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evidence that EPO confers a powerful protective effect to various organs and tissues [44, 45], PG may be useful as a lead compound for the development of novel drugs that protect against ischemic injury. Finally, our findings may have implications for people with diets that are unusually high in PG, such as those consuming `health drugs' that contain this compound. Additional studies are required to further investigate the protective or pathological consequences of PG intake.

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References

1 Semenza, G. L. (2000) HIF-1 and human disease: one highly involved factor. Genes Dev **14**, 1983-1991

2 Giaccia, A., Siim, B. and Johnson, R. (2003) HIF-1 as a target for drug development. Nat Rev Drug Discov. **2**, 803-811

3 Pugh, C. and Ratcliffe, P. (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med. **9**, 677-684

4 Hoeben, A., Landuyt, B., Highley, M. S., Wildiers, H., Van Oosterom, A. T. and De Bruijn, E. A. (2004) Vascular endothelial growth factor and angiogenesis. Pharmacol. Rev. **56**, 549-580

5 Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci U S A **92**, 5510-5514

6 Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G., Jr. (2001) HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science **292**, 464-468

7 Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W. and Ratcliffe, P. J. (2001) Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex

by O_2-regulated prolyl hydroxylation. Science **292**, 468-472

8 Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J. and Ratcliffe, P. J. (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell **107**, 43-54

9 Bruick, R. and McKnight, S. (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. Science **294**, 1337-1340

10 Mahon, P. C., Hirota, K. and Semenza, G. L. (2001) FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev **15**, 2675-2686

11 Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L. and Bruick, R. K. (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev **16**, 1466-1471

12 Laughner, E., Taghavi, P., Chiles, K., Mahon, P. C. and Semenza, G. L. (2001) HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1α (HIF-1α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol. **21**, 3995-4004

13 Fukuda, R., Hirota, K., Fan, F., Jung, Y. D., Ellis, L. M. and Semenza, G. L. (2002) Insulin-like growth Factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase

signaling in colon cancer cells. J Biol Chem **277**, 38205-38211.

14 Page, E. L., Robitaille, G. A., Pouyssegur, J. and Richard, D. E. (2002) Induction of hypoxia-inducible factor-1α by transcriptional and translational mechanisms. J Biol Chem **277**, 48403-48409

15 Kasuno, K., Takabuchi, S., Fukuda, K., Kizaka-Kondoh, S., Yodoi, J., Adachi, T., Semenza, G. L. and Hirota, K. (2004) Nitric oxide induces hypoxia-inducible factor 1 activation that is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling. J Biol Chem **279**, 2550-2558

16 Bergeron, M., Gidday, J. M., Yu, A. Y., Semenza, G. L., Ferriero, D. M. and Sharp, F. R. (2000) Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. Ann Neurol **48**, 285-296

17 Patel, T. H., Kimura, H., Weiss, C. R., Semenza, G. L. and Hofmann, L. V. (2005) Constitutively active HIF-1α improves perfusion and arterial remodeling in an endovascular model of limb ischemia. Cardiovasc Res **68**, 144-154

18 Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L. and Fujii-Kuriyama, Y. (1999) Molecular mechanisms of transcription activation by HLF and HIF-1α in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J. **18**, 1905-1914

19 Liu, Q., Berchner-Pfannschmidt, U., Moller, U., Brecht, M., Wotzlaw, C., Acker, H., Jungermann, K. and Kietzmann, T. (2004) A Fenton reaction at the endoplasmic reticulum is involved in the redox control of hypoxia-inducible gene expression. Proc Natl Acad Sci U S A

101, 4302-4307

20 Semenza, G. L., Jiang, B. H., Leung, S. W., Passantino, R., Concordet, J. P., Maire, P. and Giallongo, A. (1996) Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem **271**, 32529-32537

21 Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D. and Semenza, G. L. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol **16**, 4604-4613

22 Jiang, B. H., Zheng, J. Z., Leung, S. W., Roe, R. and Semenza, G. L. (1997) Transactivation and inhibitory domains of hypoxia-inducible factor 1α. Modulation of transcriptional activity by oxygen tension. J Biol Chem **272**, 19253-19260

23 Nagai, T., Ibata, K., Park, E., Kubota, M., Mikoshiba, K. and Miyawaki, A. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat. Biotechnol. **20**, 87-90

24 Harada, H., Hiraoka, M. and Kizaka-Kondoh, S. (2002) Antitumor effect of TAT-oxygen-dependent degradation-caspase-3 fusion protein specifically stabilized and activated in hypoxic tumor cells. Cancer Res. **62**, 2013-2018

25 Ang, S. O., Chen, H., Hirota, K., Gordeuk, V. R., Jelinek, J., Guan, Y., Liu, E., Sergueeva, A. I., Miasnikova, G. Y., Mole, D., Maxwell, P. H., Stockton, D. W., Semenza, G. L. and Prchal, J. T. (2002) Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. Nat Genet **32**, 614-621

26 Itoh, T., Namba, T., Fukuda, K., Semenza, G. L. and Hirota, K. (2001) Reversible inhibition of hypoxia-inducible factor 1 activation by exposure of hypoxic cells to the volatile anesthetic halothane. FEBS Lett **509**, 225-229

27 Hirota, K. and Semenza, G. L. (2001) Rac1 activity is required for the activation of hypoxia-inducible factor 1. J Biol Chem **276**, 21166-21172

28 Wang, G. L. and Semenza, G. L. (1993) Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood **82**, 3610-3615

29 Reddan, J., Giblin, F., Sevilla, M., Padgaonkar, V., Dziedzic, D., Leverenz, V., Misra, I., Chang, J. and Pena, JT. (2003) Propyl gallate is a superoxide dismutase mimic and protects cultured lens epithelial cells from H_2O_2 insult. Exp Eye Res. **76**, 49-59

30 Kawanishi, S., Oikawa, S. and Murata, M. (2005) Evaluation for safety of antioxidant chemopreventive agents. Antioxid Redox Signal **7**, 1728-1739

31 Cai, Z., Manalo, D. J., Wei, G., Rodriguez, E. R., Fox-Talbot, K., Lu, H., Zweier, J. L. and Semenza, G. L. (2003) Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. Circulation **108**, 79-85

32 Mole, D. R., Schlemminger, I., McNeill, L. A., Hewitson, K. S., Pugh, C. W., Ratcliffe, P. J. and Schofield, C. J. (2003) 2-oxoglutarate analogue inhibitors of HIF prolyl hydroxylase. Bioorg Med Chem Lett **13**, 2677-2680

33 McDonough, M. A., McNeill, L. A., Tilliet, M., Papamicael, C. A., Chen, Q. Y., Banerji, B., Hewitson, K. S. and Schofield, C. J. (2005) Selective inhibition of factor inhibiting

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

hypoxia-inducible factor. J Am Chem Soc **127**, 7680-7681

34 Fukuda, R., Kelly, B. and Semenza, G. L. (2003) Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E_2 is mediated by hypoxia-inducible factor 1. Cancer Res **63**, 2330-2334

35 Zhou, J., Callapina, M., Goodall, G. J. and Brüne, B. (2004) Functional integrity of nuclear Factor κB, phosphatidylinositol 3'-Kinase, and mitogen-activated protein kinase signaling allows tumor necrosis factor α-evoked Bcl-2 expression to provoke internal ribosome entry site-dependent translation of hypoxia-inducible factor 1α. Cancer Res **64**, 9041-9048

36 Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J. and Whitelaw, M. L. (2002) Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science **295**, 858-861

37 Zhou, Y., Kim, Y., Li, X., Baerson, S., Agarwal, A., Hodges, T., Ferreira, D. and Nagle, D. (2004) Hypoxia-inducible factor-1 activation by (-)-epicatechin gallate: potential adverse effects of cancer chemoprevention with high-dose green tea extracts. J Nat Prod. **67**, 2063-2069

38 Thomas, R. and Kim, M. H. (2005) Epigallocatechin gallate inhibits HIF-1α degradation in prostate cancer cells. Biochem Biophys Res Commun **334**, 543-548

39 Tsukiyama, F., Nakai, Y., Yoshida, M., Tokuhara, T., Hirota, K., Sakai, A., Hayashi, H. and Katsumata, T. (2006) Gallate, the component of HIF-inducing catechins, inhibits HIF prolyl hydroxylase. Biochem Biophys Res Commun **351**, 234-239

40 Bruick, R. and McKnight, S. (2001) Building better vasculature. Genes Dev **15**, 2497-2502

BJ Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

41 Kelly, B., Hackett, S. F., Hirota, K., Oshima, Y., Cai, Z., Berg-Dixon, S., Rowan, A., Yan, Z., Campochiaro, P. A. and Semenza, G. L. (2003) Cell-type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in non-ischemic tissue by a constitutively-active form of hypoxia-inducible factor 1. Circ. Res. **93**, 1074-1081

42 Bernaudin, M., Nedelec, A., Divoux, D., MacKenzie, E., Petit, E. and Schumann-Bard, P. (2002) Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. J Cereb Blood Flow Metab **22**, 393-403

43 Taupin, D. and Podolsky, D. K. (2003) Trefoil factors: initiators of mucosal healing. Nat Rev Mol Cell Biol **4**, 721-732

44 Baker, J. E. (2005) Erythropoietin mimics ischemic preconditioning. Vascul Pharmacol **42**, 233-241

45 Maiese, K., Li, F. and Chong, Z. Z. (2005) New avenues of exploration for erythropoietin. JAMA **293**, 90-95

Biochemical Journal Immediate Publication. Published on 30 Nov 2007 as manuscript BJ20070824

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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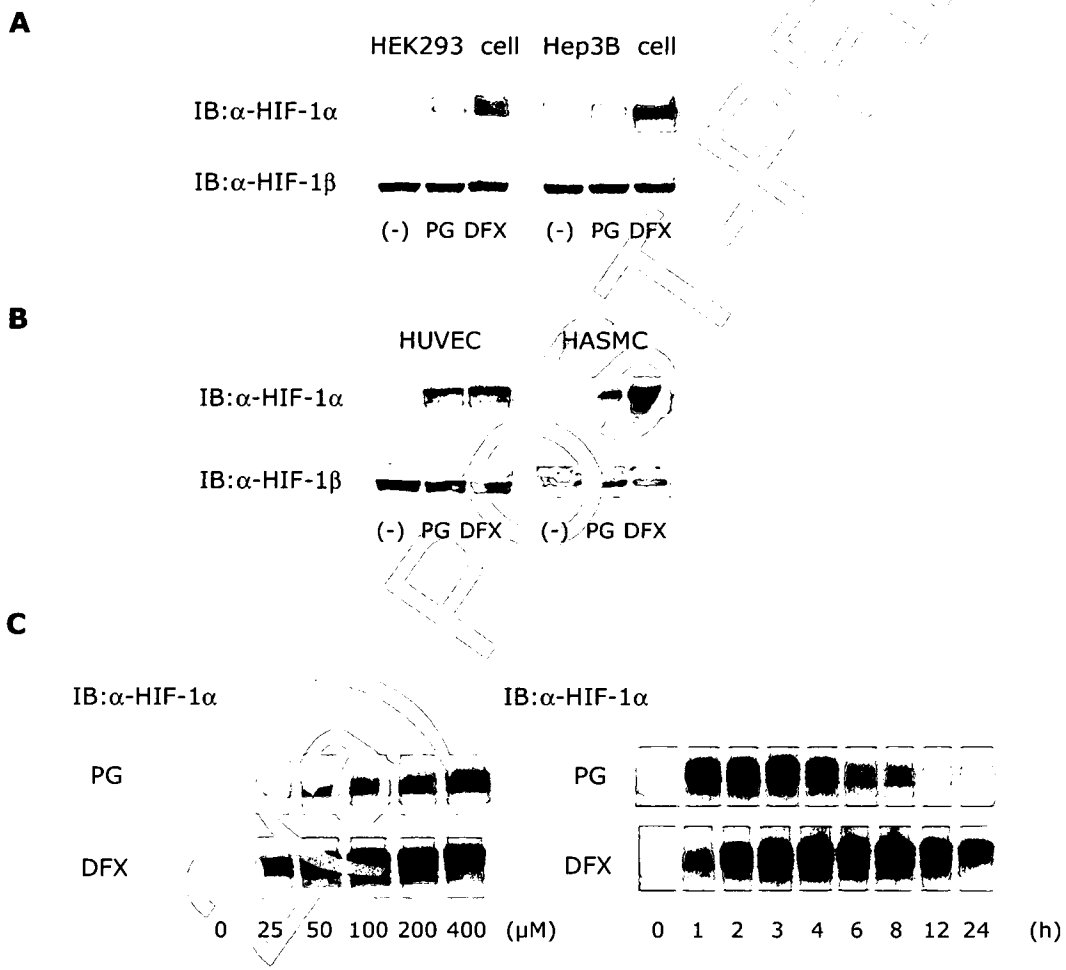

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figure 1

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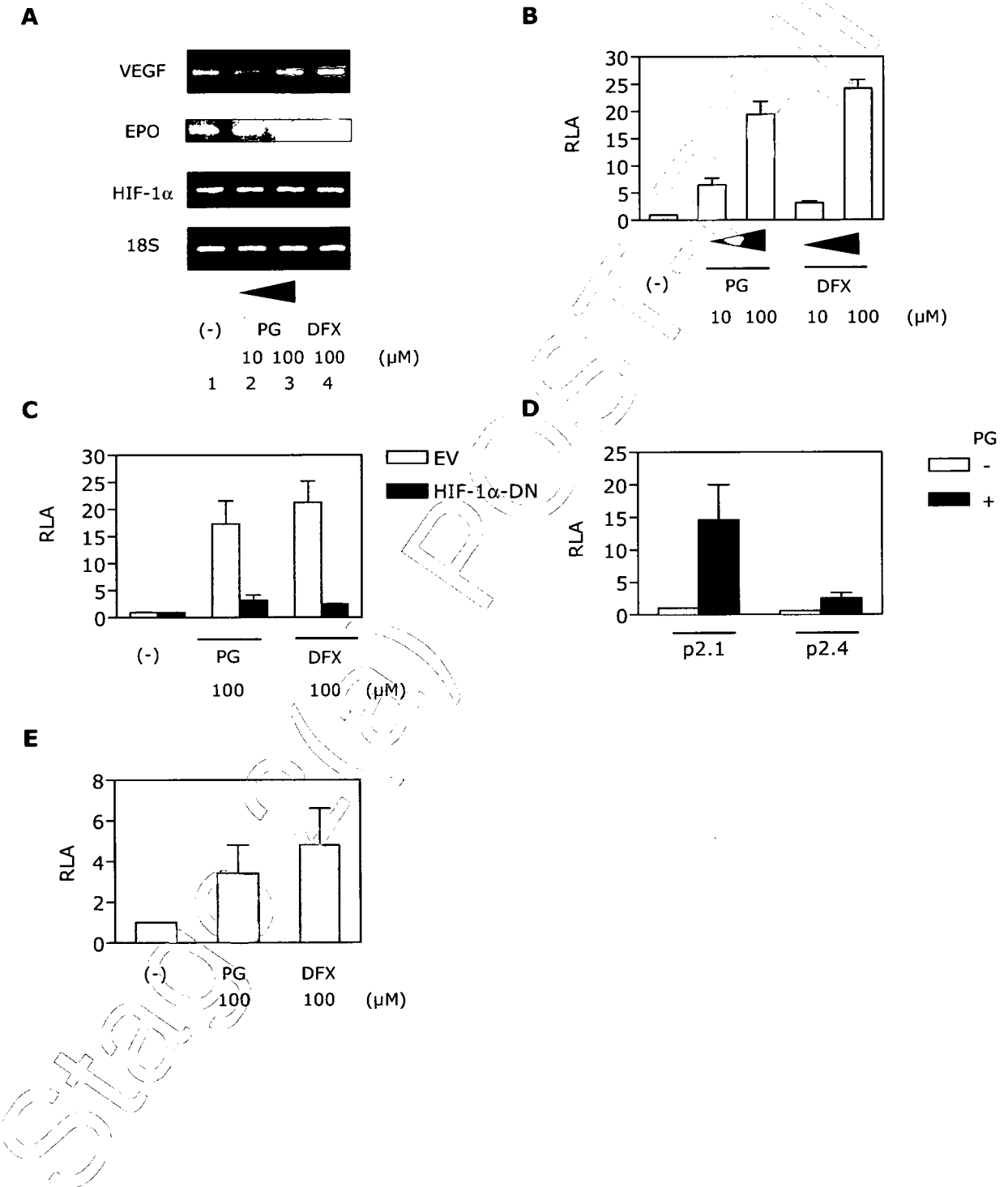

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figure 2

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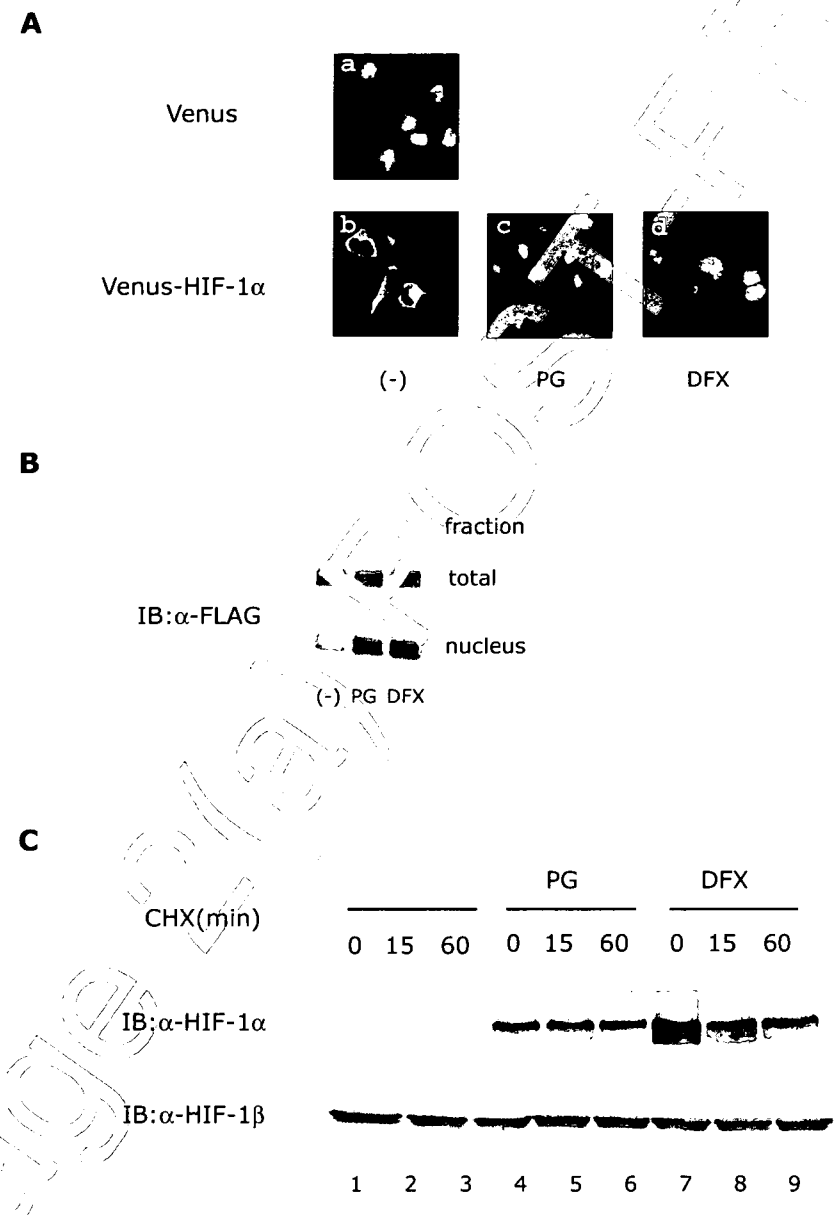

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figure 3

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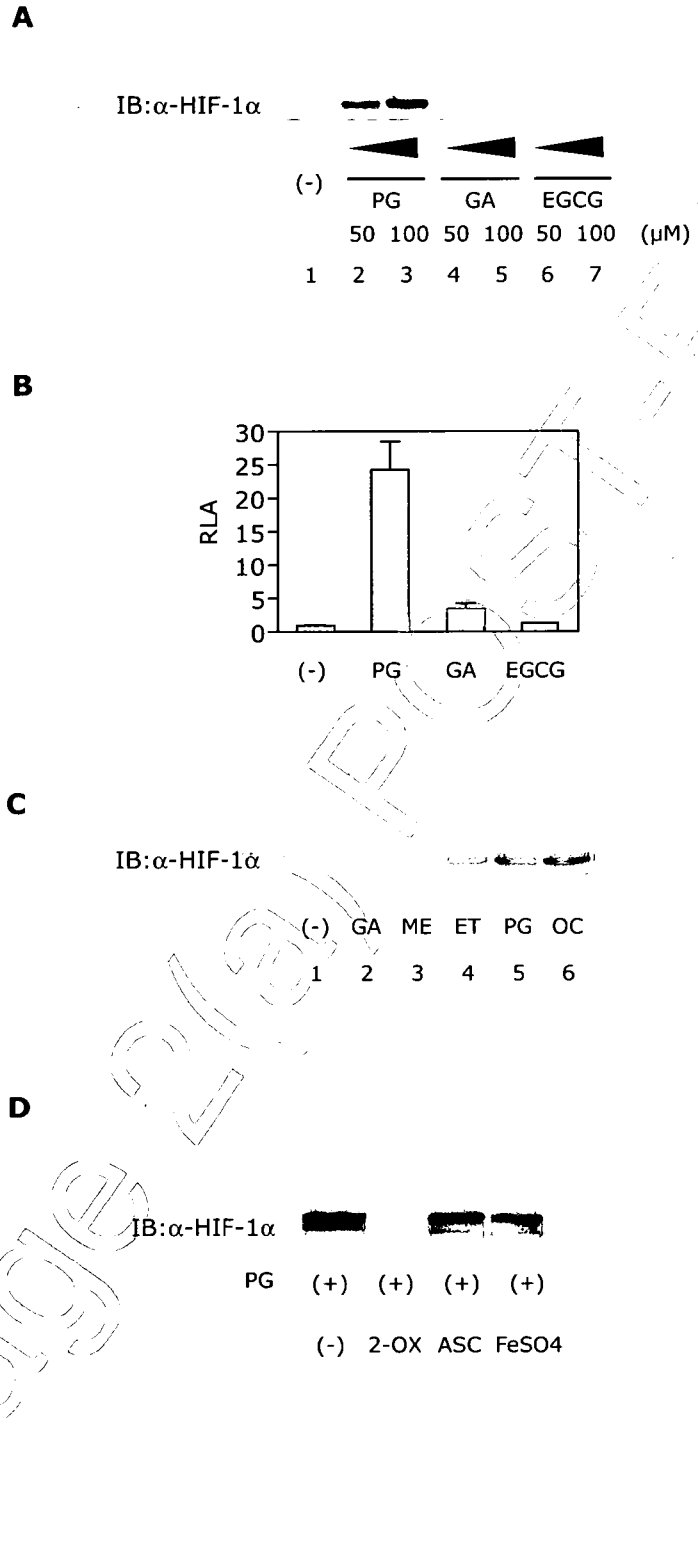

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figure 4

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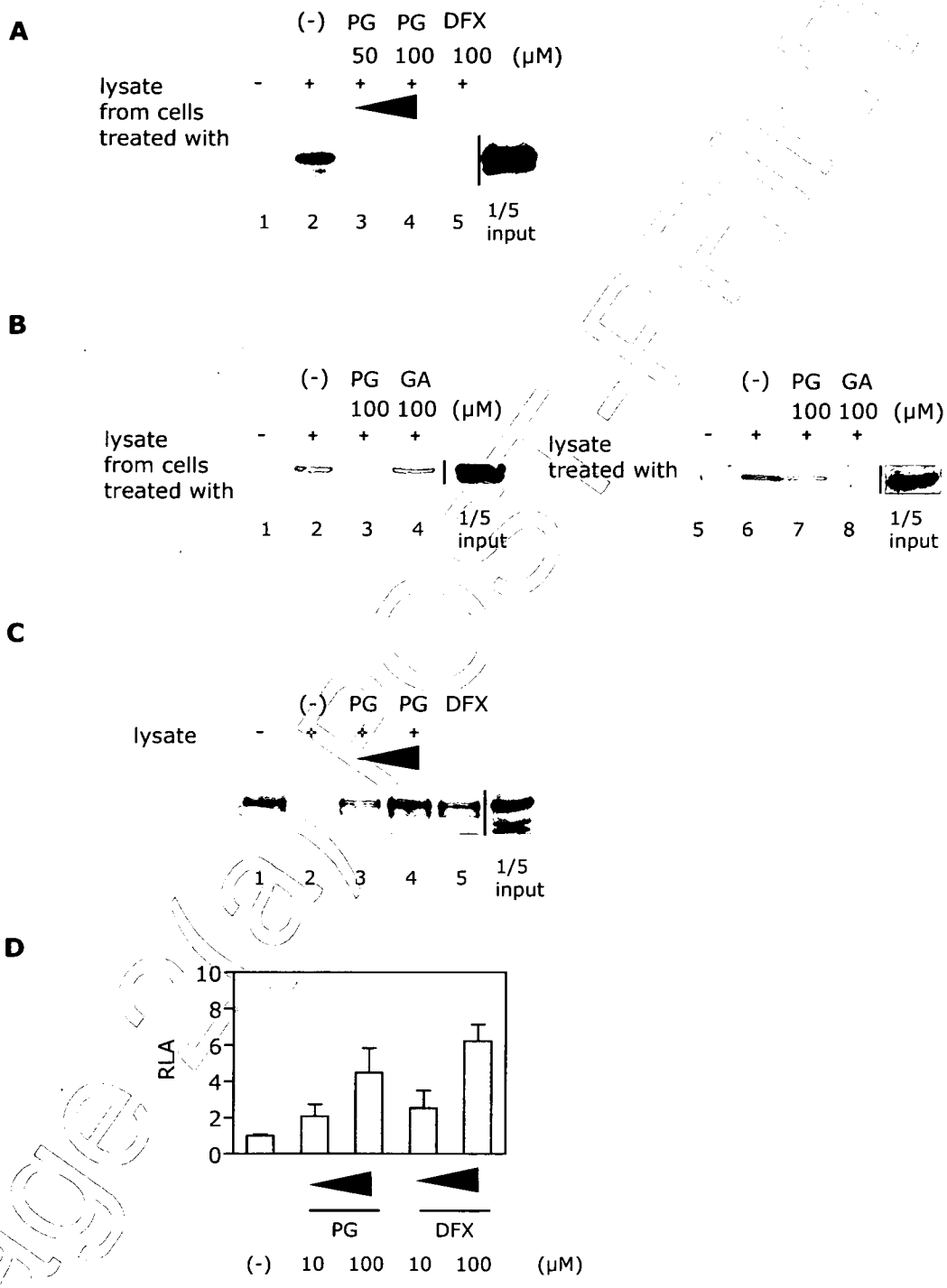

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figure 5