RL encoding *Renilla* luciferase and one of the following plasmids encoding firefly luciferase: HRE reporter p2.1 (*B, C, D*), mutant HRE reporter p2.4 (*D*), or *VEGF* promoter reporter pVEGF-KpnI-Luc (*E*). Cells were exposed to vehicle (-), 10-100 μM PG or 10-100 μM DFX for 16 h and then harvested for luciferase assays. In *C*, cells were co-transfected with p2.1, pSV40-RL, and 200 ng of expression vector encoding either no protein (EV) or a dominant negative form of HIF-1α (HIF-1α-DN). The total amount of expression vectors was adjusted to 200 ng with empty

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vector. The ratio of firefly:*Renilla* luciferase activity was determined and normalized to the value obtained from non-treated cells transfected with empty vector to obtain the relative luciferase activity (RLA). Results shown represent mean ± S.D. of three independent transfections. * $p<0.05$ vs. pretreatment (B, C, and E:one–way ANOVA, D:Student *t*-test)

Fig. 3. Effect of PG or DFX on HIF-1α protein stability

A, HEK293 cells were plated on chamber slides and transfected with pVenus (*a*) or pVenus-HIF-1α (*b-d*). Cells were untreated (*a*, *b*) or treated with 100 μM PG (*c*) or 100 μM DFX (*d*) for 1 h. *B*, pcDNA-FLAG-HIF-1α plasmid was introduced into HEK293 cells. Total cell lysate and nuculear fraction of the lysate were prepared to be subjected to immunoblot with anti-FLAG antibody. *C*, HEK293 cells were exposed to solvent (lanes 1-3), 100 μM PG (lanes 4-6), or 100 μM DFX (lanes 7-9) for 4 h and then cycloheximide (CHX) was added to a final concentration of 100 μM. The cells were incubated for 0-60 min, and whole cell lysates were subject to immunoblot assay using anti-HIF-1α (upper panel) and anti-HIF-1β (lower panel) antibodies.

Fig. 4. Effect of gallates, antioxidants, and kinase inhibitors on HIF-1α protein levels and HRE-dependent gene expression in HEK293 cells

A, HEK293 cells were exposed to vehicle (lane 1) or 50 or 100 μM PG (lanes 2 and 3), GA (lanes 4 and 5), or EGCG (lanes 6 and 7) for 4 h and harvested for immunoblot assay using anti-HIF-1α antibody. *B*, HEK293 cells were transfected with pSV40-RL encoding *Renilla*

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luciferase and reporter p2.1. Cells were exposed to vehicle (-), 100 μM PG, GA or EGCG for 16 h and then harvested for luciferase assays. * $p<0.05$ vs. pretreatment (one–way ANOVA) *C*, HEK293 cells were treated with vehicle, or 100 μM of gallic acid (GA), methyl gallate (ME), ethyl gallate (EG), propyl gallate (PG), or octyl gallate (OC) for 4 h and harvested for immunoblot assay using anti-HIF-1α antibody. *D*, HEK293 cells were exposed to 100 μM PG with vehicle, 15 mM 2-oxoglutarate (2-OX), 100 μM ascorbate (ASC), or 100 μM FeSO4 for 4 h and harvested for immunoblot assay using anti-HIF-1α antibody. Cells were harvested for analysis of HIF-1α protein.

Fig. 5. Effect of PG on the activity of HIF-1α hydroxylases

A and B, GST-HIF-1α(429-608) fusion protein was incubated with *in vitro*-translated and biotinylated VHL in the presence of PBS (lysate -) or lysate from cells (A and B, left panel) that were untreated (-) or treated or lysate directly treated with the indicated concentration of the reagents. Glutathione-Sepharose beads were used to capture GST-HIF-1α and the presence of VHL in the samples was determined by PAGE. One-fifth of the input biotinylated lysine-labeled IVTT-VHL protein was also analyzed. *C*, Caco 2 cells were transfected with siRNA against HIF-1a. Cells were treated with or withour PG and harvested. Total RNAs were subjected to RT-PCR for ITF, HIF-1α, and 18S. PCR products were fractionated by 3% Nusieve agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV. *D*, GST-HIF-1α(531-826) fusion protein was incubated with in vitro-translated p300 CH1 domain in the presence of PBS (lane 1) or lysate from cells that were untreated (lane 2) or treated

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with the indicated compound (lanes 3-5). Glutathione-Sepharose beads were used to capture GST-HIF-1α and the presence of p300 CH1 domain in the samples was determined by PAGE. One-fifth of the input biotinylated lysine-labeled IVTT-CH1 domain protein was also analyzed. *D*, A fusion protein containing the DNA-binding domain (amino acids 1-147) of the yeast transcription factor GAL4 fused to amino acid residues 531-826 of HIF-1α was analyzed for its ability to transactivate reporter gene pG5E1bLuc, which contains five GAL4-binding sites upstream of a minimal *E1b* gene promoter and firefly luciferase coding sequences. HEK293 cells were co-transfected with pRL-SV40 (10 ng), pG5E1bLuc (150 ng), and pGAL4-HIF-1α (200 ng). Cells were exposed to PG or DFX for 16 h and harvested. The ratio of firefly:Renilla luciferase activity was determined and normalized to the value obtained from untreated cells transfected with plasmid encoding pGAL4(1-147) to obtain the relative luciferase activity (RLA).

Fig. 6 Effect of PG on cells derived from intestinal epithelium and serum EPO expression
A, Caco-2, HT-29, SW48, and T84 cells were exposed to vehicle (-) or 50-400 μM PG for 4 h and whole cell lysates were subjected to immunoblot assay for HIF-1α protein expression. *B*, Caco-2 cells were exposed to 0-400 μM PG or 1% O_2 for 24 h and total RNA was isolated. Expression of VEGF, ITF, and HIF-1α mRNA and S18 was analyzed by RT-PCR. *C*, Caco-2 cells pretreated with a short interfering RNA (siRNA) or not were exposed to 100 μM PG or 1% O_2 for 24 h and total RNA was isolated. Expression of VEGF, ITF, and HIF-1α mRNA was analyzed by RT-PCR. *D*, Caco-2 cells were treated with 50 μM of GA or PG for indicated

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periods and transepithelial electrical resistance was measured. Results shown represent mean ± SD (n=5). * $p<0.05$ vs. pretreatment (one–way ANOVA) ; RER, relative electrical resistance. *E*, Serum EPO levels after PG administration (100mg/kg, 5 days) by oral gavage. Results shown represent mean ± SD (n=5). * $p<0.05$ vs. pretreatment (Student *t*-test)

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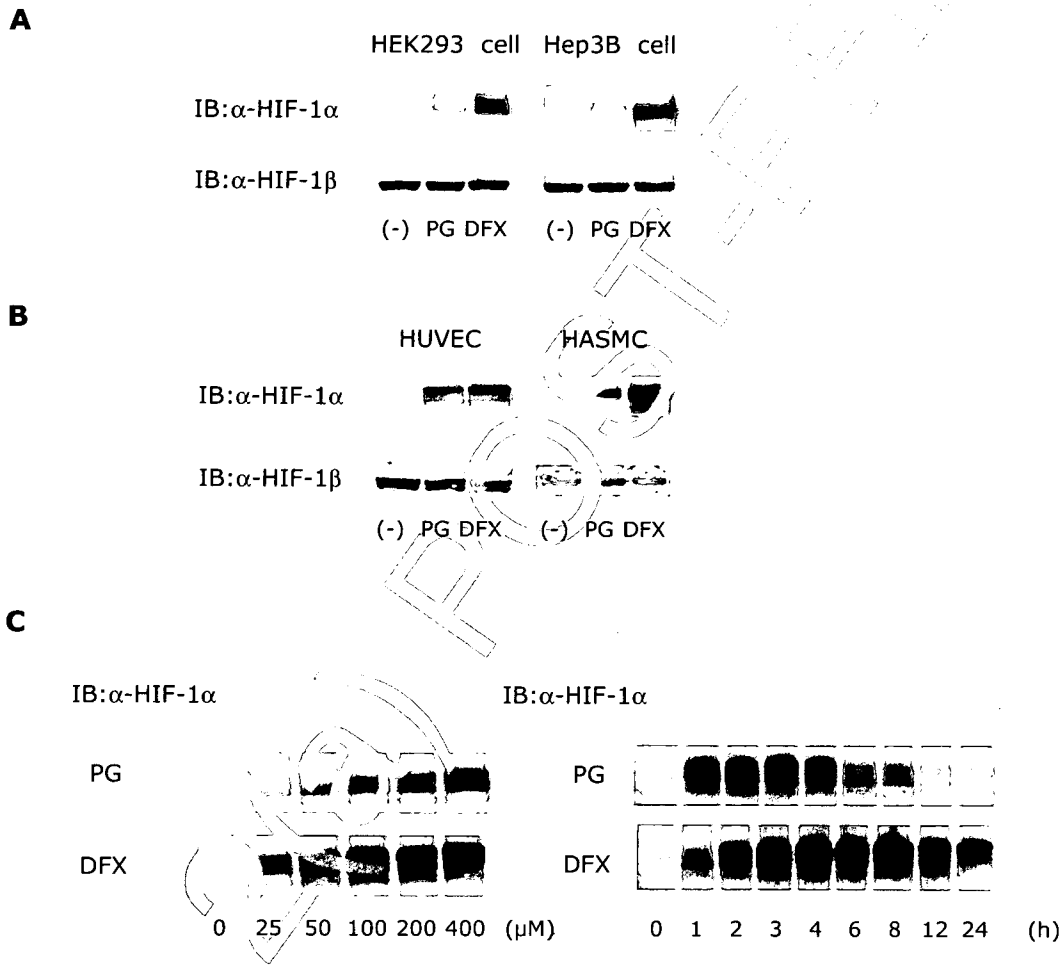

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figure 1

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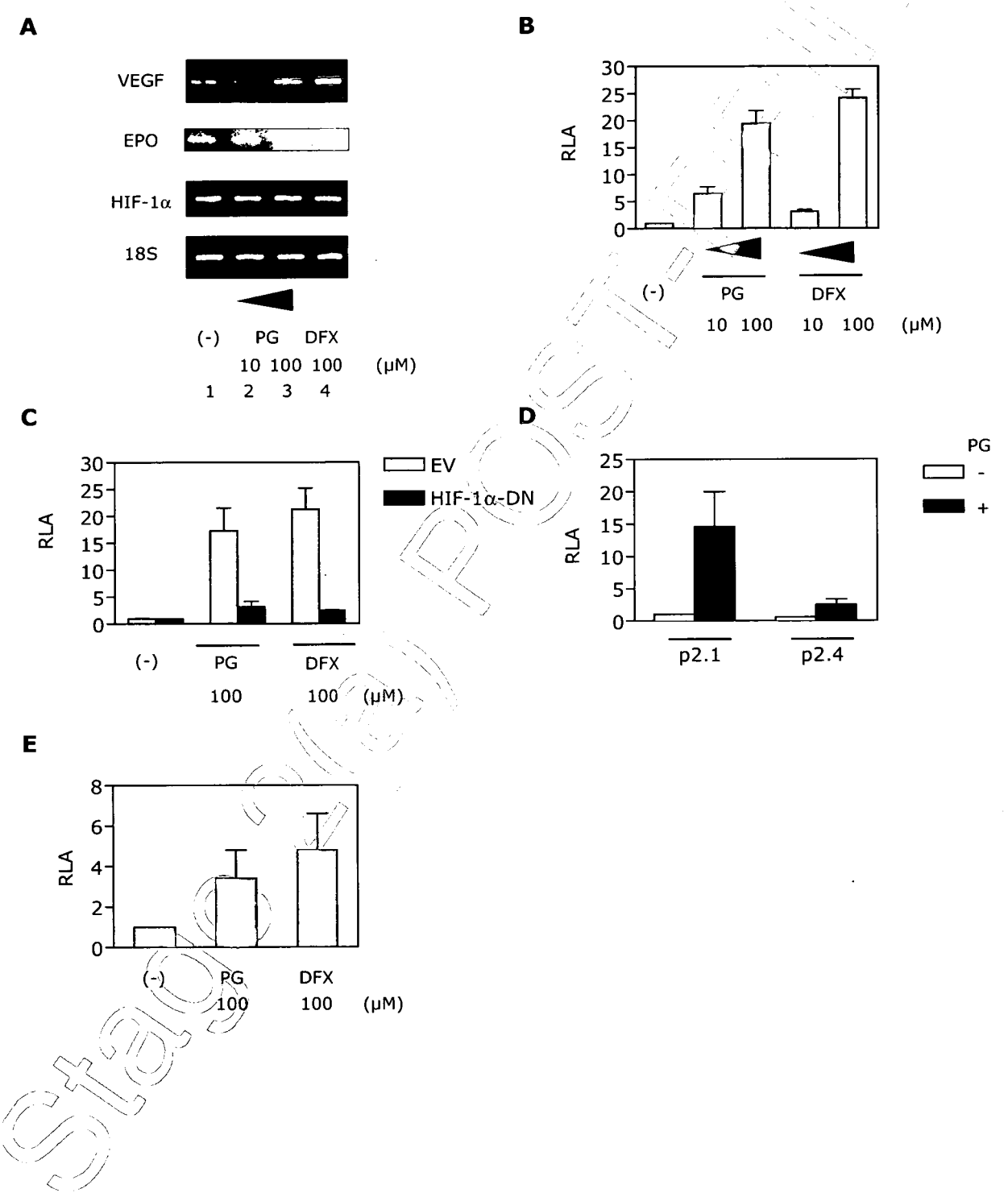

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figure 2

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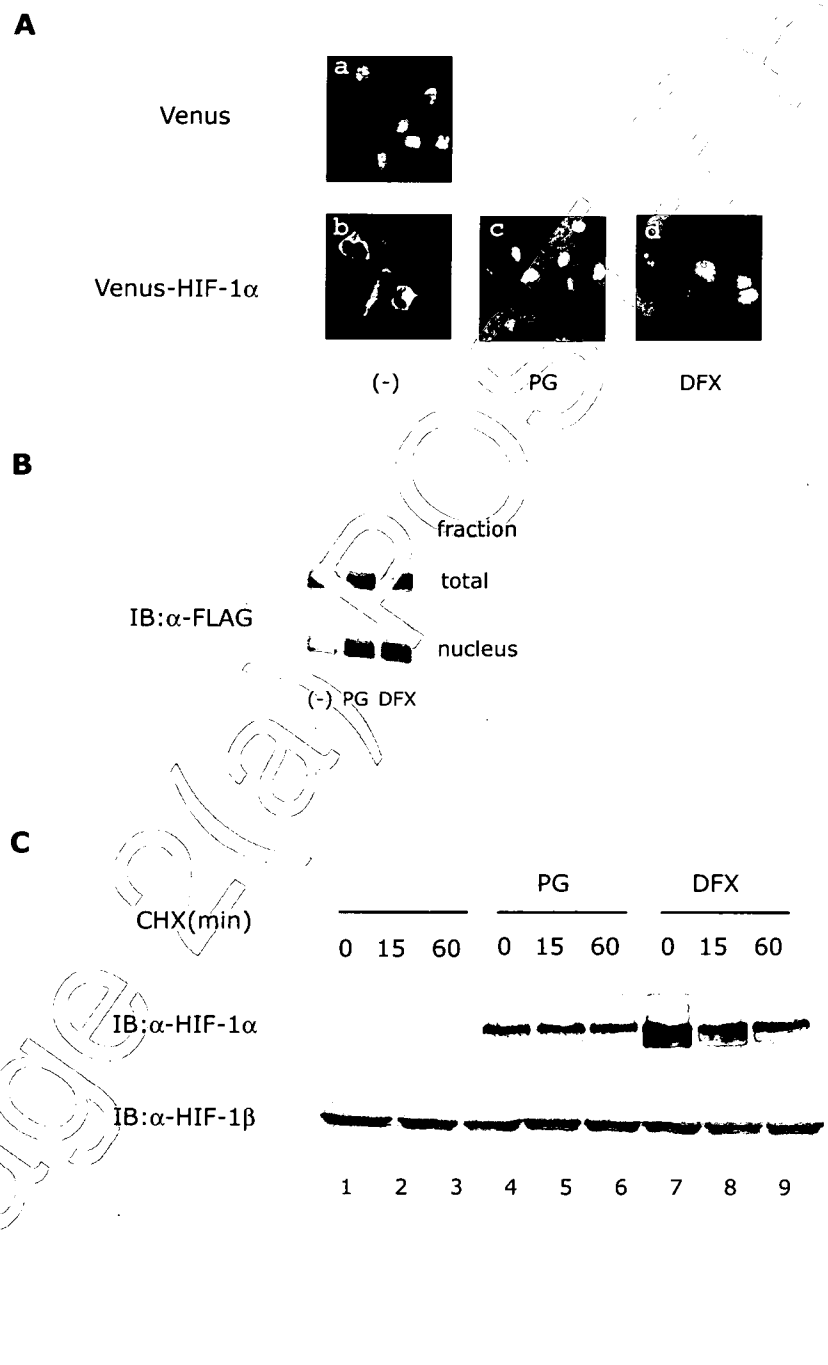

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figure 3

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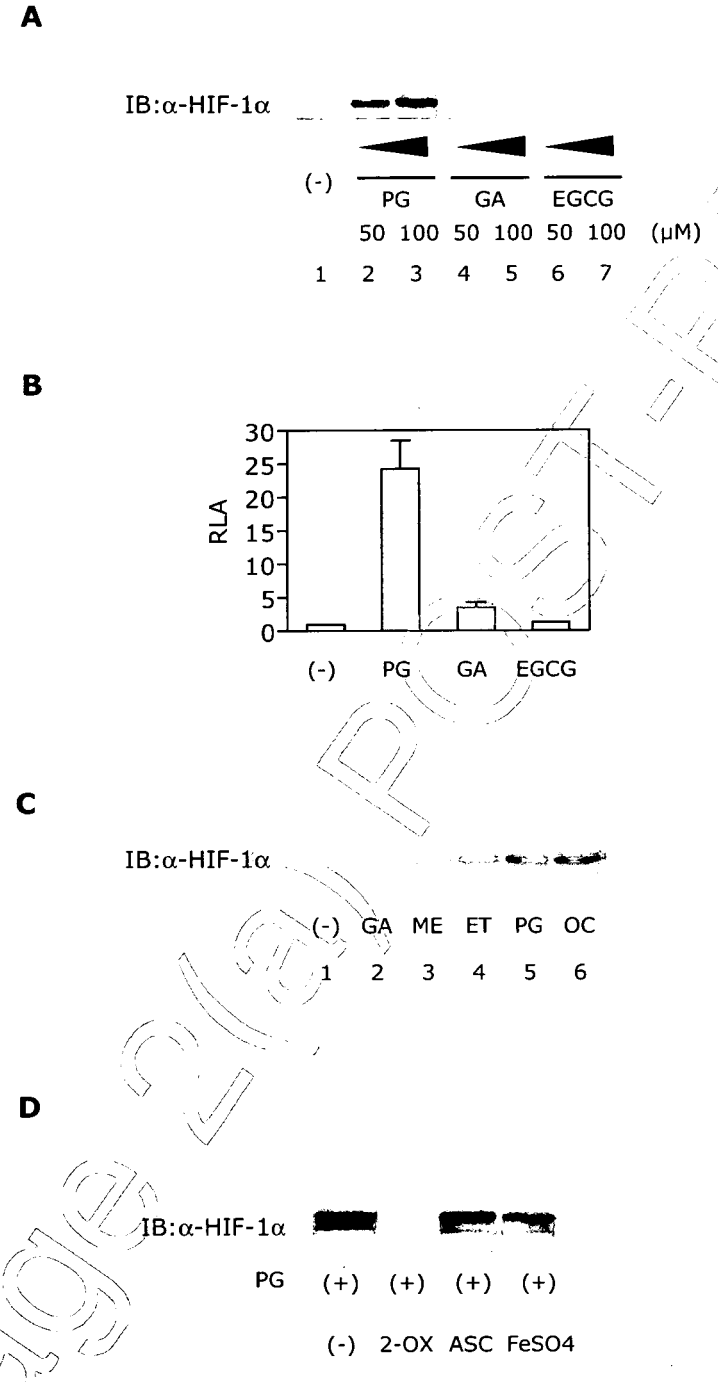

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figure 4

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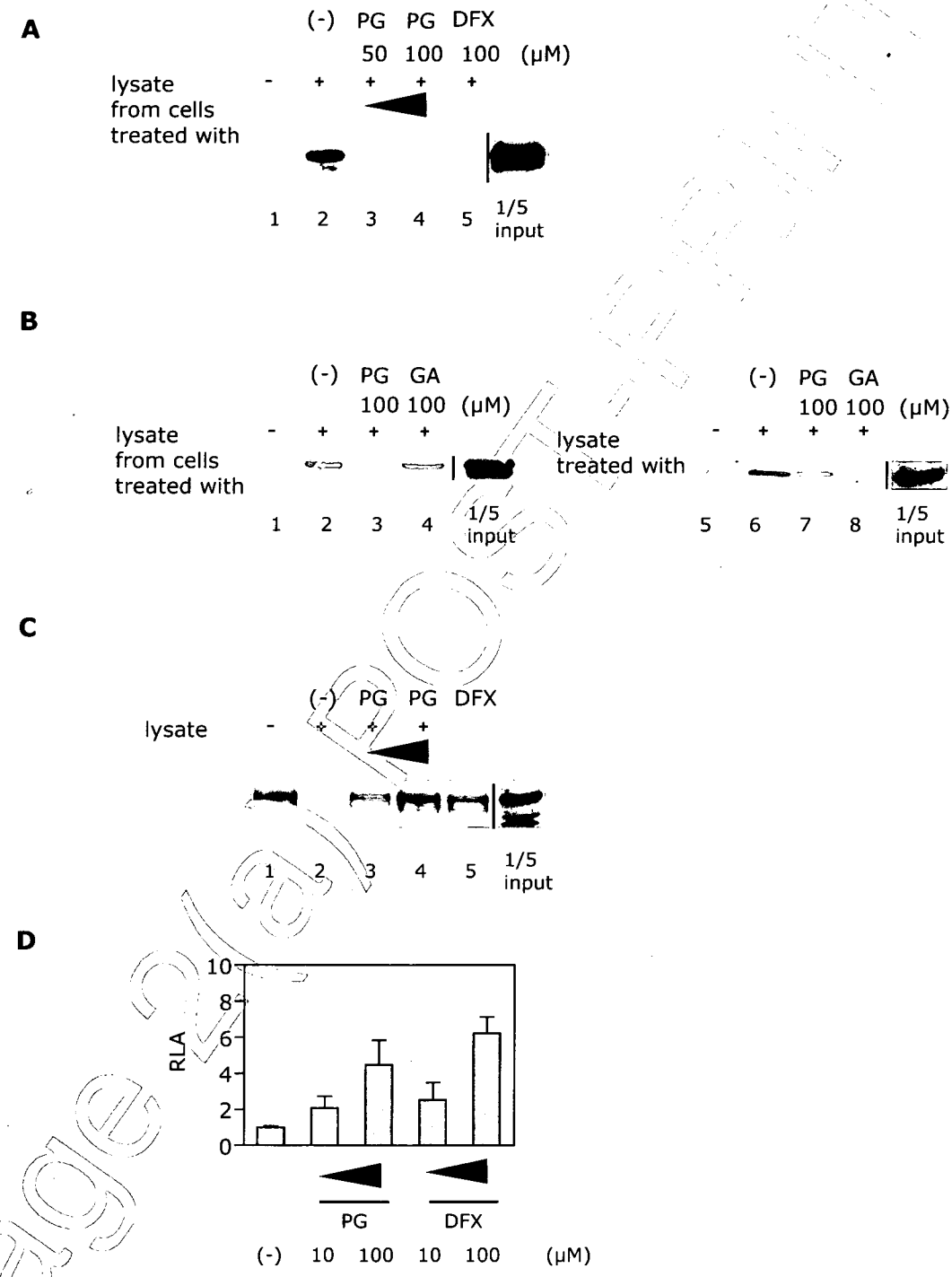

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figure 5

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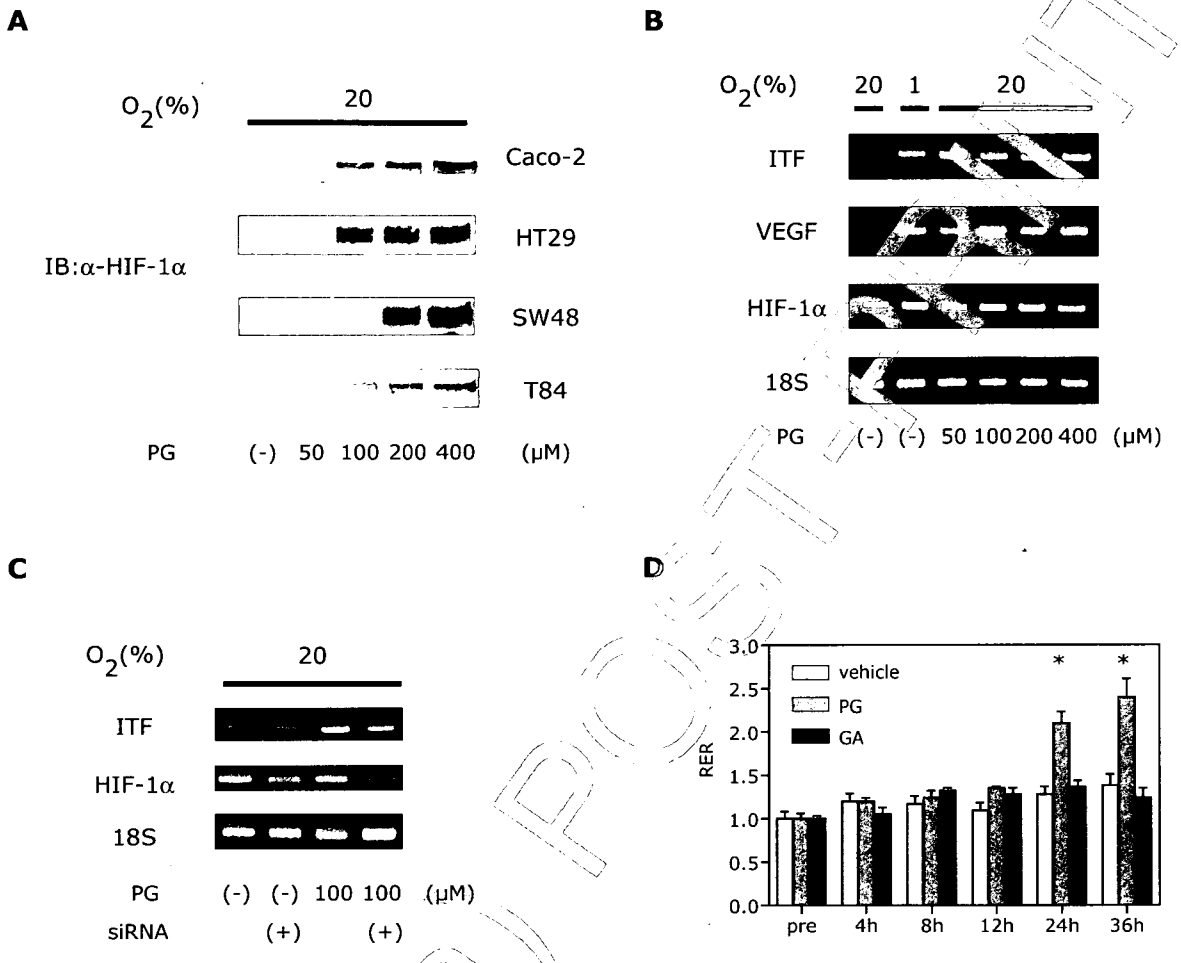

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figure 6

Near-Infrared Fluorescent Labeled Peptosome for Application to Cancer Imaging

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Nonionic amphiphilic copolypeptides, which were composed of hydrophilic poly(sarcosine) and hydrophobic poly(γ-methyl L-glutamate) blocks, were synthesized with varying chain lengths of the blocks. The polypeptides having a suitable hydrophilic and hydrophobic balance were found to form vesicular assemblies of 100 nm size in buffer, which was evidenced by the TEM observation, the DLS analysis, and the encapsulation experiment. The genuine peptide vesicles, peptosomes, were labeled with a near-infrared fluorescence (NIRF) probe. *In vivo* retention in blood experiment showed long circulation of the peptosome in rat blood as stable as the PEGylated liposome. NIRF imaging of a small cancer on mouse by using the peptosome as a nanocarrier was successful due to the EPR effect of the peptosome. Peptosome is shown here as a novel excellent nanocarrier for molecular imaging.

INTRODUCITON

Molecular imaging has attracted more and more attention in the past decades (*1–3*) to target biological molecules (*4, 5*) and tumor tissues (*6, 7*) and so on. To image the biological objects, various modalities using positron-emission tomography (PET), magnetic resonance (MR), and fluorescence have been popular. Fluorescence imaging, particularly near-infrared fluorescence (NIRF) imaging (*8*), has an advantage for living body imaging, because the near-infrared and far-red light (650–900 nm) can avoid strong absorption by red blood cells and water to allow the light to permeate a biological body in the depth of several centimeters (optical window). The NIRF imaging has several benefits: cost efficiency in preparation of the probes and hardware for detection, high sensitivity, and low risk for the living body (*9, 10*). However, NIRF probes should be carried on a vehicle for selective accumulation of the imaging probes into the tumor tissue on a minute scale for early therapy.

There have been several targeting techniques using vehicles as the imaging probes (*11–14*). Using polymers or nanocarriers to support the probes is one of the most popular techniques to provide probes with targeting functionality (*15, 16*). In tumor tissues, submicron-sized defects exist on the vascular wall because of the rapid angiogenesis, enabling permeation of macromolecules through the wall. Further, the lymph system around tumor tissue grows too slowly to exclude foreign compounds from the tumor tissue (the enhanced permeability and retention (EPR) effect) (*17, 18*). Nanocarriers the size of 100 nm are considered to target passively and therefore to accumulate into tumor tissue by the EPR effect (*19, 20*).

Nanocarriers the size of 100 nm, which will exert the EPR effect, can be constructed via self-assembly of the amphiphilic molecules. Recently, spherical molecular assemblies of micelles (*21–23*) or vesicles (*24–26*) have been reported for applications to the drug delivery system (DDS) because of their capability to carry hydrophilic, lipophilic, or conjugated drugs (*27–30*). When molecular assembly is employed as a nanocarrier for *in vivo* use, such as molecular imaging and DDS, it is required to be biocompatible and/or biodegradable. The liposome, which is a vesicular assembly of lipids and therefore is considered to be excellent in terms of biocompatibility and biodegradability, has attracted continuous attention as the nanocarrier in DDS. For *in vivo* use, the liposomes have been PEGylated (modified with poly(ethylene glycol) (PEG) on their surfaces) in order to improve their retention in blood and to suppress the clearance by the reticuloendothelial system (RES) of the liver and spleen (*31, 32*). The PEGylated liposome containing anticancer drug has already been allowed for clinical application in Europe and the United States (*33, 34*). However, several risks of the PEGylated liposome in clinical use have been also reported: the conjugated PEG chains are artificial and nondegradable, and the PEGylated liposome has several side effects on the human body, including complement activation (*35, 36*). An alternative hydrophilic nonionic polymer for the nanocarrier is therefore expected to be developed.

Polypeptides are considered to be excellent in terms of biocompatibility and biodegradability (*37*). In addition, functionalization of polypeptides is rather facile by using their side chains (*38*). Our group has already reported that amphiphilic polypeptides formed vesicular assemblies in the sizes of ca. 100 nm in the aqueous medium, and named them "peptosome" (*39–41*). As in the case of liposome, peptosome can encapsulate water-soluble compounds suitably to DDS. Further, peptosome is a polymer vesicle, which has a more robust membrane via hydrophobic interaction among hydrophobic polymer chains than a liposome of small lipids (*24*).

In the present study, we designed and synthesized AB-type amphiphilic block copolypeptides composed of poly(sarcosine) (PSar) and poly(γ-methyl-L-glutamate) (PMLG) as a hydrophilic and hydrophobic block, respectively (Figure 1). The PSar block

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