Molecular Pharmacology Fast Forward. Published on March 24, 2010 as doi:10.1124/mol.110.063933

MOL 63933

EP1 Prostanoid Receptor Coupling to $G_{i/o}$ Upregulates the Expression of Hypoxia-Inducible Factor-1α Through Activation of a Phosphoinositide-3 Kinase Signaling Pathway

Ruyue Ji, Chih-Ling Chou, Wei Xu, Xiao-Bo Chen, David F. Woodward and John W. Regan

Department of Pharmacology & Toxicology, College of Pharmacy, The University of Arizona, Tucson, AZ, U.S.A., 85721-0207 (R.J., C.L.C., W.X., X.B.C., J.W.R.)

and

Department of Biological Sciences, Allergan, Inc. Irvine, CA, 92713-9534 (D.F.W.)

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

MOL 63933

Running title: EP1 Receptor Coupling to $G_{i/o}$ Upregulates HIF-1α

Corresponding author: John W. Regan, Ph.D.; Department of Pharmacology & Toxicology, College of Pharmacy/The University of Arizona, 1703 E. Mabel St., Tucson, AZ 85721-0207; telephone, 520-626-2181; fax, 520-626-2466; email, regan@pharmacy.arizona.edu

number of text pages: 37

number of tables: 0

number of figures: 10

number of references: 40

number of words in *Abstract*: 248 (250 max)

number of words in *Introduction*: 710 (750 max)

number of words in *Discussion*: 1465 (1,500 max)

Abbreviations: GPCR, G-protein coupled receptor; PGE_2, prostaglandin-E2; HIF-1α, hypoxia-inducible factor-1α; $G_{i/o}$, the pertussis toxin-sensitive inhibitory guanine nucleotide binding protein; PI3K, phosphoinositide-3 kinase; mTOR, mammalian target of rapamycin; COX, cyclooxygenase; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; AKT, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome 10; IP, inositol phosphates; S6K1, ribosomal S6 kinase-1; APC, adenomatous polyposis coli; VEGF-C, lymphatic vascular endothelial growth factor-C.

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The EP1 prostanoid receptor is one of four subtypes whose cognate physiological ligand is prostaglandin-E2 (PGE_2). It is in the family of G-protein coupled receptors and is known to activate Ca^{2+} signaling, although relatively little is known about other aspects of EP1 receptor signaling. In HEK cells expressing human EP1 receptors, we now show that PGE_2 stimulation of the EP1 receptor upregulates the expression of hypoxia-inducible factor-1α (HIF-1α), which can be completely blocked by pertussis toxin, indicating coupling to $G_{i/o}$. This upregulation of HIF-1α occurs under normoxic conditions and could be inhibited with wortmannin, Akt inhibitor and rapamycin, consistent with the activation of a phosphoinositide-3 kinase/Akt/mTOR signaling pathway, respectively. In contrast to the hypoxia induced upregulation of HIF-1α, which involves decreased protein degradation, the upregulation of HIF-1α by the EP1 receptor was associated with the phosphorylation of ribosomal protein S6 (rpS6), suggesting activation of the ribosomal S6 kinases and increased translation. Stimulation of endogenous EP1 receptors in human hepatocellular carcinoma cells (HepG2) recapitulated the normoxic upregulation of HIF-1α observed in HEK cells, was pertussis toxin sensitive, and involved the activation of mTOR signaling and phosphorylation of rpS6. Additionally, treatment of HepG2 cells with sulprostone, an EP1 selective agonist, upregulated the mRNA expression of vascular endothelial growth factor-C, a HIF regulated gene. HIF-1α is known to promote tumour growth and metastasis and is often upregulated in cancer. Our findings provide a potential mechanism by which increased PGE_2 biosynthesis could upregulate the expression of HIF-1α and promote tumorigenesis.

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

E-type prostanoid receptors (EP) are the receptors that mediate the actions of prostaglandin-E_2 (PGE_2) and are members of the superfamily of G-protein coupled receptors (GPCR). There are four primary subtypes of EP receptors named EP1, EP2, EP3 and EP4. The EP1, EP2 and EP3 receptors were initially classified on the basis of their pharmacology and upon differences in their functional effects on various types of smooth muscle, as well as their activation of second messenger signaling pathways (Coleman, Smith & Narumiya, 1994). Thus, PGE_2 stimulation of EP1 receptors produced contractile responses that could be selectively blocked with SC-19220 and were involved in the mobilization of intracellular Ca^{2+}. PGE_2 stimulation of EP2 receptors produced smooth muscle relaxation that was associated with activation of adenylyl cyclase; whereas, stimulation of EP3 receptors produced smooth muscle contraction that was associated with inhibition of adenylyl cyclase. Pharmacologically, EP2 receptors could be selectively activated with butaprost, while EP3 receptors (and EP1) could be selectively activated with sulprostone. EP4 receptors were first recognized as EP2-like receptors that were insensitive to butaprost; thus, stimulation with PGE_2 caused smooth muscle relaxation that could not be mimicked with butaprost or sulprostone (Lawence & Jones, 1992). However, a full appreciation of their relationship to the EP2 receptors required the molecular cloning of their genes, which established that EP4 receptors were coupled to both the activation of adenylyl cyclase and the stimulation of phosphatidylinositol 3-kinase (PI3K) signaling (Regan, 2003).

Although it is well accepted that the PGE_2 stimulation of the EP1 receptor is linked to smooth muscle contraction and increased intracellular Ca^{2+}, it is unclear if this involves exclusive coupling to $G_{q/11}$ and typical activation of phospholipase-C (PLC) and phosphatidylinositol (PI) hydrolysis. For example, in CHO cells stably transfected with the mouse EP1 receptor, sulprostone induced intracellular Ca^{2+} mobilization by two pathways

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

(Katoh et al., 1995). One pathway involved transient Ca^{2+} release from internal stores that could be blocked with the PLC inhibitor, U73122; and a second involved extracellular Ca^{2+} influx that was U73122 insensitive. Co-expression of the mouse EP1 receptor with a receptor-activated Ca^{2+} channel (TPR5) in *Xenopus laevis* oocytes produced similar findings (Tabata et al., 2002). Thus, PGE_2 stimulated an initial endogenous transient inward current that followed by a large inward Ca^{2+}-dependent current, but only the former could be inhibited with antisense $G\alpha_{q/11}$ mRNA.

Further work on the signaling properties of the EP1 prostanoid receptor is limited. There is a report showing that endogenous EP1 receptors in human trophoblasts activate intracellular Ca^{2+} signaling by a mechanism involving the activation of PLC (Nicola et al., 2005). Thus, the EP1 receptor mediated release of intracellular Ca^{2+} was found to be primarily dependent on internal Ca^{2+} stores and not on extracellular Ca^{2+}. This is in contrast to earlier findings with recombinant EP1 receptors (Watabe et al., 1993) and with endogenous EP1 receptors in guinea pig trachea (Creese & Denborough, 1981) whose Ca^{2+} responses were almost entirely dependent upon extracellular Ca^{2+}. Recent evidence has suggested that stimulation of endogenous EP1 receptors can inhibit an Akt kinase signaling pathway that potentially contributes to apoptotic neuronal cell death following oxygen/glucose deprivation (Zhou et al., 2008). The molecular mechanism of this signaling is unclear, although it appears to involve an EP1 receptor mediated decrease in the phosphorylation of the lipid phosphatase, PTEN.

To further examine the signaling properties of the human EP1 prostanoid receptor we conducted an exploratory gene array screen to identify possible target genes regulated by this receptor (X.B. Chen & Regan, unpublished). Two genes related to angiogenesis were found to be upregulated following PGE_2 treatment. Hypoxia inducible factor-1α (HIF-1α) is a master transcription factor that controls the expression of many genes involved in the hypoxic response,

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

including several that regulate angiogenesis (Semenza, 2001). The cellular expression of HIF-1α is typically controlled by a posttranslational mechanism and its mRNA expression was unchanged in our microarray screen. We hypothesized, however, that posttranscriptional upregulation of HIF-1α by the EP1 receptor might be responsible for the upregulation of the angiogenic genes observed in our microarray screen. As described in the following report, we confirmed this hypothesis and found that the upregulation of HIF-1α by the human EP1 receptor involved unexpected coupling to a pertussis toxin sensitive G-protein and activation of a PI3K/Akt/mTOR signaling pathway.

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Materials. Trizol® Reagent, Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, hygromycin B, geneticin, gentamicin, pcDNA3, pCEP4 and HEK293-EBNA cells were from Invitrogen (Carlsbad, CA). iScript cDNA kit was from Biorad (Hercules, CA). Antibodies against HIF-1α were from BD Sciences (San Jose, CA). Anti-mouse IgG conjugated with horseradish peroxidase, anti-ubiquitin and anti-vinculin antibodies were from Sigma-Aldrich (St. Louis, MO). PVDF membranes were from Bio-Rad Laboratories (Hercules, CA). Cell lysis buffer and antibodies against phospho-S-6 ribosomal protein (Ser235/Ser236; product #2211) were from Cell Signaling Technology (Waltham, MA). PGE_2 and sulprostone were from Cayman Chemical Company (Ann Arbor, MI). [^{3}H]PGE_2 and myo-[2-^{3}H(N)]-inositol were from PerkinElmer (Waltham, MA). Pertussis toxin, wortmannin, rapamycin, Akt inhibitor, bisindolylmaleimide-I and BAPTA-AM were from Calbiochem (San Diego, CA). FuGENE 6 was from Roche (San Francisco, CA). The Dual Luciferase Reporter Assay System and the Renilla luciferase reporter, pRL-CMV, were from Promega (Madison, WI). TaqMan Gene Expression Assays were from Applied Biosystems (Foster City, CA) and corresponded to the following gene symbols and assay I.D. numbers: GAPDH (Hs99999905_m), HIF-1α (Hs00153153_m1), EGR-1 (Hs00152928_m1), PKG1 (Hs99999906_m1), CTGF (Hs00170014_m1), GLUT1 (Hs00892681_m1), VEGF-A (Hs00900054_m1), VEGF-C (Hs01099203_m1) and EPO (Hs01071097_m1).

Cell Culture. A cell line stably expressing the recombinant human EP1 prostanoid receptor was generated using human embryonic kidney (HEK) cells expressing the Epstein Barr nuclear antigen (EBNA). Briefly, the polymerase chain reaction (PCR) was used to amplify a product from human kidney cDNA containing the coding sequence (nucleotides 1-1209) of the human

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

EP1 receptor (Funk et al., 1993), which was then cloned into the EcoRV site of the expression vector, pcDNA3, to generate hEP1/pcDNA3. hEP1/pcDNA3 was digested with HindIII and XhoI and the small fragment containing the EP1 coding sequence was cloned into the corresponding sites of the vector, pCEP4, to yield hEP1/pCEP4. HEK-EBNA cells were transfected with hEP1/pCEP4 using calcium phosphate precipitation and were selected by resistance to hygromycin B. Clones were obtained by limiting dilution. Clonal cell lines stably expressing the EP1 receptor (HEK-hEP1) were identified by PGE_2 stimulation of IP formation and by the radioligand binding of [^{3}H]PGE_2. A control cell line expressing the "empty" pCEP4 vector (HEK-pCEP4) was prepared in a similar manner. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 250 μg/ml geneticin, 100 μg/ml gentamicin, and 200 μg/ml hygromycin B. Human hepatocellular carcinoma cells (HepG2) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in modified Eagle's medium containing 10% FBS. Cells were cultured in a humidified incubator at 37°C in 5% CO_2/95% air.

Inositol Phosphate Assay. Cells were split into 6-well plates and grown to ~90% confluence. Starting ~18 h prior to the assay, the cells were incubated with Opti-MEM containing 3.25 μCi/mL myo-[2-^{3}H(N)]-inositol. The cells were pre-treated with 10 mM LiCl, followed by 1 h of incubation with varying concentrations of PGE_2. The treated cells were harvested and inositol phosphate levels were determined by anion exchange chromatography as previously described (Fujino et al., 2000).

Western Blotting. Cells were split into 6-well plates at a density of 10^6 cells/well and incubated overnight. They were then treated with 1 μM PGE_2 for indicated times and immumoblotting was performed essentially as previously described (Fujino, Xu & Regan, 2003). In short, cell lysates

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

were prepared and measured for protein content using the Bradford assay. Approximately 100 μg of protein was electrophoresed on 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were incubated overnight at 4°C with primary antibodies and then for 1 h at room temperature with the secondary antibodies. Anti-phospho-S6K and anti-HIF-1α antibodies were used at a dilution of 1:1000 and 1:200, respectively, in 3% non-fat milk. Horseradish peroxidase conjugated secondary antibodies were used at a dilution of 1:10000 in 3% non-fat milk. After incubation with secondary antibodies, the membranes were washed and immunoreactivity was detected by enhanced chemiluminescence. To ensure equal loading of proteins, the membranes were stripped and re-probed with anti-vinculin antibodies. For immunoprecipitation experiments, lysates were incubated with anti-HIF-1α antibodies and protein-A beads overnight at 4°C. The beads were washed, resuspended in SDS-PAGE sample buffer, then electrophoresed and immunoblotted as above with either anti-ubiquitin or anti-HIF-1α antibodies.

HRE Reporter Gene Assay. Cells were split into 6-well plates, grown to ~75% confluence, and ~24 h later, the cells were co-transfected with 2 μg/well of a firefly luciferase reporter plasmid under the control of a HIF response element (pGL3/HRE-Luc27) and 10 ng/well of the Renilla luciferase reporter pRL-CMV using 5 μL FuGENE 6. Cultures were then treated with either vehicle (0.1% dimethylsulfoxide in phosphate-buffered saline solution) or 1 μM PGE_2 for 18 h. Cells were harvested and 2 μL of the lysates were taken to measure luciferase activity using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The data were normalized by calculating ratios of firefly luciferase scores to the corresponding Renilla luciferase values.

Quantitative Real-Time PCR. Total RNAs were isolated using either Trizol® Reagent or Absolutely RNA Miniprep kits according to the manufacturer's instructions. The RNA integrity

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

was verified by 1% agarose gel electrophoresis and the quantity was determined by spectrophotometry. cDNAs were prepared from ~500 ng total RNA using the iScript cDNA synthesis kit. mRNA expression was determined using the TaqMan Gene Expression Assay primers listed in **Materials** above. PCR reactions were subjected to 40 cycles of 95°C for 15 s and 60°C for 45 s in an ABI 7500 real time PCR system. Threshold values (Ct) were determined automatically by the system software and relative mRNA expression was analyzed by the comparative ΔΔCt method. Data were normalized to the mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

Results

[^{3}H]PGE$_2$ binding and stimulation of inositol phosphates (IP) formation by the recombinant human EP1 receptor expressed in HEK cells. Although it is generally assumed that EP1 receptors are coupled to $G_{q/11}$, previous studies have failed to show a robust stimulation of intracellular IP formation. For example, PGE_2 stimulation of the mouse EP1 receptor expressed in CHO cells only increased IP formation ~1.2-fold (Watabe et al., 1993); whereas agonist stimulation of the ovine FP receptor, another $G_{q/11}$ coupled prostanoid receptor, increased IP formation nearly 10-fold (Fujino et al., 2000). Some authors have even indicated that EP1 receptors do not stimulate IP formation and that the coupling of these receptors to intracellular Ca^{2+} mobilization may involve an unknown G-protein (Hata & Breyer, 2004; Sugimoto & Narumiya, 2007). To further characterize the signaling properties of these receptors, we prepared HEK cells stably expressing human EP1 prostanoid receptors and initially examined the radioligand binding of [^{3}H]PGE_2 and PGE_2 stimulated IP formation in these cells. Figure 1A shows the results of PGE_2 competition curves for the whole cell specific binding of [^{3}H]PGE_2 to HEK cells stably expressing either the empty vector control plasmid (HEK-pCEP4) or plasmid encoding the human EP1 receptor (HEK-hEP1). In cells stably transfected with the empty vector there was no significant displacement of [^{3}H]PGE_2 by PGE_2, whereas in the EP1 expressing cells PGE_2 competed for the binding of [^{3}H]PGE_2 in a simple monophasic manner with an IC_{50} of 3.6 ± 0.2 nM.

Figure 1B shows the results for the stimulation of total intracellular IP formation by PGE_2 in control HEK-pCEP4 cells and in HEK cells expressing the human EP1 receptor (HEK-hEP1). In contrast to the control cells in which no response was observed, PGE_2 treatment of HEK-hEP1

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

cells produced a robust dose-dependent stimulation of IP formation with an EC_{50} of 4.8 ± 0.2 nM; consistent with the coupling of these receptors to $G_{q/11}$.

Normoxic upregulation of HIF-1α expression by PGE_2 in HEK cells expressing the human EP1 receptor. A preliminary DNA microarray study was conducted to profile the expression of genes that were potentially regulated by PGE_2 interaction with the human EP1 prostanoid receptor (X.B. Chen & Regan, unpublished). Briefly, HEK-hEP1 cells were treated with 1 μM PGE_2 for various periods of time and changes in gene expression relative to vehicle treated cells were examined using Affymetrix human genome arrays. Among the genes that were found to be most strongly upregulated were two related to the regulation of angiogenesis. Although the gene expression (mRNA) of HIF-1α was unchanged by treatment with PGE_2, we hypothesized that changes in the protein expression of HIF-1α might be responsible for global changes in angiogenic gene expression. We, therefore, used Western blot analysis to examine potential PGE_2 stimulated upregulation of HIF-1α expression in HEK-hEP1 cells maintained under the routine normoxic conditions of cell culture (95% air/5% CO_2). Figure 2A shows an immunoblot for the expression of HIF-1α and vinculin in HEK-hEP1 cells following treatment with 1 μM PGE_2 for various periods of time between 0 (untreated) and 12 h. A low level of HIF-1α expression was present at 0 h, which was unchanged after 1 h treatment with PGE_2, but after 3, 6 and 12 h of treatment, a marked upregulation of HIF-1α was observed. On the other hand, the expression of vinculin remained essentially constant at all time points.

A luciferase reporter gene construct under the control of a HIF response element (HRE) was used to examine if the upregulation of HIF-1α following PGE_2 stimulation of the EP1 receptor could potentially stimulate transcriptional activity of HIF-1 target genes. Figure 2B shows that in HEK cells expressing the human EP1 receptor PGE_2 stimulated HRE mediated

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

luciferase activity nearly 3-fold, but had no significant effect in control cells expressing the empty pCEP4 vector. These results indicate that the upregulation of HIF-1α by agonist stimulation the EP1 receptor results in functional interactions with HIF-1β and activation of the HRE.

Upregulation of HIF-1α expression mediated by the EP1 receptor does not involve an increase in gene transcription. Quantitative real time PCR was used to examine the expression of HIF-1α mRNA in HEK-hEP1 cells treated with 1 μM PGE_2 and the results are shown in Figure 2C. In contrast to the upregulation of HIF-1α protein expression observed in Figure 2A, there were no changes in the expression of HIF-1α mRNA at 1 and 3 h following treatment with PGE_2. These results indicate that PGE_2 stimulated upregulation of HIF-1α expression mediated by the EP1 receptor does not involve changes in gene transcription, which is consistent with known mechanisms for the upregulation of HIF-1α in response to hypoxia.

Upregulation of HIF-1α expression mediated by the EP1 receptor appears to be translational and does not involve a decrease in 26S proteasome activity or decreased ubiquitination of HIF-1α. It is well established that under normoxic conditions HIF-1α is constitutively expressed and subsequently degraded by the activity of the 26S proteasome so that accumulation does not occur and the levels of HIF-1α protein expression remain very low (Semenza, 2001). Recognition of HIF-1α by the 26S proteasome requires the ubiquitination of HIF-1α by the von Hippel-Lindau protein (pVHL)/ubiquitin E3 ligase complex, which in turn depends upon the hydroxylation of regulatory proline residues in HIF-1α by oxygen sensitive prolyl hydroxylases (Schofield & Ratcliffe, 2004). Thus, the upregulation of HIF-1α expression mediated by the EP1 receptor under normoxic conditions could potentially involve decreased protein degradation,

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

either by a gobal decrease in 26S proteosome activity, or by a specific decrease in the hydroxylation and/or ubiquitination of HIF-1α.

We, therefore, used immunoprecipitation and immunoblot analysis with antibodies against HIF-1α and ubiquitin to examine the ratio of ubiquitinated HIF-1α to total HIF-1α following treatment of HEK-hEP1 cells with either vehicle or 1 μM PGE_2 for 6 h. For these experiments the cells were pretreated for 4 h with 25 μM MG132 to inhibit the constitutive degradation of HIF-1α by the 26S proteosome. Figure 3A shows a representative immunoblot and a bar graph of the pooled data from three experiments following immunoprecipitation with antibodies against HIF-1α and then immunoblotting with antibodies against either ubiquitin or HIF-1α. As shown in the bar graph, treatment with PGE_2 resulted in similar, ~1.5-fold increases in the expression of ubiquitinated HIF-1α and total HIF-1α; thus, the ratio of ubiquitinated HIF-1α to total HIF-1α did not change following treatment with PGE_2. These findings indicate that the EP1 receptor induced upregulation of HIF-1α is not a consequence of decreased ubiquitin mediated degradation resulting from either decreased prolyl hydroxylation of HIF-1α or decreased activity of the E3 ubiquitin ligase.

The 26S proteasome inhibitor, MG132, was also used to examine if the upregulation of HIF-1α could be attributed solely to a generalized decrease in the activity of 26S proteasome. Figure 3B shows an immunoblot of the time courses for the PGE_2 stimulated upregulation of HIF-1α expression in HEK-hEP1 cells under control conditions or following pretreatment with MG132. It is immediately apparent that pretreatment with MG132 caused a dramatic upregulation in the expression of HIF-1α at all time points including the zero time point. This result confirms that under normoxic conditions HIF-1α expression in HEK cells is largely under

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

the control of a classic mechanism involving constitutive proteasome mediated degradation. However, it is also evident that even after inhibiting the 26S proteasome, the expression of HIF-1α was upregulated following 3 and 6 h of treatment with 1 μM PGE_2. Three additional experiments were conducted to examine the effect of MG132 on the expression of HIF-1α following the treament of HEK-hEP1 cells with either vehicle or 1 μM PGE_2 for 6 h. For these experiments, the expression of HIF-1α was analyzed by immunoblot analysis and then quantified by densitometry. As shown in Figure 3C, treatment with PGE_2 resulted in a statistically significant 1.6-fold increase in the expression of HIF-1α following the inhibition of the 26S proteosome with MG132. Although decreased degradation of HIF-1α cannot be completely excluded, these findings strongly suggest that an additional mechanism, such as increased translation, is responsible for the upregulation of HIF-1α by the EP1 receptor.

Upregulation of HIF-1α expression mediated by the EP1 receptor involves the activation of PI3K, Akt and mTOR signaling. An important mechanism of translational control involves the activity of the ribosomal S6 kinases, which in turn, are regulated by the activity of the PI3K, Akt and the mammalian target of rapamycin (mTOR) signaling pathways (Dufner & Thomas, 1999). We, therefore, decided to examine the potential involvement of PI3K, Akt and mTOR signaling in the EP1 receptor mediated upregulation of HIF-1α expression through the use of wortmannin, Akt inhibitor and rapamycin, which are selective inhibitors of PI3K, Akt and mTOR, respectively. Figure 4A shows that pretreatment of HEK-hEP1 cells with any one of these inhibitors completely blocked the upregulation of HIF-1α protein expression mediated by PGE_2 and suggest that the EP1 receptor mediated upregulation of HIF-1α occurs as a result of increased translation that is driven by the activation of PI3K, Akt and mTOR signaling.

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

Pretreatment of HEK-hEP1 cells with pertussis toxin blocks the PGE_2 stimulated upregulation of HIF-1α expression by the EP1 receptor indicating coupling to $G_{i/o}$. Given the coupling of the EP1 receptor to $G_{q/11}$ and its ability to stimulate intracellular Ca^{2+} mobilization, we examined the effects of the intracellular Ca^{2+} chelator, BAPTA/AM, and the protein kinase C (PKC) inhibitor, BIM, on the PGE_2 stimulated upregulation of HIF-1α by the human EP1 receptor. Unexpectedly, as shown in Figure 4B, pretreatment of HEK-hEP1 cells with either BIM or BAPTA/AM did not significantly inhibit the PGE_2 stimulated upregulation of the expression of HIF-1α, suggesting that coupling to $G_{q/11}$ and activation of Ca^{2+}/PKC signaling were not involved in mediating this response. We have recently found that the human EP4 prostanoid receptor can activate PI3K signaling by coupling to a pertussus toxin sensitive G-protein (Fujino & Regan, 2007). In addition, a number of GPCRs that were traditionally considered to couple exclusively to $G_{q/11}$ have been found to couple to $G_{i/o}$ and activate PI3K signaling (e.g., Voss et al., 2007). We, therefore, decided to use the $G_{i/o}$ inhibitor, pertussis toxin, to examine the potential involvement of $G_{i/o}$ in upregulation of HIF-1α expression mediated by the human EP1 receptor. Figure 4C shows that pretreatment of HEK-hEP1 cells with pertussis toxin completely blocked the PGE_2 stimulated upregulation of HIF-1α as compared with the vehicle treated control cells. These data indicate that EP1 prostanoid receptor mediated upregulation of HIF-1α requires coupling to $G_{i/o}$.

PGE_2 stimulation of the EP1 receptor induces phosphorylation of ribosomal protein S6 and requires $G_{i/o}$ mediated activation of PI3K signaling. Ribosomal protein S6 (rpS6) is one of the primary targets of the ribosomal S6 kinases and is a key regulator of translation and cell growth (Ruvinsky & Meyuhas, 2006). The activity of the ribosomal S6 kinases is in turn regulated both directly and indirectly by the activation of PI3K, Akt and/or mTOR signaling. Two key sites of

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

rpS6 phosphorylation that reflect activation of the ribosomal S6 kinases are Ser235/Ser236. We, therefore, examined the phosphorylation of rpS6 on Ser235/Ser236 following the treatment of HEK-hEP1 cells for various periods of time with 1 μM PGE_2. As shown by the immunoblot in Figure 5A, PGE_2 treatment of HEK-hEP1 cells increased the phosphorylation of rpS6 over basal levels after just 1 h and was maintained for up to 24 h. The evidence that the PGE_2 induced phosphorylation of rpS6 precedes the upregulation of HIF-1α is supportive for a putative role of activation of the ribosomal S6 kinases in the translation control of HIF-1α expression following activation the human EP1 receptor.

Next, HEK-hEP1 cells were pretreated with either pertussis toxin or wortmannin to examine the potential involvement of coupling to $G_{i/o}$ and PI3K signaling in the PGE_2 induced phosphorylation of rpS6. Figures 5B and 5C, respectively, show that pretreatment of cells with either pertussis toxin or wortmannin decreased the PGE_2 stimulated phosphorylation of rpS6 as compared with the vehicle treated control cells and suggests that coupling to $G_{i/o}$ and activation of PI3K signaling by PGE_2 stimulation of the EP1 receptor results in the activation of ribosomal S6 kinase activity.

EP1 and EP2, but not EP3 and EP4, prostanoid receptors are expressed in the HepG2 hepatocellular carcinoma cell line. HepG2 cells were used to examine the potential of natively expressed endogenous EP1 receptors to couple to $G_{i/o}$ and upregulate the expression of HIF-1α. HepG2 cells were chosen because it has been previously shown that prostaglandin synthesis contributes to the growth of these cells by a mechanism involving the activation of Akt, although the specific prostanoid receptors contributing to this response are unknown (Leng et al., 2003). In addition, it is well known that the expression of HIF-1α is important in cancer growth and metastasis (Semenza, 2001) and we were interested in the potential normoxic upregulation of

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

HIF-1α by PGE_2 in cancer cells. PCR with primers specific for the EP1, EP2, EP3 and EP4 receptors was used to examine the expression of the EP prostanoid receptor subtypes in RNA prepared from HepG2 cells and from SH-SY5Y cells, a neuroblastoma cell line known to express endogenous EP1 receptors (Hoshino et al., 2007). As shown in Figure 6, HepG2 cells expressed the EP1 and EP2 subtypes, but not the EP3 and EP4 subtypes. SH-SY5Y cells, on the other hand, expressed all four EP receptor subtypes.

Sulprostone stimulates the upregulation of HIF-1α protein expression, but not mRNA, in HepG2 cells. HepG2 cells were used to examine the potential upregulation of HIF-1α protein and mRNA expression by sulprostone, a selective agonist of the EP1 and EP3 receptors. The upper panel of Figure 7A shows an immunoblot of the expression of HIF-1α and vinculin following treatment of HepG2 cells with 1 μM sulprostone for various periods of time. In untreated cells (0 h) and following 1 h of treatment with sulprostone there was a low basal expression of HIF-1α, which increased progressively following 3, 6, 12 and 24 h of treatment. Treatment of HepG2 cells with sulprostone did not affect the expression of vinculin. The lower panel of Figure 7A shows the results of quantitative real time PCR for the expression of HIF-1α mRNA. As can be seen, there were no significant changes in the expression of HIF-1α mRNA following the treatment of HepG2 cells with sulprostone. These findings mirror the results obtained in HEK-hEP1 cells and show that sulprostone does not upregulate the expression of HIF-1α in HepG2 cells by increased transcription. In separate studies we have found that the human EP1 receptor also mediates the upregulation of early growth response factor-1 (EGR-1) in HEK-hEP1 cells by increased transcription (Regan & Ji, unpublished). As a positive control for the measurement of transcriptional changes, we also examined the upregulation of EGR-1 protein and mRNA expression by sulprostone in HepG2 cells. The immunoblot in the upper

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

panel of Figure 7B shows that the expression of EGR-1 in HepG2 cells was increased following 1, 2 and 3 h of treatment with 1 μM sulprostone. However, in contrast to the results obtained for HIF-1α, the lower panel of Figure 7B shows that EGR-1 mRNA expression increased ~3.5-fold after 1 h of treatment with sulprostone and then returned to baseline by 3 h.

Pretreatment of HepG2 cells with pertussis toxin or rapamycin blocks the sulprostone stimulated upregulation of HIF-1α expression by the EP1 receptor. HepG2 cells were pretreated with pertussis toxin and then stimulated with sulprostone to determine if the upregulation of HIF-1α observed with PGE_2 treatment involved coupling of an EP1 receptor to $G_{i/o}$. As shown by the immunoblot in Figure 8A, treatment of HepG2 cells with sulprostone under control conditions resulted in a marked increase in the expression of HIF-1α with no observable change in the expression of vinculin. Furthermore, pretreatment of HepG2 cells with pertussis toxin completely blocked the sulprostone stimulated increase in HIF-1α expression indicating coupling of the endogenous EP1 receptor to $G_{i/o}$.

Cells were pretreated with rapamycin and then stimulated with sulprostone to examine the potential involvement of mTOR signaling in the upregulation of HIF-1α expression mediated by the endogenous EP1 receptors in HepG2 cells. The immunoblot in Figure 8B shows that pretreatment of HepG2 cells with rapamycin completely blocked the sulprostone stimulated increase in the expression of HIF-1α, suggesting that the mechanism involved in the upregulation of HIF-1α expression by the endogenous EP1 receptor in HepG2 cells is similar to that characterized for the recombinant EP1 receptor expressed in HEK cells and involves the activation of mTOR signaling. We, therefore, examined a time course for the phosphorylation of ribosomal protein S6 (rpS6) following the treatment of HepG2 cells with sulprostone. As shown in Figure 8C treatment of HepG2 cells with 1 μM sulprostone resulted in an initial

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

phosphorylation of rpS6 after 3 h of treatment that increased after 6 h and was maintained up to 12 h. This sulprostone mediated phosphorylation of rpS6 at Ser235/Ser236 is consistent with EP1 receptor stimulation of mTOR signaling and activation of the ribosomal S6 kinases.

Treatment of HepG2 cells with sulprostone results in the upregulation of VEGF-C mRNA expression. Numerous studies have documented the HIF-1α dependent upregulation of dozens of genes in a wide variety of cell types following hypoxia (Semenza, 2001). These genes include regulators of angiogenesis, such as vascular endothelial growth factors A and C (VEGF-A and VEGF-C), regulators of glycolytic metabolism, such as phosphoglycerate kinase 1 (PKG1) and the glucose transporter (GLUT1), as well as other growth factors and hormones such as connective tissue growth factor (CTGF) and erythropoietin (EPO). Using quantitative real time PCR we examined the mRNA expression of these six genes following the treatment of HepG2 cells with sulprostone to determine if activation of endogenous EP1 receptors under normoxic conditions could regulate the expression of any known HIF-1 regulated target genes. As shown in Figure 9 treatment of HepG2 cells with 1 μM sulprostone resulted in clear time dependent increase in the mRNA expression of VEGF-C, with essentially no effect on the expression of the other genes. Figure 10 shows the relative transcript levels of these six genes at time 0, prior to treatment of the cells with sulprostone. The mRNA expression of PKG1, CTGF and GLUT1 were high, approximately half the transcript level of GAPDH, which may have precluded further upregulation of these genes by a receptor dependent mechanism. The expression of VEGF-A was approximately 5% of that of GAPDH, while the transcript levels of VEGF-C and EPO were more than a thousand times lower than the mRNA expression of GAPDH.

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

The EP1 prostanoid receptor is one of the four primary receptor subtypes for PGE_2, the others being the EP2, EP3 and EP4 receptor subtypes. Interestingly in terms of its amino acid sequence indentity with the other prostanoid receptors it is actually more closely related to the FP and TP receptors, followed by the EP3, EP2 and EP4 receptors (Regan et al, 1994). Of the four EP receptors, the EP1 also has the lowest binding affinity for PGE_2 and like the FP and TP receptors the EP1 is generally regarded to couple to $G_{q/11}$ as opposed to the EP2 and EP4 receptors that couple primarily to G_s and the EP3 receptors that couple primarily to $G_{i/o}$. As noted previously, however, the coupling of the EP1 receptor to $G_{q/11}$ has occasionally been questioned because of its generally poor ability to stimulate the formation of intracellular inositol phosphates (IP). In the present study, however, we have shown that PGE_2 clearly stimulated intracellular IP accumulation in HEK cells expressing the human EP1 receptor, consistent with coupling of this receptor to $G_{q/11}$. We also show that the human EP1 receptor can couple to $G_{i/o}$ to upregulate the expression of HIF-1α through the activation of PI3K, Akt and mTOR signaling. This upregulation of HIF-1α by the EP1 receptor occurs under conditions of normoxia and involves activation of the ribosomal S6 kinases and increased translation.

The expression of HIF-1α is classically regulated by increased protein stability, resulting from decreased protein degradation in response to hypoxia (Semenza, 2001). Thus, HIF-1α is constitutively expressed in most cells, but under normoxic conditions it undergoes continuous ubiquitin-mediated degradation following hydoxylation of regulatory proline residues. The prolyl hydrolases that carry out the hydroxylation of HIF-1α utilize molecular oxygen and are also sensitive to the concentration of O_2 in the cell (Schofield & Ratcliffe, 2004). Decreases in the partial pressure of O_2 during hypoxia decrease the activity of these enzymes and result in the

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

stabilization and upregulation of HIF-1α expression. Although less fully appreciated, HIF-1α upregulation is known to occur under normoxic conditions in response to receptor mediated activation by a variety of cytokines and hormones (Zhou & Brune, 2006). The signaling pathways involved in this receptor mediated upregulation of HIF-1α under normoxic conditions typically involve the activation of PI3K and Akt signaling and/or MAP kinase signaling. However, in contrast to the decreased degradation of HIF-1α that occurs during hypoxia, the cytokine receptor mediated upregulation of HIF-1α that occurs during normoxia is typically the result of increased translation and/or transcription. Although PGE_2 has been previously reported to upregulate the expression of HIF-1α under normoxic conditions in lung and gastric carcinoma cells, the specific mechanism of this activation was not investigated and was considered to be a downstream consequence of the upregulation of COX-2 expression (Jung et al., 2003; Huang et al., 2005).

Like PGE_2, many of the cytokines that can upregulate the expression of HIF-1α are also involved in inflammatory responses. Well-characterized examples include IL-1β and TNF-α, which have been shown to upregulate HIF-1α under normoxic conditions. For example in human synovial fibroblasts, IL-1β and TNF-α increased HIF-1α mRNA and functional expression of HIF-1 (Thornton et al., 2000). HIF-1α translocates to the nucleus and combines with HIF-1β to form HIF-1, the functionally active transcription factor. HIF-1 is a master transcription factor that has been shown to affect the expression of gene families, including those involved in angiogenesis, erythropoiesis, energy metabolism and epithelial-mesenchymal transition (Semenza, 2001). Increasingly HIF-1 is being recognized as a critical factor in the inflammatory response. This was clearly demonstated by a targeted deletion of HIF-1α in the myeloid cells of mice in which the inflammatory response was greatly decreased (Cramer et al., 2003). In

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

addition, the expression of a variety of hypoxia-responsive genes was significantly diminished under both normoxic and hypoxic conditions indicating the critical role of HIF-1α in both normoxic and hypoxic gene regulation.

Another key player in the inflammatory response is cyclooxygenase-2 (COX-2), which is strongly upregulated in the earliest phases of inflammation and remains elevated throughout the inflammatory response (Smith et al., 2000). Typically the expression of microsomal prostaglandin-E synthase-1 (mPGES-1) is simultaneously upregulated leading to marked increase in the tissue concentration of PGE_2. A similar upregulation of COX-2 has also been well documented in a wide variety of tumors lending strong support to the idea that chronic inflammation is a key event in tumorigenesis (Mantovani et al., 2008). Hepatocellular carcinoma (HCC) is one example of a cancer in which there is good evidence for a role of COX-2 and prostaglandin signaling in the development of hepatic inflammation and malignant transformation (Breinig et al., 2007). Thus, COX-2 is overexpressed in HCC and its expression progessively increases as the liver goes from a stage of chronic hepatitis, to cirrhosis, to a pre-malignant condition and finally to cancer. In addition, the concentation of PGE_2 is increased in HCC and exogenous PGE_2 has been shown to drive hepatic cancer cell growth and invasiveness.

Although it is generally assumed that PGE_2 contributes to tumorigenesis through a combination of increased cell survival, increased cell proliferation/motility, induction of angiogenesis and suppression of immune surveillance, there is very little known about the specific EP receptors and molecular mechanisms mediating these effects. In addition there is little known about specific mechanisms that could potentially link the role of PGE_2 in the inflammatory response to its role in carcinogenesis. In this regard our present findings provide important new information regarding the putative role of the EP1 receptor in inflammation and

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

tumorigenesis. Thus, we have shown both in HEK cells expressing recombinant EP1 receptors and in HepG2 cells expressing endogenous EP1 receptors that PGE_2 can rapidly induce the upregulation of HIF-1α under normoxic cell culture conditions. This means at the very earliest stages of inflammation, following the initial upregulation of COX-2 and PGE_2 synthesis, there is the potential for increased expression of HIF-1α and the activation of HIF-1 responsive genes. These genes even include COX-2 itself, which has previously been shown to be upregulated in HCC and associated with tumor angiogenesis (Cheng et al., 2004).

A highly significant correlation has recently been found between the expression of HIF-1α and lymphatic metastasis and VEGF-C expresssion in human esophageal cancer (Katsuta et al., 2005). Similarly, in lymph node positive invasive breast cancer, a significant correlation has been found between the expression of HIF-1α and VEGF-C and between the expression of HIF-1α and peritumoral lymphangiogenesis (Schoppmann et al., 2006). Although hypoxia and over expression of HIF-1α have been found to upregulate the gene expression of VEGF-C (Manalo et al., 2005), the exact mechanism of this upregulation is unclear since the VEGF-C promoter does not contain a known consensus binding site for HIF-1. It is intriquing, therefore, that we have found that EP1 receptor activation in HepG2 cells can upregulate the protein expression of HIF-1α and the mRNA expression of VEGF-C and that the upregulation of HIF-1α appears to precede the upregulation of VEGF-C. In this regard a previous study has found that overexpression of COX-2 upregulates the expression of VEGF-C in human lung adenocarcinoma cells by a mechanism involving the activation of EP1 receptors (Su et al, 2004). These findings suggest the possibility that upregulation of VEGF-C in cancer involves either a permission interaction between the upregulation of HIF-1α and EP1 receptor activation or a direct

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

mechanistic relationship, possibly involving an EP1 receptor mediated upregulation of HIF-1α, followed by a HIF-1α mediated upregulation of VEGF-C.

We have shown for the first time that the EP1 receptor mediated upregulation of HIF-1α occurs by a mechanism involving coupling of the EP1 receptor to $G_{i/o}$ and activation of a PI3K/Akt/mTOR signaling pathway. It is well established that the PI3K/Akt/mTOR pathway is dysregulated in many types of cancer (Shaw & Cantley, 2006) and our findings provide a further mechanism by which the EP1 receptor could influence known oncogenic signaling pathways in addition to its upregulation of HIF-1α itself. Thus, inappropriate activation of PI3K/Akt/mTOR signaling could drive cellular growth through increased translation and promote cell survival by inhibiting apoptosis. Our findings with the HepG2 cells have specific implications for a role of EP1 receptors in HCC. Thus, previous studies have shown that HIF-1α is overexpressed in patients with HCC (Huang et al., 2005) and that stimulation of EP1 receptors in human HCC cells can enhance tumor cell invasion (Han et al., 2006); however, the potential mechanistic relationship between these observations is unknown. Our results suggest that the upregulation of COX-2, that is known to occur in HCC, could lead to the upregulation of HIF-1α and VEGF-C through PGE_2 stimulation of the EP1 receptor and thereby drive tumor angiogenesis and metastasis. The EP1 prostanoid receptor clearly merits interest as a potential therapeutic target for the treatment of inflammation and cancer.

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

We thank Dr. Garth Powis (University of Texas M. D. Anderson Cancer Center) for generously providing the pGL3/HRE-Luc reporter plasmid and the Arizona Cancer Center Genomics Facility Core, and Dr. George Watts specifically, for their help with qPCR and the interpretation of microarray data.

References

Breinig M, Schirmacher P and Kern MA (2007) Cyclooxygenase-2 (COX-2) A therapeutic target in liver cancer? *Curr Pharm Design* **13**:3305-3315

Cheng ASL, Chan HLY, To KAF, Leung WK, Chan KK, Liew CT and Sung JJY (2004) Cyclooxygenase-2 pathway correlates with vascular endothelial growth factor expression and tumor angiogenesis in hepatitis B virus-associated hepatocellular carcinoma. *Internat J Oncol* **24**:853-860

Coleman RA, Smith WL and Narumiya S (1994) VIII. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* **46** 205-229

Cramer T, Yamanishi Y and Johnson RS (2003) Hif-1α is essential for myeloid cell-mediated inflammation. *Cell* **112**:645-657

Creese BR and Denborough MA (1981) The effects of imidazole on contractility and cyclic AMP levels of guinea-pig tracheal smooth muscle. *Clin Exp Pharmacol Physiol* **9**:145-155

Dufner A and Thomas G (1999) Ribosomal S6 kinase signaling and the control of translation. *Exp Cell Res* **253**:100-109

Fujino H, Srinivasan D, Pierce KL and Regan JW (2000) Differential regulation of prostaglandin F2α receptor isoforms by protein kinase C. *Mol Pharmacol* **57**:353-358

Fujino H, Xu W and Regan JW (2003) Prostaglandin E2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. *J Biol Chem* **278**:12151-12156

Fujino H, Chen XB, Regan JW and Murayama T (2007) Indomethacin decreases EP2 prostanoid receptor expression in colon cancer cells. *Biochem Biophys Res Commun* **359**:568-573

Funk CD, Furci L, FitzGerald GA, Grygorczyk R, Rochette C, Bayne MA, Abramovitz M, Adam M and Metter KM (1993) Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtypes. *J Biol Chem*. **268**:26767-26772

Han C, Michalopoulos GK and Wu T (2006) Prostaglandin E2 receptor EP1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. *J Cell Physiol* **207**:261-270

Hata AN and Breyer RM (2004) Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacol Ther* **103**:147-166

Hoshino T, Nakaya T, Homan T, Tanaka K, Sugimoto Y, Araki W, Narita M, Narumiya S, Suzuki T and Mizushima T (2007) Involvement of prostaglandin E2 in production of amyloid-beta peptides both in vitro and in vivo. *J Biol Chem* **45**:32676-32688

Huang GW, Yang LY and Lu WQ (2005) Expression of hypoxia-inducible factor 1a and vascular endothelial growth factor in hepatocellular carcinoma: impact on neovacularization and survival. *World J Gastroenterol* **11**:1705-1708

Huang SP, Wu MS, Shun CT, Wang HP, Hsieh CY, Kuo ML and Lin JT (2005) Cyclooxygenase-2 increases hypoxia-inducible factor-1 and vascular endothelial growth factor to promote angiogenesis in gastric carcinoma. *J Biomed Sci* **12**:229-241

Jung YJ, Isaacs JS, Lee S, Trepel J and Neckers L (2003) IL-1β-mediated up-regulation of HIF-1α via an NFκB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *FASEB J* **17**:2115-2117

Katoh H, Watabe A, Sugimoto Y, Ichikawa A and Negishi M (1995) Characterization of the signal transduction of prostaglandin E receptor EP1 subtype in cDNA-transfected Chinese hamster ovary cells. *Biochim Biophys Acta* **1244**:41-48

Katsuta M, Miyashita M, Makino H, Nomura T, Shinji S, Yamashita K, Tajiri T, Kudo M, Ishiwata T and Naito Z (2005) Correlation of hypoxia inducible factor-1a with lymphatic metastasis via vacular endothial growth factor-C in human esophageal cancer. *Exp Mol Path* **78**:123-130

Lawence RA and Jones RL (1992) Investigation of the prostaglandin E (EP-) receptor subtype mediating relaxation of the rabbit jugular vein. *Br J Pharmacol* **105**:817-824

Leng J, Han C, Demetris AJ, Michalopoulos GK and Wu T (2003) Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through AKT activation: evidence for AKT inhibition in celecoxib-induced apoptosis. *Hepatology* **38**:756-768

Manalo DJ, Rowan A, Lavoie T, Natarajan L, Kelly BD, Ye SQ, Garcia JGN and Semenza GL (2005) Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* **105**:659-669

Mantovani A, Allavena P, Sica A and Balkwill F (2008) Cancer-related inflammation. *Nature* **454**:436-444

Nicola C, Timoshenko AV, Dixon SJ. Lala PK and Chakraborty C (2005) EP1 receptor-mediated migration of the first trimester human extravillous trophoblast: the role of intracellular calcium and calpain. *J Clin Endocrinol Metab* **90**:4736-4746

Regan JW (2003) EP2 and EP4 prostanoid receptor signaling. *Life Sci* **74**:143-153

Regan JW, Bailey TJ, Donello JE, Pierce KL, Pepperl DJ, Zhang D, Kedzie KM, Fairbairn CE, Bogardus AM and Woodward DF (1994) Molecular cloning and expression of human EP3 receptors: evidence of three variants with differing carboxyl termini. *Br J Pharmacol* **112**:377-385

Schofield CJ and Ratcliffe PJ (2004) Oxygen sensing by Hif hydroxylases. *Nat Rev Mol Cell Biol* **5**:343-354

Schoppmann SF, Fenzl A, Schindl M, Bachleitner-Hofmann T, Nagy K, Gnant M, Horvat R, Jakesz R and Birner P (2006) Hypoxia inducible factor-1a correlates with VEGF-C expression and lymphangiogenesis in breast cancer. *Breast Cancer Res Treat* **99**:135-141

Semenza GL (2001) Hypoxia-inducible factor-1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med* **7**:345-350

Shaw RJ and Cantley LC (2006) Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* **441**:424-430

Smith WL, DeWitt DL and Garavito RM (2000) Cyclooxygenases: Structural, cellular and molecular biology. *Annu Rev Biochem* **69**:145-182

Shoji Y, Takahashi M, Kitamura T, Watanabe K, Kawamori T, Maruyama T, Sugimoto Y, Negishi M, Narumiya S, Sugimura T and Wakabayashi K (2004) Downregulation of prostaglandin E receptor subtype EP3 during colon cancer development. *Gut* **53**:1151–1158

Su JL, Shih JY, Yen ML, Jeng YM, Chang CC, Hsieh CY, Wei LH, Yang PC and Kuo ML (2004) Cyclooxygenase-2 induces EP1 and HER-2/Neu Dependent vascular endothelial growth factor-C up-regulation: a novel mechanism of lymphangiogenesis in lung adenocarcinoma. *Cancer Res* **64**:554-564

Sugimoto Y and Narumiya S (2007) Prostaglandin E receptors. *J Biol Chem* **282**:11613-11617

Tabata H, Tanaka S, Sugimoto Y, Kanki H, Kaneko S and Ichikawa A (2002) Possible coupling of prostaglandin E receptor EP1 to TRP5 expressed in Xenopus laevis oocytes. *Biochem Biophys Res Comm* **298**:398-402

Thornton RD, Lane P, Borghaei RC, Pease EA, Caro J and Mochan E (2000) Interleukin 1 induces hypoxia-inducible factor 1 in human gingival and synovial fibroblasts. *Biochem J* **350** Pt **1**:307-312

Voss B, McLaughlin JN, Holinstat M, Zent R and Hamm HE (2007) PAR1, but not PAR4, activates human platelets through a $G_{i/o}$/phosphoinositide-3 kinase signaling axis. *Mol Pharmacol* **71**:1399-1406

Watabe A, Sugimoto Y, Honda A, Irie A, Namba T, Negishi M, Ito S, Narumiya S and Ichikawa A (1993) Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor. *J Biol Chem* **268**:20175-20178

Welsh SJ, Bellamy WT, Briehl MM and Powis G (2002) The redox protein thioredoxin-1 (Trx-1) increases hypoxia inducible factor 1α protein expression: Trx-1 overexpression results in increased vascular endothelial growth factor production and enhanced tumor angiogenesis. *Cancer Res* **62**:5089-5095

Zhou J and Brune B (2006) Cytokines and hormones in the regulation of hypoxia inducible factor-1alpha (HIF-1 alpha). *Cardiovasc Hematol Agents Med Chem* **4**: 189-197

Zhou P, Qian L, Chou T and Ladecola C (2008) Neuroprotection by PGE2 receptor EP1 inhibition involves the PTEN/AKT pathway. *Neurobiol Dis*. **29**:543-551

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

Financial support for this work was provided in part to J.W.R. by the National Institutes of Health (NIH) (EY11291), by Allergan Inc. and by the University of Arizona College of Pharmacy. Portions of this work were also made possible by grants to the Arizona Cancer Center Genomics Facility Core (5P30 ES06694-14900209).

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Specific whole cell binding of [^{3}H]PGE_2 (**A**) and PGE_2 stimulation of total inositol phosphates formation (**B**) in HEK cells stably expressing either empty vector (HEK-pCEP4) or vector encoding the human EP1 prostanoid receptor (HEK-hEP1). **A**, PGE_2 competition curves for the specific binding of [^{3}H]PGE_2 were done essentially as previously described (Regan et al., 1994) by incubating whole cells for 1 h at room temperature in a final concentration of 2.5 nM [^{3}H]PGE_2 in the presence of various concentrations of nonradioactive PGE_2. Data are the means ± S.E.M. of triplicate measurements from a representative experiment that was repeated three times. **B**, total inositol phosphates (IP) were determined as described in Materials and Methods on HEK-pCEP4 and HEK-hEP1 cells that were incubated for 1 h at 37°C with the indicated concentrations of PGE_2. Data are the means ± S.E.M. of duplicate measurements from a representative experiment that was repeated three times.

Figure 2. Time courses for the PGE_2 stimulated upregulation of HIF-1α protein (**A**), mRNA expression (**C**), and stimulation of HIF-1 reporter gene activity (**B**) in HEK cells stably expresssing the human EP1 receptor. **A**, HEK-hEP1 cells were incubated with 1 μM PGE_2 at 37°C for the indicated times and were subjected to immunoblot analysis using antibodies against human HIF-1α or vinculin as described in Materials and Methods. A representative immunoblot is shown from one of three independent experiments. **B**, HIF responsive luciferase reporter gene activity in control HEK-pCEP4 cells or HEK-hEP1 cells following treatment with either vehicle (veh) or 1 μM PGE_2. Cells were transiently transfected with a reporter plasmid under the control of a HIF-1 response element (HRE) and luciferase activity was determined as described in Materials and Methods. Data are the means ± S.E.M. of quadruplicate measurements from a

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

representative experiment that was repeated three times. *** $p < 0.001$; compared to corresponding vehicle treatment; 2-way ANOVA, followed by Bonferroni post test. **C**, HEK-hEP1 cells were incubated with 1 μM PGE_2 at 37°C for the indicated times and then RNA was isolated and used for quantitative real-time PCR with primers specific for either HIF-1α or GAPDH as described in Materials and Methods. Data were analyzed by the comparative ΔΔCt method relative to the expression of GAPDH. Data are the means ± S.E.M. (n = 6) of the pooled data from two independent experiments each done in triplicate.

Figure 3. Immunoblot analysis showing the PGE_2 stimulated upregulation of ubiquinated HIF-1α or total HIF-1α (**A**) and time course of the upregulation of HIF-1α (**B**) in HEK cells stably expressing the human EP1 receptor under control conditions or following pretreatment with the proteosome inhibitor, MG132. **A**, HEK-hEP1 cells were pretreated with MG132 for 4 h and were then treated with either vehicle (veh) or 1 μM PGE_2 for 6 h at 37°C. Lysates were prepared and HIF-1α was immunoprecipitated (IP) with antibodies against HIF-1α and then immunoblotted (IB) with antibodies against either ubiquitin or HIF-1α as described in Materials and Methods. Shown is a representative immunoblot and a bar graph of the pooled data from three independent experiments analyzed by densitometry. Data are the means ± S.E.M.; *** $p < 0.001$ compared to corresponding vehicle; 1-way ANOVA, followed by Bonferroni post test. **B**, HEK-hEP1 cells were pretreated with either vehicle (control) or MG132 for 4 h and were then treated with 1 μM PGE_2 for indicated times at 37°C. Lysates were prepared and subjected to immunoblot analysis with antibodies against either HIF-1α or vinculin as described in Materials and Methods. **C**, HEK-hEP1 cells were pretreated with MG132 for 4 h and were then treated with either vehicle (veh) or 1 μM PGE_2 for 6 h at 37°C. Lysates were prepared and subjected to immunoblot

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

analysis with antibodies against either HIF-1α or vinculin as above and were then analyzed by densitometry with the expression of HIF-1α normalized to the expression of vinculin. Shown is a bar graph of the pooled data from three independent experiments. Data are the means ± S.E.M.; ** $p < 0.01$ compared to vehicle; 1-way unpaired t-test.

Figure 4. Immunoblots showing the PGE_2 stimulated upregulation of HIF-1α expression in HEK cells stably expressing the human EP1 receptor under control conditions or following pretreatment with either the PI3K inhibitor, wortmanin; Akt inhibitor; or the mTOR inhibitor, rapamycin (**A**); or following pretreatment with either the protein kinase C inhibitor, BIM; or the Ca^{2+} chelator, BAPTA-AM (**B**); or following pretreatment with the $G_{i/o}$ inhibitor, pertussis toxin (**C**). **A**, HEK-hEP1 cells were pretreated with either vehicle (control) or 10 μM wortmanin (wort), 10 μM Akt inhibitor (Akt I) or 4 μM rapamycin (rapa) for 30 min at 37°C and were then treated with either vehicle (veh) or 1 μM PGE_2 for 6 h at 37°C. **B**, HEK-hEP1 cells were pretreated with either vehicle (control) or 10 μM bisindolylmaleimide-I (BIM) or 10 μM BAPTA-AM (BAPTA) for 30 min at 37°C and were then treated with either vehicle (veh) or 1 μM PGE_2 for 6 h at 37°C. **C**, HEK-hEP1 cells were pretreated with either vehicle (control) or 5 nM pertussis toxin (PTX) overnight and were then treated with either vehicle (veh) or 1 μM PGE_2 at 37°C for 6 h. Cell lysates were prepared and subjected to imunoblot analysis with antibodies against HIF-1α or vinculin as described in Materials and Methods. Data are representative of at least three independent experiments for each antibody and condition.

Figure 5. Immunoblots showing the PGE_2 stimulated phosphorylation of ribosomal protein S6 (phospho-rpS6) in HEK cells stably expressing the human EP1 receptor following a time course

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

of PGE_2 treatment (**A**); or following pretreatment of cells with the $G_{i/o}$ inhibitor, pertussis toxin (**B**); or following pretreatment of cells with the PI3K inhibitor, wortmanin (**C**). **A**, HEK-hEP1 cells were treated with 1 μM PGE_2 for indicated times at 37°C and lysates were prepared and subjected to immunoblot analysis either with antibodies against phospho-rpS6 (Ser235/Ser236) or vinculin as described in Materials and Methods. **B**, HEK-hEP1 cells were pretreated with either vehicle (control) or 5 nM pertussis toxin (PTX) overnight and were then treated with either vehicle (veh) or 1 μM PGE_2 at 37°C for 6 h. Lysates were prepared and subjected to immunoblot analysis as above. **C**, HEK-hEP1 cells were pretreated with either vehicle (control) or 10 μM wortmanin (wort) for 30 min and then treated with 1 μM PGE_2 at 37°C. Lysates were prepared and subjected to immunoblot analysis as above. Data are representative of at least three independent experiments for each antibody and condition.

Figure 6. Photographs of ethidium-stained 2% agarose gels showing the products obtained following reverse transcription and the polymerase chain reaction (RT-PCR) with primers specific for the EP1 (**A**), EP2 (**B**), EP3 (**C**) and EP4 (**D**) prostanoid receptor subtypes and template RNA isolated from HEK cells stably expressing either the EP1, EP2, EP3 or EP4 receptor subtypes and from human hepatocellular carinoma cells (HepG2) and SH-SY5Y human neuroblastoma cells. RT-PCR was performed as described previously (Fujino et al., 2007) with an initial incubation at 94°C for 5 min, followed by 35 cycles of 94 °C for 20s, 60°C for 30s and 72°C for 60s. The EP receptor primers were exactly according to Shoji (Shoji et al., 2004). Molecular size standards are in the far left lanes and non-template control (NTC) reactions are in the far right lanes. Representative gels are shown from one of three independent experiments.

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7. Time courses for the upregulation of protein and mRNA expression for HIF-1α (**A**) and early growth response factor-1 (**B**) following the treatment of HepG2 cells with sulprostone, an EP1/EP3 selective agonist. Cells were treated with 1 μM sulprostone at 37°C for the indicated times and then subjected to immunoblot analysis with antibodies against either HIF-1α, early growth response factor-1 (EGR-1) or vinculin; or to quantitative real time PCR analysis with primers specific for either HIF-1α, EGR-1 or GAPDH as described in Materials and Methods. Shown are representative immunoblots from one of at least three independent experiments for each antibody and condition. PCR data were analyzed by the comparative ΔΔCt method relative to the expression of GAPDH at each time point and were then normalized to expression at time 0 for each gene. Bar graphs represent the mean ± S.E.M. (n = 6) of the pooled data from two independent experiments each performed in triplicate; *** $p < 0.001$ compared to time 0; 1-way ANOVA, followed by Bonferroni post test.

Figure 8. Immunoblots showing the upregulation of HIF-1α expression (**A**,**B**) or phosphorylation of ribosomal protein S6 (**C**) by sulprostone in HepG2 cells either alone (**C**) or following pretreatment of cells with either the $G_{i/o}$ inhibitor, pertussis toxin (**A**), or the mTOR inhibitor, rapamycin (**B**). **A**, HepG2 cells were pretreated with either vehicle (control) or 5 nM pertussis toxin (PTX) overnight at 37°C and were then treated with either vehicle (veh) or 1 μM sulprostone (SP) for 6 h at 37°C. Lysates were prepared and subjected to immunoblot analysis with antibodies against HIF-1α and vinculin as described in Materials and Methods. **B**, HepG2 cells were pretreated with either vehicle (control) or 4 μM rapamycin (rapa) for 30 min and were then treated with either vehicle (veh) or 1 μM sulprostone (SP) for 6 h at 37°C. Lysates were prepared and subjected to immunoblot analysis as above. **C**, HepG2 cells were treated with 1 μM

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

sulprostone (SP) for indicated times at 37°C and lysates were prepared and subjected to immunoblot analysis either with antibodies against phosphorylated ribosomal protein S6 (phospho-rpS6) or vinculin as described in Materials and Methods. Data are representative of at least three independent experiments for each antibody and condition.

Figure 9. Quantititative real-time PCR analysis for the expression of the indicated human genes following treatment of HepG2 cells with 1 μM sulprostone for 1, 3 or 6 h. Gene symbols and names are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKG1, phosphoglycerate kinase 1; CTGF, connective tissue growth factor; GLUT1, facilitated glucose transporter member 1; VEGF-A, vascular endothelial growth factor A; VEGF-C, lymphatic vascular endothelial growth factor C; EPO, erythropoietin. Data for each time point were analyzed by the comparative ΔΔCt method relative to the expression of GAPDH and were then normalized to the 0 time point for each gene. Shown are the means ± S.E.M. (n = 4) of the pooled data from two independent experiments each performed in duplicate. *** $p < 0.001$; * $p < 0.05$; compared to time 0; 1-way ANOVA, followed by Bonferroni post test.

Figure 10. Quantititative real-time PCR analysis for the relative expression of the indicated human genes in HepG2 cells at time 0, prior to treatment with sulprostone. Gene symbols and names are provided in the legend to Figure 9. The data obtained at time 0 in the experiments depicted in Figure 9 were re-analyzed by the comparative ΔΔCt method relative to the expression of GAPDH and were then normalized to the expression of GAPDH. Values for VEGF-C and EPO were 0.0000025 ± 0.0000002 and 0.0002 ± 0.00003, respectively. Shown are the means ±

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

S.E.M. (n = 4); *** $p < 0.001$; compared to GAPDH; 1-way ANOVA, followed by followed by Bonferroni post test.

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

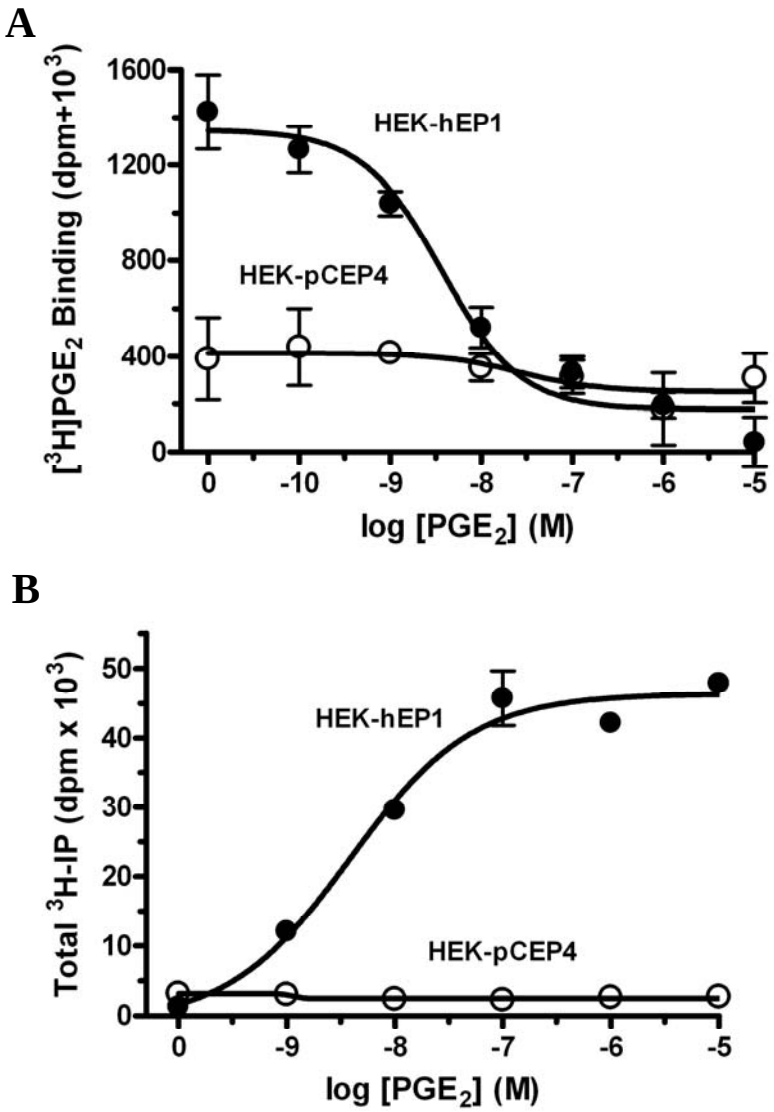

Figure 1

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

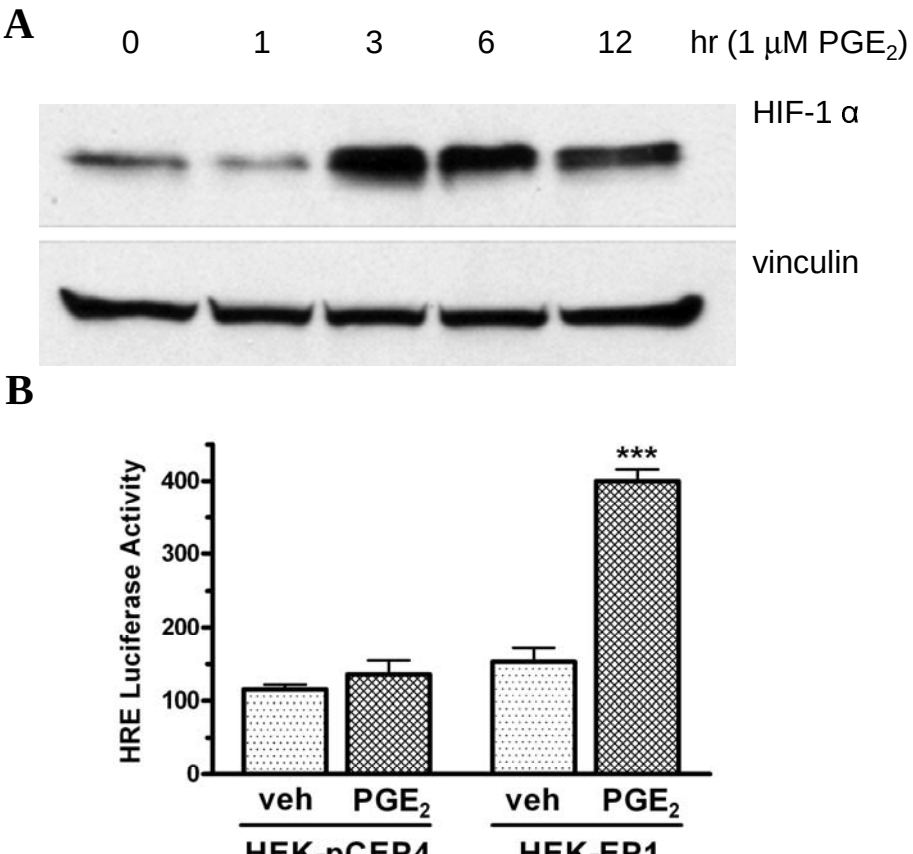

Figure 2

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

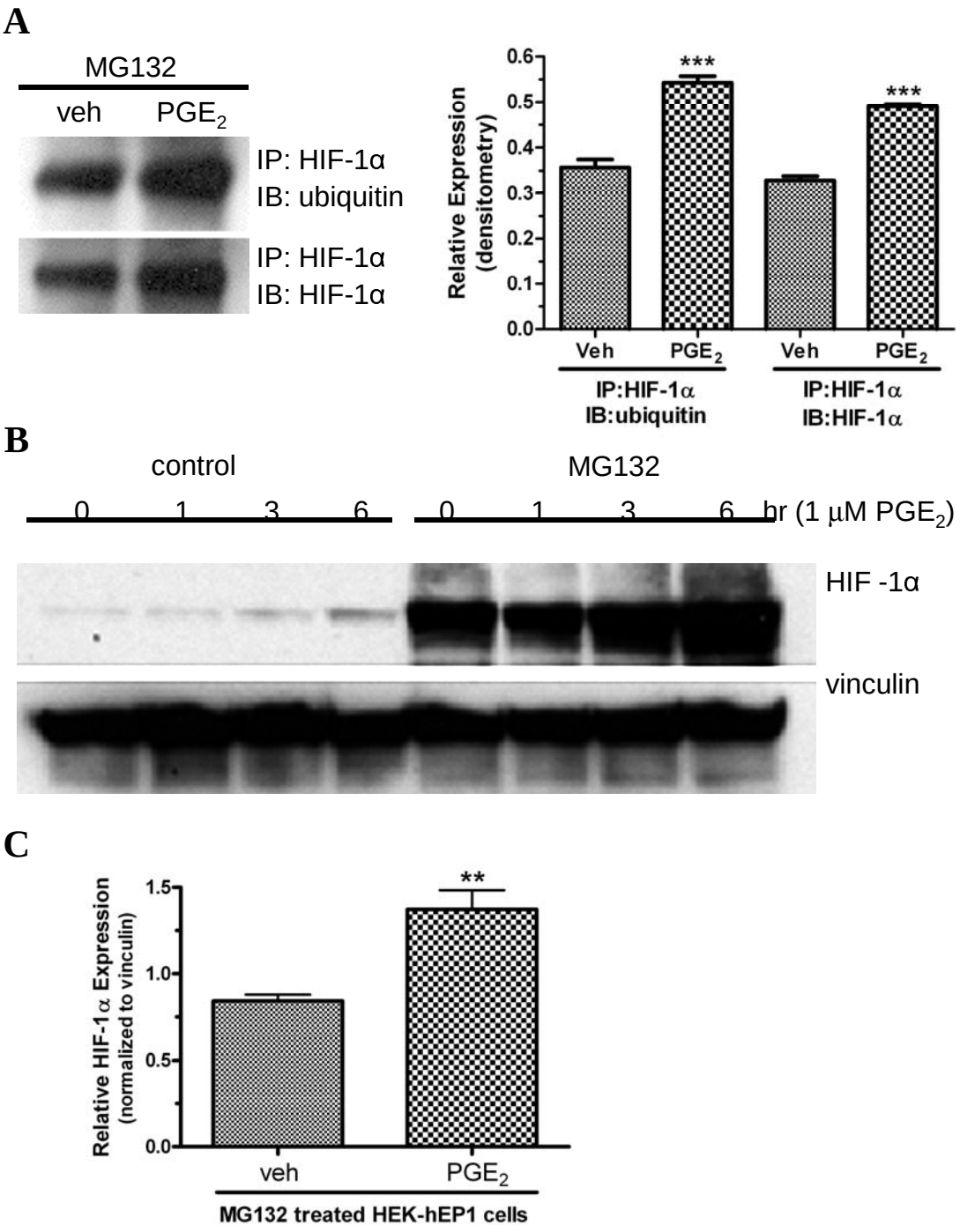

Figure 3

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

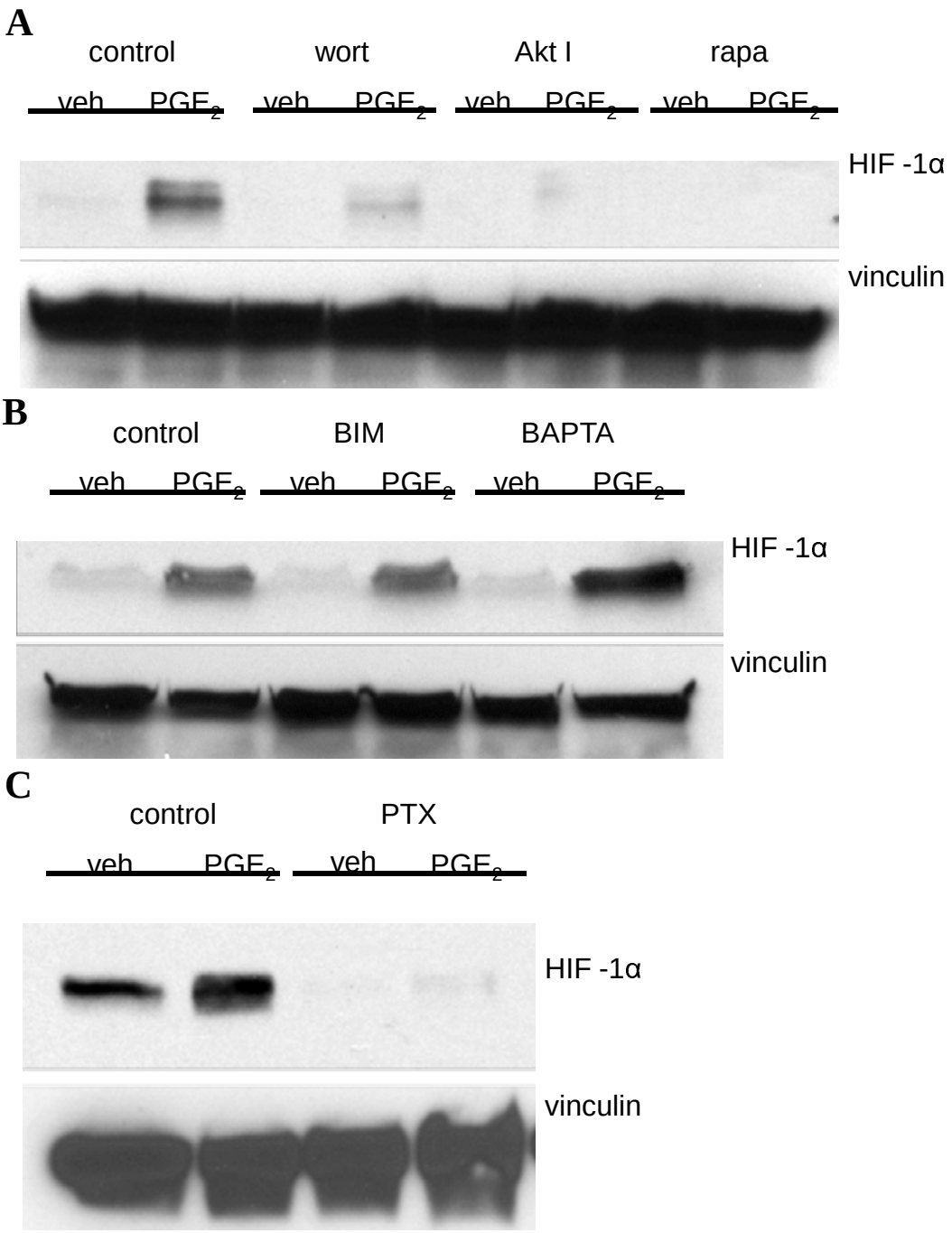

Figure 4

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

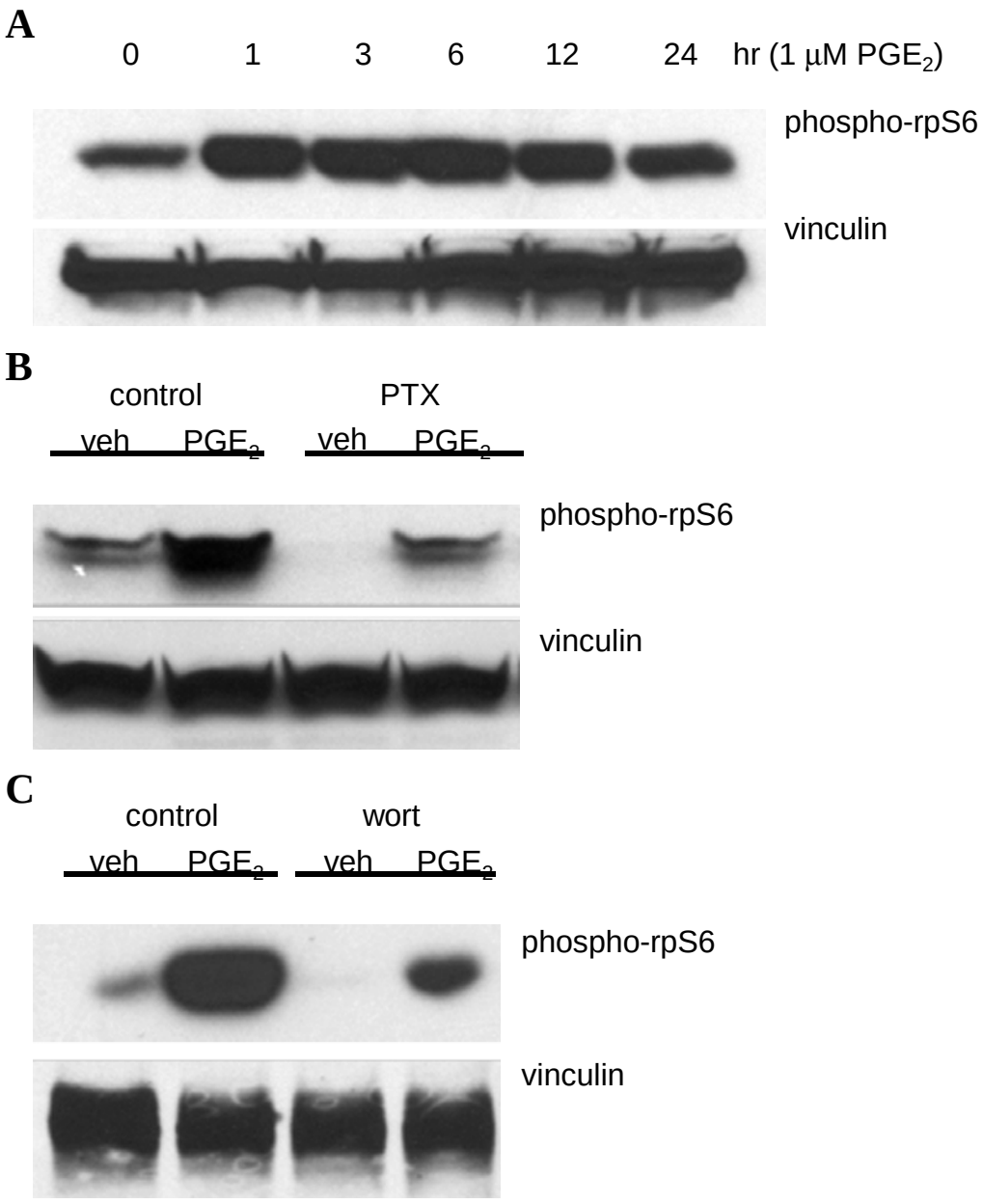

Figure 5

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

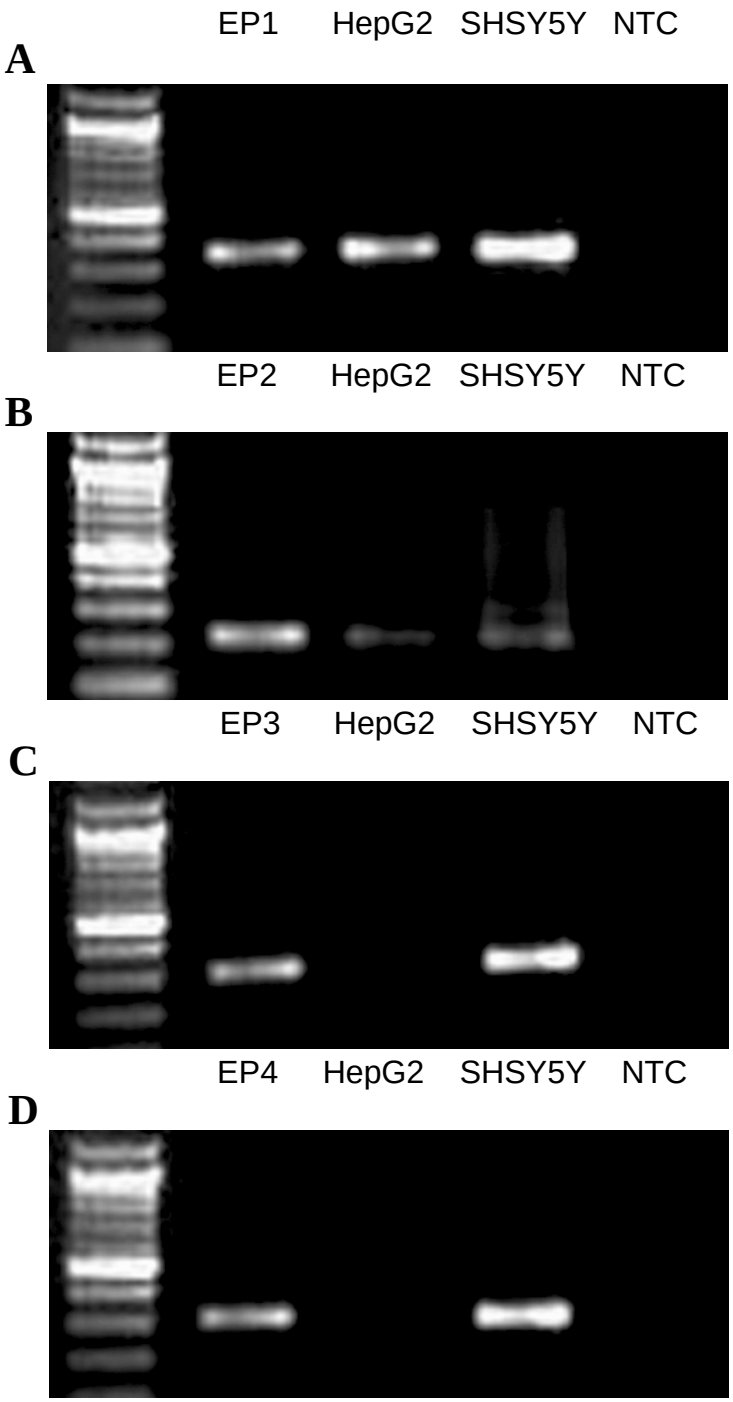

Figure 6

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

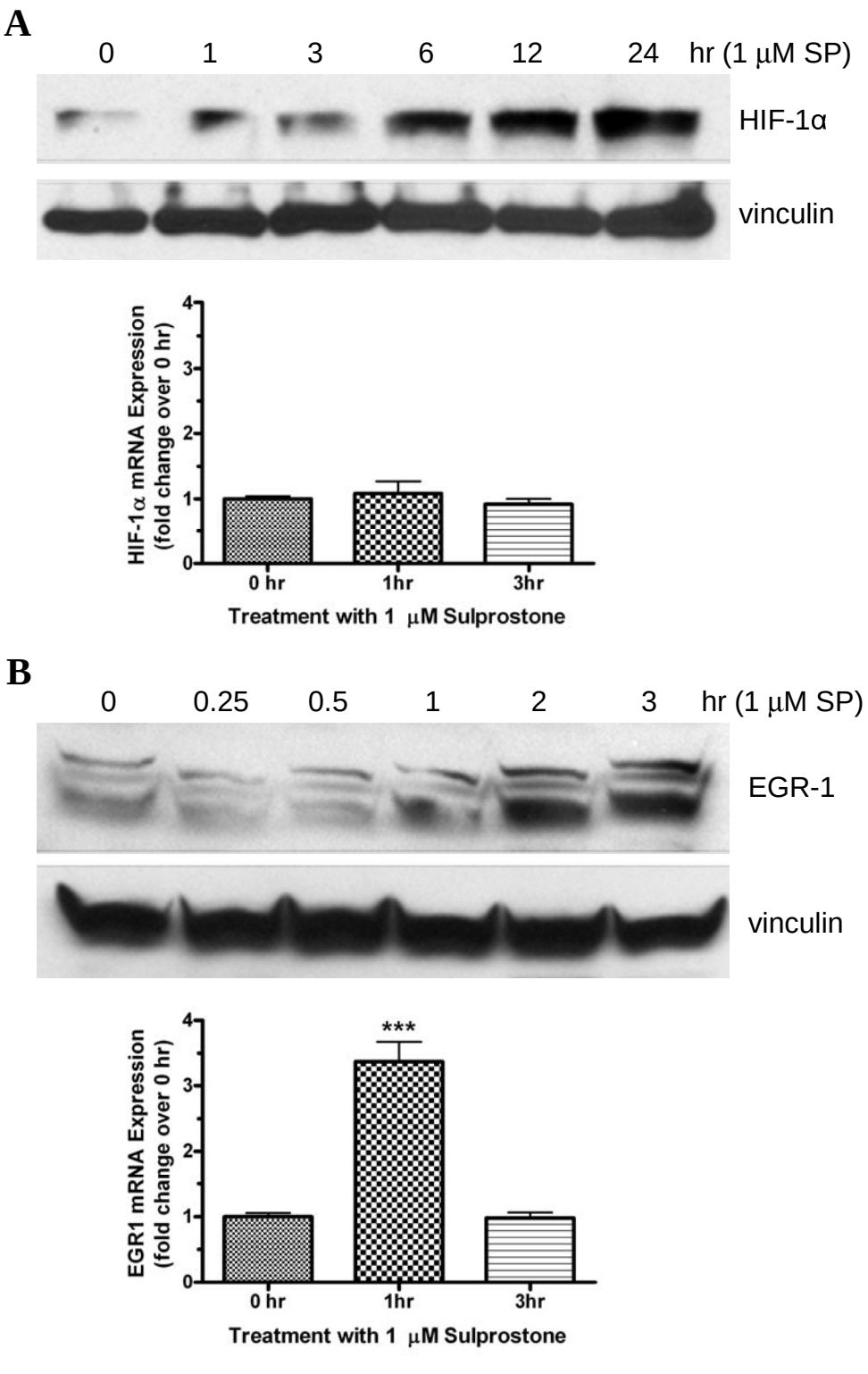

Figure 7

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

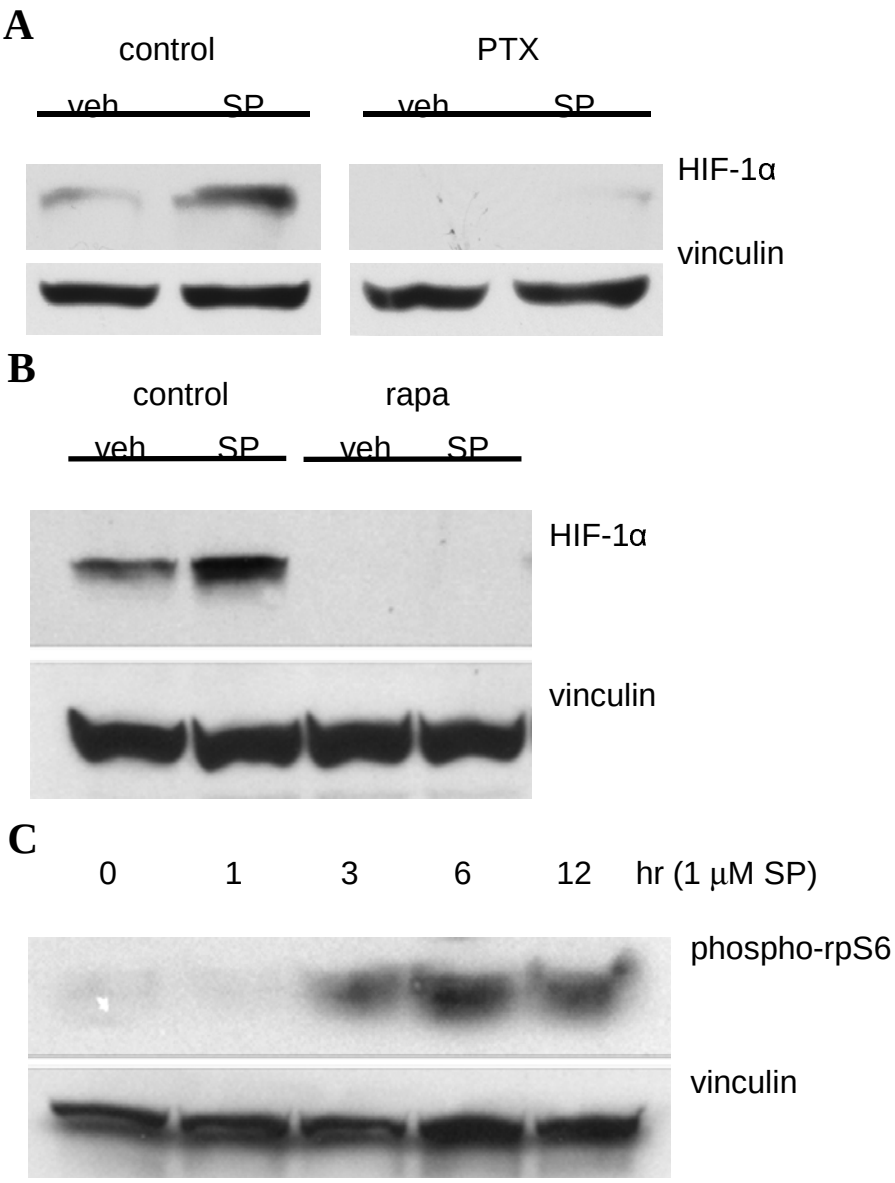

Figure 8

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

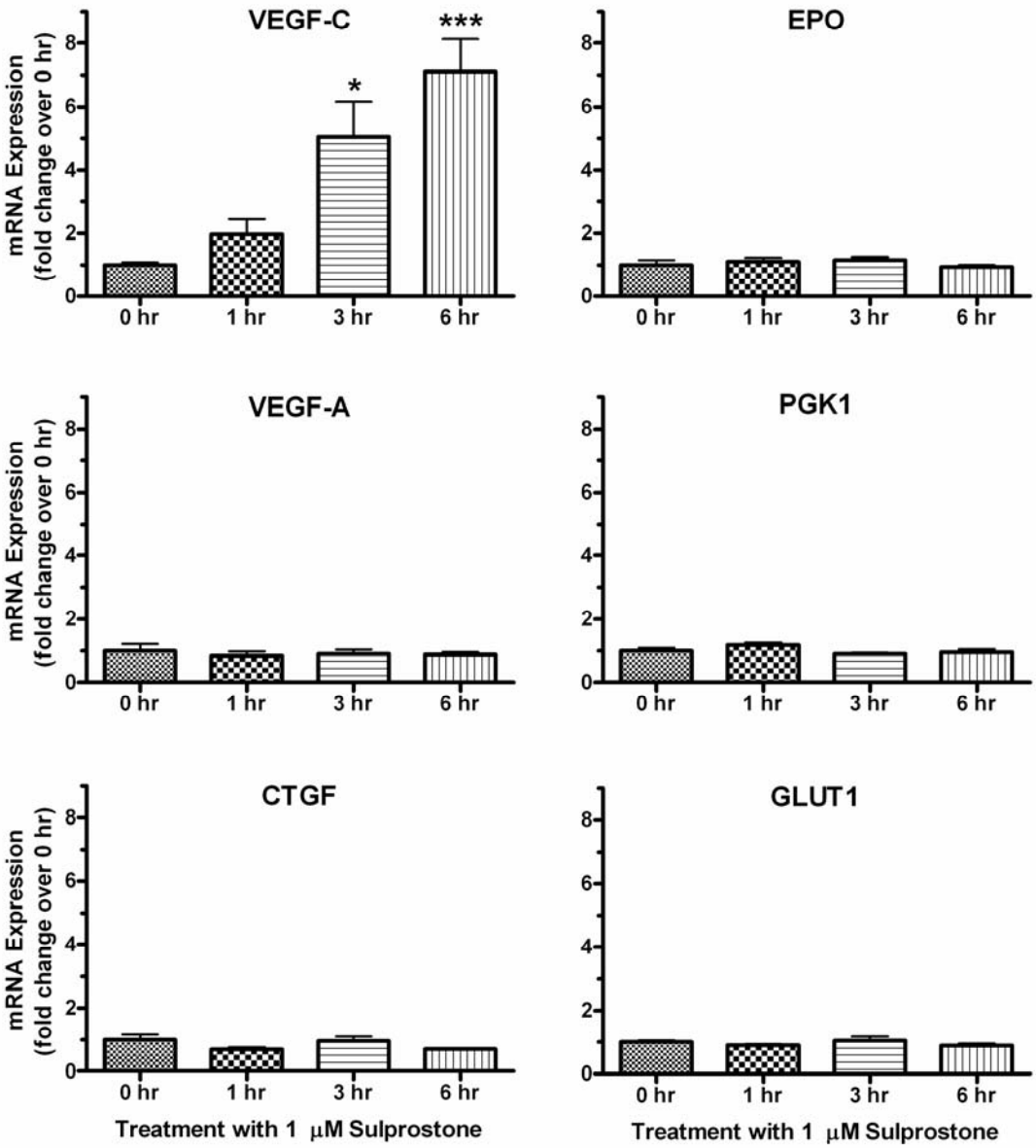

Figure 9

Molecular Pharmacology Fast Forward. Published on March 24, 2010 as DOI: 10.1124/mol.110.063933
This article has not been copyedited and formatted. The final version may differ from this version.

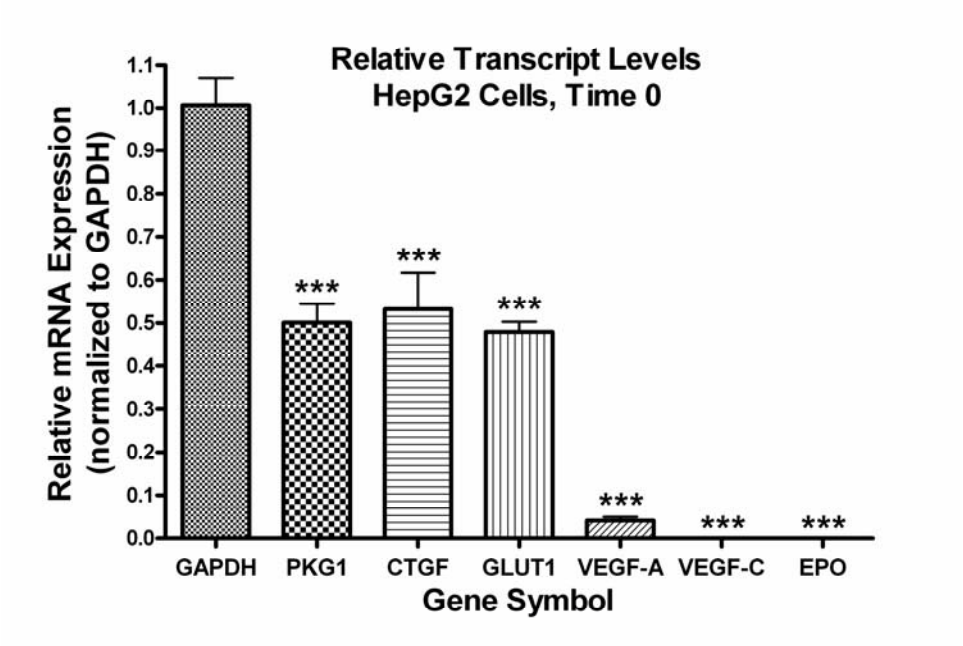

Figure 10