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EP_2 receptor activation by prostaglandin E_2 leads to induction of HO-1 via PKA and PI3K pathways in C6 cells

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ABSTRACT

Recently we proposed that COX-2 induction precedes expression of HO-1 in ischemic preconditioned rat brain. In the current study, we investigated the molecular mechanism by which prostaglandin E_2, one of COX-2 metabolites, induces HO-1 in rat C6 brain cells. We demonstrated that concentration of PGE_2 increased HO-1 expression in C6 cells *in vitro*. The effects of PGE_2 were mimicked by PGE_2 receptor EP_2 agonists, 11-deoxy PGE_2, and cAMP analog, dibutyl-cAMP. HO-1 expression by PGE_2 was inhibited by LY294002, PI3K inhibitor and H89, PKA inhibitor. The EP_2-specific antagonist, AH8006 also inhibited PGE_2-mediated HO-1 expression in a concentration-dependent manner. Finally, PGE_2 inhibited GOX-induced apoptosis as assayed by FACS analysis or DNA strand breaks assay, and this cell death was reversed by ZnPPIX, HO-1 inhibitor. In addition to HO-1 induction, PGE_2 also increased phosphorylation of Bad by PKA- and PI3K-depednent manner. Taken together, we conclude that PGE_2 induces HO-1 protein expression through PKA and PI3K signaling pathways via EP_2 receptor in C6 cells. The induction of HO-1 along with increase of p-Bad by PGE_2 is responsible for anti-apoptosis against oxidant stress.

Ischemic preconditioning (PC) is an endogenous protective mechanism invoked by a brief, sublethal ischemic insult. The molecular mechanisms of the preconditioning induced ischemic tolerance (PTIC) are not yet identified, but the process has been demonstrated in several organs, including the brain [1,2]. We recently proposed that there are kinetic changes in COX-2 expression after PC with a 10 min middle cerebral artery occlusion (MCAO), followed by different periods of reperfusion in the rat brain, and these altered levels of COX-2 could be associated with production of the cytoprotective protein, HO-1 [3,4]. Indeed, we showed that both COX-2 and HO-1 are induced in rat brains previously subjected to ischemic PC by MCAO for 10 min, followed by reperfusion [3,4]. Furthermore, pretreatment with rofecoxib, a COX-2-selective inhibitor, increased infarct size and abolished PCIT-induced COX-2 and HO-1 expression *in vivo*, although ischemic PC significantly reduced the brain infarct size against severe ischemia (24 h MCA occlusion) in the rat [3,4]. Given that COX-2 expression was upregulated after ischemic PC [5,6] and inhibition of COX-2 activity after PC abolished ischemic PC [3–5] and COX-2-induced PGE_2 showed neuroprotection against oxygen/glucose deprivation [7], we investigated the mechanism by which PGE_2 induces HO-1 protein expression *in vitro*. The current study provides evidence that PGE_2 induces HO-1 through PKA and PI3K signaling pathways, via the EP_2 receptor *in vitro*. Furthermore, PGE_2 inhibited oxidative stress (GOX)-induced apoptosis in C6 cells through induction of HO-1. In addition to HO-1 induction, PGE_2 also increased phosphorylation of Bad by PI3K and PKA pathways, which may contribute to anti-apoptotic effect against oxidative stress in the C6 cells.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were purchased from Gibco BRL (Rockville, MD). The anti-HO-1 antibody was purchased from Transduction Laboratories (Lexington, KY). Enhanced chemiluminescence Western blotting detection reagent was from Amersham (Buckinghamshire, UK). PGE_2, 11-deoxy PGE_2, ZnPP IX, LY294002, and AH8006 were supplied by Calbiochem (La Jolla, CA). All other chemicals, including dibutyl-cAMP (Bt_2-cAMP), and H89 were purchased from Sigma–Aldrich (St. Louis, MO).

Cell culture. C6 cells were purchased from ATCC (Manassas, VA) and cultured as described previously [4]. In brief, cells were plated at 2×10^5 cells/well in 6- or 24-well culture plates with DMEM (Dulbecco's modified Eagle medium) containing 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells

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were maintained at 37 °C under 5% CO_2 in a humidified atmosphere. Upon reaching confluence, cells were treated with various concentrations of PGE_2 in a 5% CO_2 atmosphere. The incubation time was adjusted depending on the experimental purpose.

HO-1 activity. To determine HO enzyme activity, confluent cells were incubated in 100 mm culture dishes for 9 h with PGE_2. Some experiments were performed in the presence of H89, LY294002 or AH8006. After incubation, the cells were washed twice with 1× phosphatebuffered saline (PBS, pH 7.4) and centrifuged (100,000*g*, 5 min, 4 °C). The cell pellet was suspended in 2 mM $MgCl_2$ in 100 mM phosphate buffer (pH 7.4), frozen at −70 °C, thawed three times, and finally sonicated on ice before centrifugation at 100,000*g* for 15 min at 4 °C. The supernatant (400 μL) was added to an NADPH-generating system containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-1-dehydrogenase, 2 mg protein of rat liver cytosol (prepared from the 105,000*g* supernatant fraction), 100 mM potassium phosphate buffer (pH 7.4), and PGE_2 in a final volume of 200 μL. The reaction was incubated for 1 h at 37 °C in the dark and terminated by the addition of 600 μL chloroform. The extracted bilirubin concentration was calculated by the difference in absorption between 464 and 530 nm, using a quartz cuvette (= 40 mM/cm). HO-1 activity was represented as picomoles of bilirubin formed per milligram of protein per hour.

Western blot analysis. Cells were lysed in PRO-PREP protein extract solution. The sample was centrifuged at 100,000*g* for 20 min at 4 °C. The protein concentration was determined using the Bradford method. An equal volume of 2× SDS sample buffer (0.1 M Tris–CI, 20% glycerol, 4% SDS, and 0.01% bromophenol blue) was added to an aliquot of the supernatant fraction from the lysates, and the samples were boiled for 5 min. Seventy micrograms of protein were subjected to 10% SDS–polyacrylamide gel electrophoresis for 90 min at 110 V. The separated proteins were transferred to polyvinylidene difluoride membranes for 2 h at 20 mA using SD Semi-dry Transfer Cells (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 2 h at room temperature with 5% nonfat milk in Tris-buffed saline (TBS, pH 7.0) containing 0.05% Tween 20 (TBS-T). The membranes were incubated with an anti-HO-1 antibody overnight at 4 °C, using a dilution of 1:500 in 5% skim milk in TBS-T. Bound antibody was detected by horseradish peroxidase conjugated anti-goat or anti-rabbit IgG. The membranes were washed and then developed using a Western blotting luminol reagent system (iNtRON Biotechnology, Houston, TX) and autoradiography.

DNA isolation and gel electrophoresis. The appearance of DNA laddering was detected using agarose gel electrophoresis [8]. Briefly, samples were scraped in 500 μL lysis buffer (Puregene DNA isolation kit) and quickly homogenized with a microfuge tube pestle. They were then centrifuged at 13,000*g* for 5 min. The supernatant, containing the DNA, was precipitated with isopropanol. After centrifugation at 13,000*g* for 5 min, the resulting DNA pellets were washed with 75% ethanol and dissolved in DNA hydration solution. The content of DNA was detected at 260 nm by spectrophotometry (Lambda EZ 210), and 10 μg DNA was loaded into a 1.8% agarose gel containing 0.5 μg/mL ethidium bromide. DNA electrophoresis was carried out at 80 V for 1 h. DNA laddering, which is an indicator of apoptotic nucleosomal DNA fragmentation, was visualized and photographed under ultraviolet transillumination.

FACS analysis. A total of 1×10^7 cells were seeded in 100 mm culture dishes and incubated at 37 °C. The cells were harvested at indicated times after mitosis. Cells were stained with propidium iodide as described by us [9]. Briefly, cells were trypsinized, washed twice in PBS, followed by fixation in 70% ethanol on ice for a minimum of 2 h. Cells were washed 2 additional times in PBS, and then stained for 30 min at 37 °C in 50 μg/mL PI solution containing 200 μg/mL RNase A and 0.1% Triton X-100. Following incubation in the dark for 30 min at room temperature, cell cycle profiles were determined using a Beckton-Dickinson flow cytometer (Becton Dickinson Biosciences, San Jose, CA). A minimum of 10,000 cells was counted and analyzed with CellQuest® Software.

Statistics. All data are expressed as mean ± SD unless otherwise indicated. Comparisons of parameters among the four groups were made by one-way ANOVA for repeated measures, followed by the Scheffé test. A probability value <0.05 indicated statistical significance.

Results

PGE_2 produces HO-1 protein expression through the PKA signaling pathway

To understand the signaling pathway by which PGE_2 mediates HO-1 induction in C6 cells, the cyclic AMP analog, Bt_2-cAMP was used. This reagent was chosen because PGE_2 increases cAMP [10]. PGE_2 increases HO-1 protein expression in a concentration-dependent manner (Fig. 1). HO-1 was also induced in response to

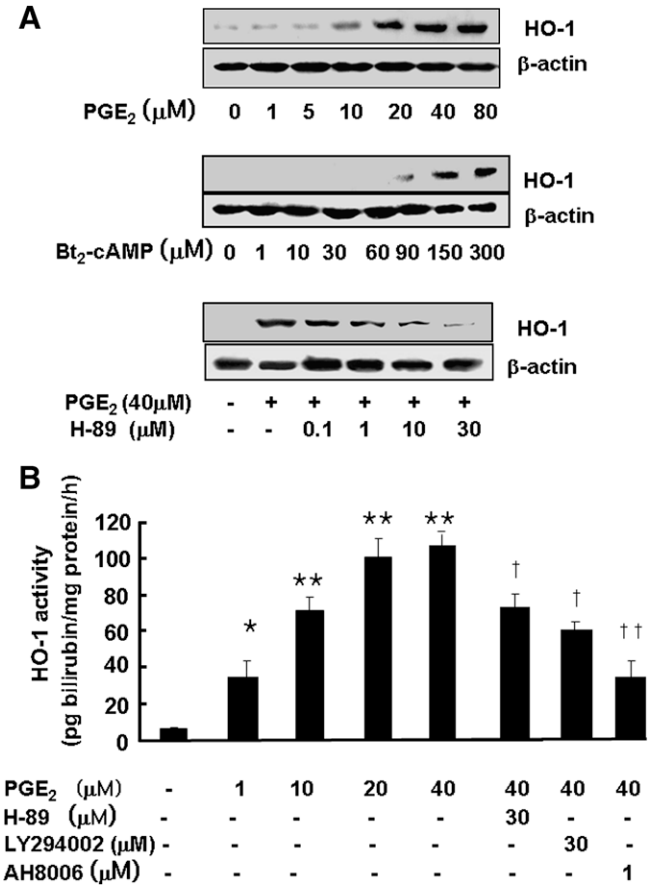

Fig. 1. PKA- and PI3K-dependent HO-1 induction by PGE_2 in C6 cells. (A) Upper panel shows that PGE_2 increases HO-1 protein expression in a concentration-dependent manner. After treatment with the indicated concentrations of PGE_2 or dibutyl-cAMP (Bt_2-cAMP, middle panel) for 8 h, a Western blot was performed. The lower panel shows that PGE_2-induced HO-1 protein expression was diminished by H89, and this effect was dependent on concentration. The indicated concentrations of the PKA inhibitor, H89, were added 30 min prior to the PGE_2, and a Western blot was performed after an 8 h incubation. (B) PGE_2-mediated increases in HO-1 activity were significantly inhibited by the PKA inhibitor, H89, the PI3K inhibitor, LY294002, or the EP_2 receptor antagonist, AH8006. Data represent mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to untreated controls. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared to corresponding controls.

increasing concentrations of Bt_2-cAMP. However, Bt_2-cAMP is required at much higher concentrations to induce HO-1, compared to PGE_2. To further investigate the signals involved in PGE_2-mediated induction of HO-1, we utilized the PKA inhibitor, H89 and PI3K inhibitor, LY294002. The increased HO-1 activity by PGE_2 was significantly inhibited by LY294002 and H89 (Fig. 1B).

PGE_2 induces HO-1 protein expression through activation of the EP_2 receptor

Among four subtypes of the PGE_2 receptor, the EP_2 and EP_4 receptors are known to be coupled to a stimulatory G-protein, which leads to the activation of adenylate cyclase by PGE_2 [11]. However, EP_2 receptor activation is known to be related to neuronal survival [12–14]. Thus, for the current experiences, EP_2-specific agonist and antagonist were used. Fig. 2A shows that the PGE_2 agonist, 11-deoxy PGE_2, increased HO-1 expression in a concentration-

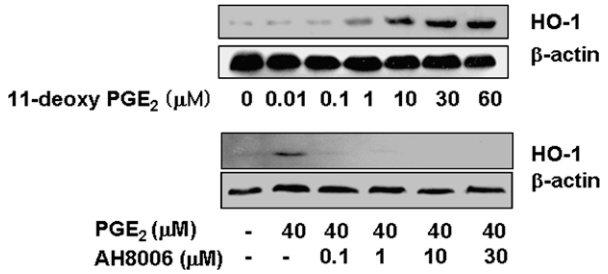

Fig. 2. PGE_2 induces HO-1 protein expression via stimulation of the EP_2 receptor in C6 cells. The upper panel shows that the prostaglandin EP_2-specific agonist, 11-deoxy PGE_2, increases HO-1 protein expression in a concentration-dependent manner. After an 8 h treatment with the indicated concentrations of 11-deoxy PGE_2, a Western blot was performed. The lower panel shows that PGE_2-induced HO-1 protein expression was diminished by the EP_2-specific antagonist, AH8006. The effect of the inhibition was directly related to the concentration of the AH8006.

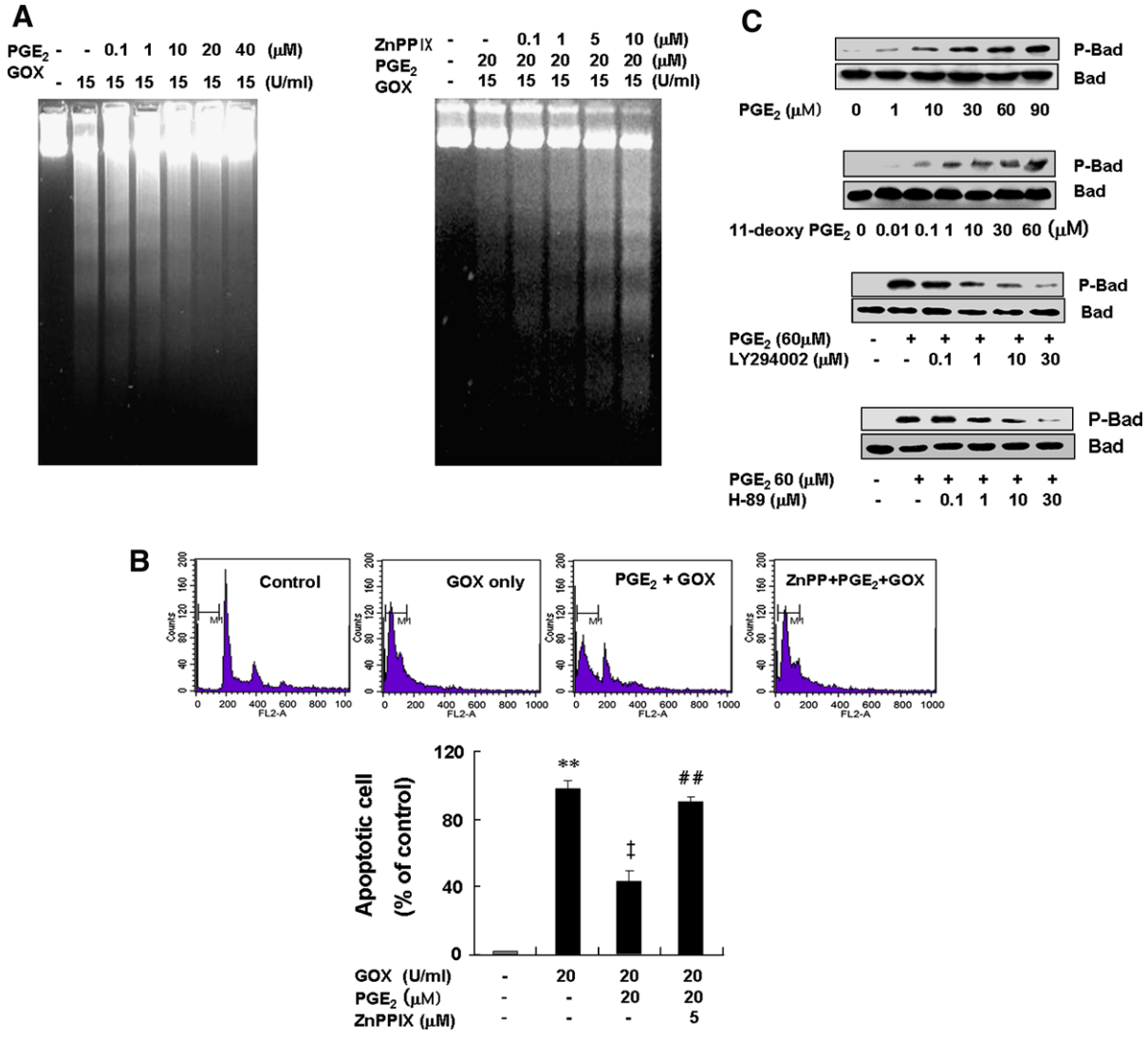

Fig. 3. HO-1 activity is involved in prevention of GOX-induced DNA strand breaks by PGE_2. (A) a fixed concentration of GOX was used along with different concentrations of PGE_2. After a 12 h incubation, genomic DNA was isolated and subjected to electrophoresis. As the concentration of PGE_2 increases, DNA strand breaks were diminished (left). Cells were treated with different concentrations of ZnPP IX, a HO-1 inhibitor, along with fixed concentrations of PGE_2 and GOX. After a 12 h incubation, genomic DNA was isolated and subjected to electrophoresis. As the concentration of ZnPP IX increases, PGE_2-mediated DNA laddering was again increased. (B) FACS analysis was performed in cells treated with the same protocol described in the method. (C) PGE_2 increased phosphorylation of Bad which was inhibited by LY294002 and H89. EP_2-specific agonist, 11-deoxy PGE_2 also increased p-Bad expression. Data represent mean ± SD of three independent experiments. $^{**}P < 0.01$ compared to untreated controls. $^{**}P < 0.05$, compared to control; $^{‡}P < 0.05$, compared to GOX-treated group; $^{\#\#}P < 0.05$ compared to corresponding controls.

dependent manner. Furthermore, PGE_2-induced HO-1 expression was significantly inhibited by the EP_2-specific antagonist, AH8006, and this effect was dependent on the concentration of the inhibitor (Fig. 2A). Accordingly, elevated HO-1 activity by PGE_2 was significantly reduced by AH8006 (Fig. 1B).

PGE_2 inhibits GOX-induced apoptosis which can be reversed by ZnPP IX

We next asked whether PGE_2 could protect cells from oxidative-stress induced injury by regulating HO-1 expression. HO-1 plays many important roles in the general cellular defense mechanisms against oxidative stress in mammalian cells. GOX is frequently used as an oxidative stress inducing agent, and it induces C6 cell apoptosis [15]. Thus, we investigated whether PGE_2 protects from GOX-induced apoptosis. Fig. 3A shows that GOX-induced DNA strand breaks were gradually diminished with increasing concentrations of PGE_2. The diminished DNA ladders by PGE_2 slowly became obvious again as the concentration of ZnPPIX increased. This result was further confirmed by FACS analysis (Fig. 3B), which showed that about 86% of cells underwent apoptosis by GOX (20 U/mL), but pretreatment with PGE_2 significantly reduced the fraction of dead cells to 40%. However, co-treatment with ZnPPIX significantly reduced the anti-apoptotic effect of PGE_2 (Fig. 3B). We obtained similar results with TUNEL analysis (data not shown). Interestingly, PGE_2 and 11-deoxy PGE_2 also concentration dependently increased expression of p-Bad, and PGE_2-induced increase of p-Bad was inhibited by both LY294002 and H89, indicating that activation of PKA as well as PI3K pathway is also involved in the expression of p-Bad (Fig. 3C).

Discussion

We clearly demonstrated that PGE_2 increased HO-1 expression by activation of EP_2 specific receptors through PI3K and PKA pathways and significantly decreased GOX-induced apoptotic cell death in C6 cells. It has been reported that COX-2 induction can function as a mediator of cellular protection in the brain and in the heart of animals using an ischemia/reperfusion model [12,14]. These reports emphasize the fact that COX-2 metabolites serve a critical role for a cell's protective mechanism during oxidative stress. However, a detailed mechanism has yet to be elucidated. Previously, we reported that HO-1 activity is closely related to protection of cells during oxidative stress *in vitro* [16] or *in vivo* [17]. In the CNS, HO-1 is induced in brain tissue that has been exposed to ischemic insults [4] and intraparenchymal or subarachnoid hemorrhage [18,19]. Although COX-2 derived PGE_2 has been reported to protect brain cells from oxidative stress, it is not yet clear whether it is due to HO-1 expression. Furthermore, there is no report so far that PGE_2 induces HO-1 expression via EP_2 receptor activation. To prove that HO-1 induction accounts for the protective effects of PGE_2 against oxidative stress (GOX) in C6 cells, we used ZnPPIX, a HO-1 inhibitor. The anti-apoptotic effect of PGE_2 toward GOX-induced stress in C6 cells was examined using DNA strand breaks and FACS analysis. Almost same results were obtained from both analysis that ZnPPIX inhibited the anti-apoptotic effect of PGE_2, suggesting that PGE_2 promotes C6 cell survival against oxidative stress through a HO-1-dependent mechanism. Next, we addressed which subtype(s) of PGE_2 receptor is activated to induce HO-1 expression by PGE_2, since PGE_2 exerts its biological actions by binding to PGE_2 receptors (EPs), located mainly on the plasma membrane [11]. Although production of cAMP by PGE_2 is linked to a G-protein via the EP_2 and EP_4 receptors [11,20], we focused on the EP_2 receptors, because EP_2, but not EP_4, has a central issue for NMDA receptor-mediated cytotoxicity [10,13,14,21]. In fact, it has been reported recently that the PGE_2 receptor agonist, misoprostol, reduced brain injury in the murine MCAO-reperfusion model [13], and activation of the EP_2 receptor was neuroprotective in paradigms of NMDA toxicity and oxygen glucose deprivation [7]. In contrast, there are compelling evidences that PGE_2 enhanced NMDA-mediated cell death via the EP_2 receptor in cultured cortical neurons [10,21]. Thus, the role of PGE_2 over the EP_2 activation and brain function is controversial, particularly in conditions such as ischemia/reperfusion injury where COX-2 activity is vulnerable. Recently, we proposed that minimal induction of COX-2 after PC is a necessary and sufficient event for the production of HO-1 in the preconditioned rat brain [4]. There are many reports that the EP_2 receptor is involved in protection of neurons, using a model of ischemia and reperfusion [12,14]. We found that activation of EP_2 receptor by PGE_2 caused to induction of HO-1 via PI3K and PKA signal pathways. This finding may be important for understanding the molecular mechanism of PCIT. Although we did not measure cAMP levels in the current study, it has been reported that PGE_2 increases cAMP in C6 cells [22]. It is well established that PKA pathway is involved in HO-1 expression [23,24], so it may be possible that Bt_2-cAMP increased HO-1 expression in C6 cells. However, the concentration required to induction of HO-1 by Bt_2-cAMP was relative high compared to concentration of PGE_2. This may imply that PGE_2 induces HO-1 induction by another mechanism such as activation of PI3K pathway. Indeed, we found that HO-1 activity was significantly reduced by LY294002. We suggested previously that PI3K pathway is involved in the induction of HO-1 by PGE_2 [4]. Finally, PKA has been shown to phosphorylate numerous substrates and to elicit a wide range of cellular responses, including inactivation of the pro-apoptotic protein Bad [25,26]. Inactivation of this protein is closely related to anti-apoptosis in many cells. With this respect, it should be noted that PGE_2 also increased p-Bad with activation of PKA and PI3K pathways (Fig. 3C). It is interesting to note that PGD_2 also causes HO-1 induction in human retinal pigment epithelial cells [27]. Thus, COX-2 induction in the brain during an inflammation or oxidative stress state may play an important role in the protection of brain cells. Although we showed that HO-1 induction

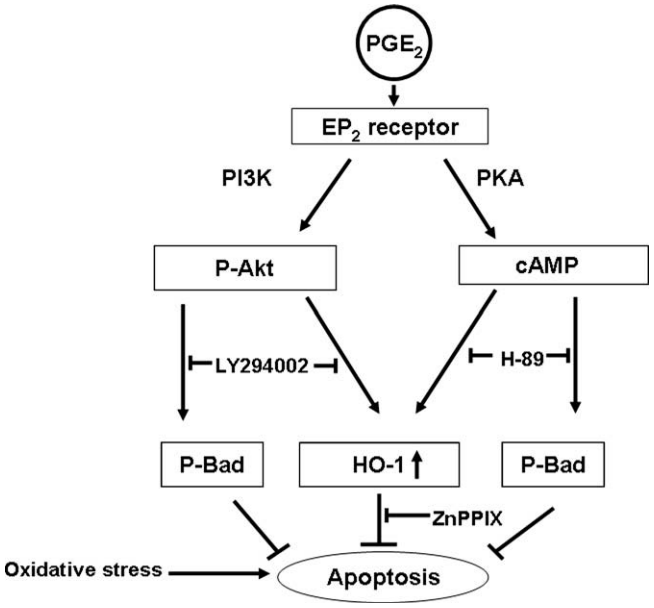

Fig. 4. Schematic representation of PGE_2-mediated anti-apoptosis against oxidative stress in C6 cells. PGE_2, one of mayor metabolites of COX-2, activates PI3K and PKA pathways by binding to EP_2 receptor located in the cell membrane. The activated signal produces increase of p-Akt and cAMP which lead to induction of HO-1 protein, respectively. At the same time, both increased cAMP and p-Akt can phosphorylate Bad protein. Thus, increase of HO-1 and p-Bad expression together may protect cells from oxidative stress. Arrow indicates stimulation and bar indicates inhibition.

is responsible for anti-apoptotic effect of PGE_2 against GOX-induced stress, we believe that PGE_2 exerts its anti-apoptotic effects by modulating expression of p-Bad as well (Fig. 4). However, the possible cross talk between HO-1 and p-Bad in relation to anti-apoptosis by PGE_2 remains to be resolved.

In summary and conclusion, we demonstrated that PGE_2, one of COX-2 metabolites, leads to HO-1 protein expression in brain cells through EP_2 receptor activation via the PKA and PI3K pathway. This induction of HO-1 was related to anti-apoptosis of C6 cells against oxidative stress. Thus, we concluded that PGE_2 may be important in the preconditioned brain for triggering production of the cytoprotective protein HO-1.

Acknowledgments

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