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Prostaglandins antagonistically control Bax activation during apoptosis

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Abbreviations: Bax: Bcl-2-associated X protein; COX-2: cyclooxygenase 2; L-PGDS: lipocalin-like prostaglandin D₂ synthase; mPGES-1: microsomal prostaglandin E₂ synthase 1; PGE₂: prostaglandin E₂; 15-keto PGE₂: 15-keto prostaglandin E₂.

The Bax protein (Bcl-2-associated X protein) is pivotal for the apoptotic process. Bax, which resides in an inactive form in the cytosol of healthy cells, is activated during the early stages of apoptosis and becomes associated with mitochondria through poorly understood mechanisms. Here we show that a family of bioactive lipids, namely prostaglandins, regulates Bax-dependent apoptosis. The prostaglandin E₂ (PGE₂) or its derivative PGA₂ binds to Bax, induces its change of conformation, and thereby triggers apoptosis. A cysteine present in the loop between the two transmembrane alpha helices of Bax, Cys126 is critical for its activation. PGD₂ inhibited PGE₂ binding to Bax and PGE₂-induced apoptosis, as well as cell death induced by staurosporine and UV-B in various cell lines. This result is consistent with the fact that apoptosis is accompanied during these treatments by an increase in PGE₂. This process is distinct, yet cooperative, from that involving the BH3-only protein Bid. Our results establish that the PGE₂/PGD₂ balance is involved in a new early mechanism of control in the activation of Bax during apoptosis.

INTRODUCTION

Members of the Bcl-2 family of proteins determine both the initiation and the execution of mitochondrial outer membrane permeabilization (MOMP) and the subsequent apoptosis. These proteins share a similar solution structure, and are subdivided into 2 groups: anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W and Mcl-1) and pro-apoptotic proteins, which control apoptosis through complex interaction with each other. Pro-apoptotic proteins are further sub-divided into multidomain proteins (namely Bax, Bak and Bok) and BH3-only proteins (e.g. Bim, Bad, Bid, Puma or Noxa) whose homology with Bcl-2 is limited to the BH3 domain. BH3-only proteins act as upstream sensors that selectively respond to specific signals and promote the pro-apoptotic function of Bax and/or Bak. Both *in vivo* and *in vitro* experiments have proved that the conformation of monomeric Bax/Bak change upon induction of apoptosis, leading to their oligomerization in the mitochondrial membrane and MOMP¹⁻³.

How Bax and Bak are activated is a matter of debate^{2,4}. A widely accepted model proposes that some BH3-only proteins called 'activators' (tBid, Bim and Puma) induce the Bax/Bak apoptotic conformational change through transient physical interaction in a 'hit and run' manner. Another group named 'enabler' (also called de-repressor or sensitizer) can release Bax and Bak from anti-apoptotic proteins, which in turn trigger MOMP¹⁻³. The existence of a direct interaction of Bax/Bak with BH3-only proteins has been challenged, suggesting the existence of alternative mechanisms responsible for the activation of Bax or Bak. A change in pH or temperature has also been shown to facilitate the transition between the inactive and active states of Bax, supporting a role for non-protein interactions in apoptosis⁵⁻⁷. However, the physiological occurrence for this type of regulation has not been provided in these studies.

We have recently shown that microsomal prostaglandin E₂ synthase 1 (mPGES-1), the enzyme responsible for the synthesis of PGE₂ downstream of cyclooxygenase 2 (COX-2), facilitates a Bax-dependent apoptosis in human gliomas⁸. The exact role of prostaglandins during apoptosis remains unclear, especially in the case of PGE₂, which has been described, to affect differently programmed cell death depending upon the cellular types and/or contexts (see for example⁹⁻¹¹). However, most studies have essentially been focused on extracellular PGE₂ and its binding to EP receptors. Here, we describe the role of intracellular PGE₂ and its closely related prostaglandin PGD₂ on Bax activation.

RESULTS

PGE₂-derived prostaglandins specifically induce Bax activation.

We have recently shown that PGE₂ microinjection induced a Bax-dependent cell death in human glioma primary cultures⁸. The microinjection of PGE₂ into the cytoplasm of a rat fibroblast cell line (i.e. Rat-1) induced a substantial and rapid cell death (i.e. more than 40% cell death within 6 h) (Fig. S1). This result implies that the cell death inducing activity of PGE₂ is not restricted to malignant cells, to the nervous system or to human cell lines and thus could be a general feature during apoptosis. To address this question, we have analyzed the impact of cytoplasmic microinjections of PGE₂-related prostaglandins on the induction of apoptosis (Fig. S2). In vivo, PGE₂ is rapidly transformed by a non-enzymatic dehydration into PGA₂ or is metabolized into an inactive form (i.e. 15-keto prostaglandin E₂, 15-keto PGE₂) by 15-hydroxy-prostaglandin dehydrogenase (15-PGDH)¹². As shown in Fig. 1A, the microinjection into an established glioma cell line (U251) of PGA₂ and 15-keto PGE₂ induced cell death, as efficiently as PGE₂, while the microinjection of U46619 (the non metabolic version of PGH₂, the precursor of PGE₂), or that of the closely related molecule latanoprost lactone diol did not affect cell viability. These results suggest that a “non-active” physiological form of PGE₂ (i.e. 15-keto PGE₂) is still capable of activating apoptosis but not its precursor (i.e. PGH₂). Of note, PGD₂, the other major metabolite of PGH₂ in the central nervous system, did not induce cell death in glioma cells, as previously described⁸. Consistent with the microinjection experiments, neither PGH₂ nor PGD₂ and its metabolite PGJ₂ resulted in the mitochondrial relocalization of Bax in a cell free assay while PGE₂ and PGA₂ (Fig. 1B) and 15-keto PGE₂ (data not shown) triggered Bax translocation to the organelle. PGE₂ did not trigger the release of cytochrome c from mitochondria unless Bax is present (Fig. S3).

To determine the binding sites of PGE₂ on Bax, we used a linear peptide scan we previously described¹³. In this assay, the whole sequence of Bax was decomposed into 12-mer peptides (overlapping by 10 residues) covalently bound to a nitrocellulose membrane, which was incubated with biotinylated PGE₂. Fig. 1C shows that biotinylated PGE₂ was able to interact with several α -helices (H α) in Bax but showed a maximal binding to the H α 2 (BH3 domain), the loop formed between the H α 5 and H α 6 and last H α present in its C-terminal end, H α 9. These domains have been shown to play an important role in the interaction of Bax with mitochondrial membranes and/or Bax oligomerization and interaction with Bcl-Xl¹⁴⁻¹⁷. Bax-induced release of mitochondrial cytochrome c is often related to its oligomerization through the BH3 domain and the exposure of the H α present in its C-terminal end³. However, since PGE₂ did not trigger Bax oligomerization (Fig. 1D) and the deletion of the H α 9 of Bax did not impair its ability to interact with PGE₂ (Fig. 1E), we conclude that these domains are not fundamental for PGE₂-induced activation of Bax.

PGE₂ binds the Ha5-a6 hairpin of Bax and the resulting activation of Bax requires the cysteine 126 residue.

Given the widely reported affinity of cyclopentenone prostaglandins for cysteine residues^{18,19}, we questioned the ability of cysteine reagents, such as the competitive inhibitor N-acetylcysteine (NAC) or the cysteine blocker iodoacetamide, to interfere with the PGE₂-Bax interaction. As shown in Fig. 2A, both reagents inhibit the interaction suggesting that the free access to the regions including one or both cysteine residues of Bax was crucial for the physical interaction with PGE₂. Consistent with this observation, the addition of NAC to the culture medium of the glioma cell line (LN18) prior to PGE₂ microinjection protected against cell death (Fig. 2B). We thus performed site-directed mutagenesis experiments to assign the role of both cysteine residues in Bax-to-PGE₂ binding. Bax deficient DU-145 cell are not sensitive to microinjected PGE₂-induced cell death (Fig. 2C). The transfection of wild-type or C62S-mutated Bax rendered these cells sensitive to microinjected PGE₂-induced cell death, while that of C126S-mutated and the double-mutated Bax (C126S and CCSS respectively) inhibited the sensitivity toward PGE₂ (Fig. 2C). Of note, cells transfected with any of the mutants were still responsive to apoptotic inducers staurosporine and UV-B, although the C126S mutation provoked a non-significant decrease in the maximal cell death (Fig. S4). Since cysteine-mutants of Bax were still able to interact with PGE₂ (Fig. S5), we conclude that other residues close to C126 also may be involved in the interaction and thus that this cysteine residue is essentially involved in the subsequent activation of Bax induced by PGE₂ rather than its binding. This was further substantiated by the fact that the ability of PGE₂ to induce a change in the conformation of Bax was suppressed in the C126S mutant (Fig. 3B, lane 0).

Mass spectrometry showed no covalent binding between Bax and PGE₂, or its derivative PGA₂ (data not shown). We performed a molecular modelling study of a non-covalent binding between the known structure of Bax and PGE₂ (Fig. 2D). To explore the PGE₂ binding mode in Bax, we first examined the solvent accessible surface area of the protein structure. A narrow hydrophobic pocket is present in Bax in close proximity to C126. This region consists of the C-terminal part of H α 5, the N-terminal region of H α 6 and the loop between H α 3 and H α 4 (Fig. 2D). PGE₂ was then subjected to docking using an H-bond constraint approach with the probable target C126 thiol group. The most stable docking model is shown in Fig. 2D. The carboxylate group of the α -chain in PGE₂ is involved in salt bridges with the terminal amino groups of K119 and K123 in Bax H α 5. Moreover, the ω -chain in PGE₂ was incorporated into the hydrophobic pocket, composed mainly of residues P88, L120, A124, L132 and I136 in Bax and could stabilize the molecule through hydrophobic interactions. Interestingly, this putative interaction domain of Bax with PGE₂ appears to be different from that ascribed to the BH3-only pro-apoptotic

protein Bim²⁰. Of note, the most stable docking models obtained with PGE₂, PGA₂ and 15-keto PGE₂ are very similar, which is consistent with their common activating ability (Fig. S6) and with the identical position of the keto group on the cycle (Fig. S2).

PGD₂ inhibits the interaction between PGE₂ and Bax, and the subsequent induction of cell death.

Prostaglandins from the E and D series have been widely reported to play opposite roles in their various fields of activities²¹⁻²³. We thus challenged the impact of PGD₂ on the PGE₂-induced Bax activation. PGD₂ inhibited the interaction between PGE₂ and Bax (Fig. 3A) and counteracted PGE₂ ability to activate Bax (Fig. 3B) in cell-free assays. The co-incubation of Bax and PGE₂ resulted in Bax activation and its subsequent translocation to the MOM in isolated mitochondria (Fig. 1B). The addition of increasing concentrations of PGD₂ precluded this relocalization as well as the consecutive MOMP (Fig. 3C). Similarly, the co-injection of PGD₂ and PGE₂ into glioma cells alleviated the death-inducing ability of PGE₂ (Fig. 3D). This was substantiated with the observation that the activation of Bax by microinjected PGE₂ into GBM cells, viewed by the labelling with the 6A7 antibody and relocalization to mitochondria (Fig. 3E left), was totally abolished by equimolar concentrations of microinjected PGD₂ (Fig. 3E, right). We designed a competitive assay in which biotinylated-PGE₂ was incubated with a Bax pepscan membrane in the presence of equimolar concentration of unlabelled PGD₂ (Fig. 3F). The peptide scan experiment revealed that the maximal inhibition of PGE₂ binding to Bax elicited by PGD₂ was localized to two regions in Bax, namely H α 2 and H α 5 (including the proximal H α 6), which contain the two cysteine residues of Bax, C62 and C126 (Fig. 3F). This is in agreement with the molecular modelling of the interaction between PGE₂ and Bax we hypothesized earlier.

PGE₂ is associated with cell death and PGD₂ with cell survival.

We have previously shown that over-expression of mPGES-1, the enzyme responsible for PGE₂ synthesis downstream of COX-2, sensitized GBM cells to cell death and that its inhibition decreased the apoptotic threshold⁸. Other groups, nevertheless, have reported an anti-apoptotic role for COX-2 assuming that this effect was mediated solely through the production of PGE₂. Given our previous results, we hypothesized that the anti-apoptotic effect of COX-2 reported in the literature might be linked to PGD₂ production by the other enzyme downstream of COX-2 in GBM cells, L-PGDS (lipocalin-like PGD₂ synthase). To support our contention, we selectively down-regulated mPGES-1, COX-2 or L-PGDS expression in GBM cells. Expression knock-down experiments were performed using two different shRNA sequences for each enzyme, which were efficiently

down-regulated the expression of these enzymes (Fig. S7). As shown in Fig. 4A, the down-regulation of mPGES-1 inhibited the apoptosis induced by staurosporine in glioma cells, whereas that of COX-2 enhanced it. This result supported an opposite role for these enzymes during apoptosis. The decreased expression of L-PGDS, similar to COX-2, gave an increase in cell death (Fig. 4A). We observed that the inhibition of one of the enzymes influenced the expression of the others in a rather complex manner (data not shown). However, we found that, in all cases, apoptosis was correlated to the mPGES-1/COX-2 ratio as the higher the ratio, higher the cell death (Fig. 4A). The same correlation could be made between the mPGES-1/COX-2 ratio and the DEVDase activity in the treated cells, confirming that the cell death observed was apoptosis (Fig. S8A).

Then, we studied the effect of PGD₂ on the susceptibility of glioma cells in the induction of apoptosis by various agents. PGD₂ microinjection into glioma cells significantly inhibited cell death induced by staurosporine or UV-B irradiation (Fig. 4B), whereas the pharmacological inhibition of L-PGDS by SeCl₄ sensitized the cells to staurosporine-induced apoptosis (Fig. 4C and Fig. S8B) as well as UV-induced apoptosis (data not shown). We then asked whether PGE₂ was implicated in the cell death induced by cytotoxic agents, namely etoposide or staurosporine in different cell lines. To be able to measure intracellular PGE₂ in treated cells, we defined experimental conditions under which apoptosis was induced by etoposide or staurosporine in four different cell lines, representing models of cancer cells from various tissues (i.e. HeLa, SH-SY5Y, LN18, HCT116). As shown in Fig. 4D, an increase in PGE₂ (intracellular and/or secreted) was observed after 4 hours and before any morphological signs of apoptosis under all conditions and in all cancer cells tested. Of note, the PGE₂ concentration observed in the early step of apoptosis was similar to that used to activate Bax in the cell-free assay (data not shown). The increase in PGE₂ was also observed in the Bax-deficient cell line (Fig. S9), a cell line highly resistant to apoptosis¹⁶, suggesting that it is not a consequence of cell death. These results show that the increase in PGE₂ synthesis is a rapid process that occurs during the early stages of apoptosis.

PGE₂ and Bid activate Bax by independent pathways that can cooperate

Among the different BH3-only proteins, Bid has been reported to induce the direct activation of Bax²⁴. Bid can activate Bax through either its truncated form tBid or merely its BH3^{Bid}, see for example²⁵). We thus questioned whether PGE₂ and BH3^{Bid} were redundant in Bax activation. We first observed that the microinjection of sub-lethal amounts of PGE₂ or BH3^{Bid} induced no cell death within 5 hours, whereas the co-injection of sub-lethal doses of both compounds resulted in a massive cell death within the same time frame (Fig.

5A, left), indicating cooperation between PGE₂ and BH3^{Bid} in apoptosis induction. The cell death induced was inhibited by the addition of equimolar (to PGE₂) amount of PGD₂ (Fig. 5A left, black squares). Of note, PGD₂ was unable to inhibit cell death induced by a lethal concentration of BH3^{Bid} even at very high concentrations (Fig. 5A, right). Similarly, PGE₂ induced apoptosis whether Bid expression was repressed or not (Fig. 5B). As shown in Fig. 5C, in a more physiological situation, the mPGES-1 or L-PGDS over-expression did not significantly affect the sensitivity of glioma cells to TRAIL, an apoptotic inducer targeting the activation of Bax through Bid¹⁶. Conversely, the knock-down of Bid by shRNA rendered the cells partly resistant to the staurosporine/SeCl₄-induced apoptosis whereas that of Bax totally abrogated cell death (Fig. 5D). Thus, pharmacological inhibition of PGD₂ production reversed the partial resistance to apoptosis in shBid-treated cells, but had no effect on the shBax-treated cells, suggesting a functional cooperation between PGE₂ and Bid during staurosporine-induced apoptosis (Fig. 5D).

All together these data show that PGE₂ and Bid share the same function, namely Bax activation, but do not compete with each other and can instead cooperate in this function. This is corroborated by the fact that the binding sites of Bax implicated in their respective activating functions are distinct.

DISCUSSION

Prostanoids possess both pro- and anti-apoptotic functions that seem to depend on the differentiation stage and tissue origin of the treated cells²⁶. To give a few examples, PGE₂, the main prostaglandin, has been reported to inhibit apoptosis in colon cancer¹⁰ and in radiation-treated epithelial cells¹¹ through mechanisms involving the proteins of the Bcl-2 family. On the other hands, PGE₂ has been shown to induce apoptosis in human lymphocytes and in fibroblasts through multiple pathways^{9,27}. It is also well-established that the increase in the synthesis of PGE₂ during hypoxia/ischemic shock exacerbates stroke injury²⁸.

Several enzymes have been shown to be involved in prostanoids synthesis downstream of the arachidonic acid²⁶. These enzymes have different cellular and tissue localizations and play different roles in various pathologies²⁹. mPGES-1 is a critical factor during post-ischemic neurological dysfunctions as its genetic knock-out in mice significantly reduces the size of the infarct and the extent of caspase-dependent apoptosis³⁰. mPGES-1, the terminal enzyme in the synthesis of PGE₂³¹, is encoded by a gene named pig12, which has been shown to be involved in early p53-induced apoptosis³². We have shown that mPGES-1 overexpression in human high grade glioma was correlated to a longer survival⁸, even if mPGES-1 overexpression has often been observed in tumours compared to healthy tissues, notably in brain tumours³³. The same discrepancy can be observed in cell

culture. mPGES-1 could namely promote the growth of a human glioma cell line³⁴ through PGE₂ extracellular pathway whereas we showed that it was a pro-apoptotic enzyme in human brain tumour primary cultures through the activation of Bax by intracellular PGE₂ and subsequent caspase activity⁸. We confirmed that this discrepancy was linked to PGE₂ localisation since the pharmacological sequestration of PGE₂ was sufficient to induce apoptosis⁸.

Here we show that this activating property of PGE₂ towards Bax is closely linked to the PGE₂ structure, since only the structurally related prostaglandin A₂ and 15-keto PGE₂, among all the challenged prostaglandins, retain this activating ability. We also report that the region in Bax encompassing C126 is the binding site of PGE₂. However, since the Bax C126S point mutation does not abrogate the interaction between Bax and PGE₂ and since no covalent binding could be demonstrated between PGE₂ or PGA₂ and C126, the entire interacting domain of PGE₂ in Bax has still to be determined. Despite the fact that Bax^{C126S} mutant is not a loss-of-function mutant, it is not able to respond to PGE₂ as revealed by the conformation-specific immunoprecipitation with the 6A7 anti-Bax antibody. Our interpretation of these observations is that C126 is not the only residue implicated in PGE₂ binding to Bax but it is crucial to the subsequent activation of Bax induced by PGE₂. We show that PGE₂ increases during the onset of apoptosis in various cell lines using different inducers, to a level consistent with the conditions used to activate Bax in cell-free assays (i.e. micromolar range). In contrast with the pro-apoptotic activity of PGE₂, we show that a closely related PGH₂-derived prostaglandin, namely PGD₂, is a functional antagonist of PGE₂ activity towards Bax. This might be surprising insofar as PGD₂ and its final metabolite 15deoxy Δ^{12-14} PGJ₂ are implicated in the onset of apoptosis, through the activation of DP receptors or of PPAR γ ²⁶ or of other targets¹⁹. Payne *et al.*³⁵ also reported that L-PGDS loss was observed during transition from low grade to higher grade astrocytomas and was correlated to a poorer survival. However, our results demonstrate that PGD₂ is not an absolute antiapoptotic compound, but rather a competitive inhibitor of PGE₂ in Bax activation, since it does not preclude the Bid-induced activation of Bax. Besides, we only focused on the role played by PGD₂ on Bax activation and we did not explore the overall targets of PGD₂ and 15deoxy Δ^{12-14} PGJ₂. The observation that L-PGDS inhibition induces Bax down-regulation³⁶ might be related to the important role played by PGD₂/PGE₂ ratio on cell survival. We have shown that silencing of L-PGDS increased the expression of PGES (Fig.4A), therefore the decrease of Bax could ensure cell survival of L-PGDS deficient chondrocytes.

As described in Fig. 6, we propose that apoptosis is under the control of the balance between intracellular concentrations of PGE₂ and PGD₂ and that of anti-apoptotic extracellular PGE₂. The liberation of this prostaglandin by dying cells would thus participate in the paracrine protection of adjacent cells or induce cell

death depending on the nature of the tissue (Fig. 6). This “double-agent” mechanism could partly account for the equivocal and sometimes contradictory effects of COX-2 on apoptosis described in tumour cells ²⁶. In addition, the release of PGE₂ by dying cells could also influence tumour environment and play a role in the suppression of the immune system as suggested by Kokoglu et al. ³⁷.

Small bioactive lipids such as prostaglandins thus appear as new potential candidates for the regulation of Bax activation. We show that PGE₂ and Bid do not compete with each other but can rather cooperate in Bax activation and cell death induction. Bax activation could thus be considered as the integrative result of various changes in the intracellular environment occurring in the very early stages of apoptosis, including BH3-only protein activation or release, anti-apoptotic proteins oversupply, slight local physicochemical modifications and wavering in the PGE₂/PGD₂ balance or in other lipid compounds. All of these environmental factors create a cellular context in which the most probable conformation of Bax according to thermodynamic considerations is either the inactive, globular conformation, or the active, outstretched conformation.

Our results definitely suggest that Bax activation should not only be regarded as a strict protein event but should be integrated in the overall cellular context, including bioactive lipids.

MATERIALS AND METHODS

Materials.

Antibodies were purchased from indicated companies: Bax 2D2 (amino-acids 3-16, Sigma, #B8554), Bax 6A7 (amino-acids 12-24, ABCAM, #ab5714), F₁-ATPase (Molecular Probes, #A-21350), cytochrome c (Chemicon, #MAB1501R). ³⁵S-Met (Amersham, France) labelled proteins were synthesized from cDNAs using the TNT-coupled transcription/translation system from Promega (France). Prostaglandins were purchased from Cayman Chemical (Interchim, France). Unless mentioned, chemical products and reagents were obtained from Sigma (France).

Immunoblots were quantified using the ImageJ software (NIH, USA).

Every experiment was repeated at least 3 independent times. Statistical analyses were performed using the GraphPad software (San Diego, CA 92130 USA) (unpaired two-tailed t-test, unless mentioned otherwise).

Unless mentioned, data shown represent mean values +/-SEM.

Cell free assay for Bax insertion.

Mitochondria were prepared from normal rat liver and the cell-free association of Bax with the mitochondria was performed as described previously¹⁶. ³⁵S-Met-labeled Bax (2 fmol), PGE₂ and isolated rat liver mitochondria (100 µg mitochondrial proteins) were incubated in a standard import buffer for 1h at 30°C. Mitochondria were pelleted and samples were separated by SDS-PAGE. Gels were fixed and dried, and ³⁵S-labeled Bax was detected on a PhosphorImager scanner. Image quantification was done using the ImageJ software (NIH, USA).

Peptide scan.

The experiments were performed as described in Bellot et al. (2007). Briefly, the peptide membranes were prepared on a MultiPep automated peptide synthesizer (Intavis AG, Germany). Bax sequence was divided into 12-mer peptides with a two amino-acid frame shift. After the side-chain deprotection of the peptides, the membrane was saturated by 5% BSA-PBS for 2 h at room temperature. Biotinylated prostaglandins (2 µM in PBS) were incubated overnight at 4°C. Extravidin-peroxidase was then incubated and the binding was assessed by ECL reaction. The binding intensities of prostaglandins for Bax-derived peptides were determined by quantification using the ImageJ software and converted to peptide-specific normalized units. Every experiment was repeated on four different membranes. The value attributed to each amino acid was the mean value of the spot intensities corresponding to the peptides covering this amino acid.

PGE₂-AChE Bax interaction.

GBM cell lysates in excess (25 µg protein/condition) were incubated in a BSA-saturated 96-well plate, previously coated with the 2D2 anti-Bax antibody. After 3 washes with 0.1% Tween 20-PBS, prostaglandins were incubated at the indicated concentration with acetylcholine-coupled PGE₂ (PGE₂-AChE) added. The amount of PGE₂-AChE bound to Bax was measured by the coloration of Ellman's reagent (405nm). When indicated, cell lysates were treated by NAC or iodoacetamide for 1 h at 37°C, then diluted 1:24 in 1% BSA-PBS before binding to the antibody-coated wells.

Microinjection experiments.

Microinjection was performed as described by Lalier et al.⁸. Prostaglandins were co-injected with a dextran coupled to a fluorochrome (Oregon Green, Molecular Probes). The percentage of fluorescent cells exhibiting morphological apoptotic features was evaluated every hour following microinjection using an inverted fluorescent microscope (DMIRE2, Leica France). Apoptosis was induced 1 h after injection, when indicated.

Cross-linking experiments.

Bax-containing GBM cytosolic extracts were incubated with 5 μ M PGE₂ or with Triton X100 (TX100) as a positive control for 1 h at 30°C. Cross-linking was then performed as described earlier¹⁶ and Bax was revealed by western blot.

Molecular modelling.

Molecular modelling studies were performed using SYBYL software version 7.2 (Tripos Associates Inc., St. Louis, U.S.A.) running on a Silicon Graphics Octane2 workstation. The geometry of PGE₂ was subsequently optimized using the Tripos force field including the electrostatic term calculated from Gasteiger and Hückel atomic charges. Powell's method available in Maximin2 procedure was used for energy minimization until the gradient value was smaller than 0.001 kcal/mol. Å. The NMR structure of human pro-apoptotic protein Bax was obtained from the RCSB Protein Data Bank (PDB code: 1F16) (<http://www.rcsb.org/pdb/>)¹⁷. The protein solvent accessible area with lipophilic potential was generated using the MOLCAD module in SYBYL 7.2. The program GOLD (version 3.1; CCDC, Cambridge, United Kingdom), an automated docking program (Jones et al., 1997), was used to dock PGE₂ with its target protein. For the docking calculations, all residues of the protein were fixed while PGE₂ was flexible. An initial H-bond constraint from the C-9 carbonyl group of PGE₂ to the thiol (-SH) of C126 was applied. The most stable docking model was selected according to the best scored conformation predicted by the GoldScore³⁸ and X-Score³⁹ scoring functions. The complex was energy-minimized using Powell's method available in Maximin2 procedure with the MMFF94 force field and a dielectric constant of 4.0 until the gradient value reached 0.1-kcal/mol. Å.

Confocal analysis.

For confocal analysis, the cells were fixed with 4% paraformaldehyde-0.19% picric acid-PBS for 30 min at room temperature. After washing with PBS, the cells were permeabilized with 0.1% SDS-PBS for 10 min at room temperature, then saturated by 5% gelatin-PBS for 15 min at room temperature. The cells were incubated with indicated antibodies for 1 h at room temperature in 1% gelatin-PBS. The secondary antibodies used were coupled to a fluorochrome (Alexa 568 or 633, Molecular Probes). Images were collected on a Leica TCS NT microscope with a 63x1.3 NA Fluotar objective (Leica, France).

Knock-down experiments.

Enzyme knock-down was assessed using two different shRNA sequences (Fig. S6) and its efficiencies checked by immunoblots and/or by measuring the synthesis of PGE₂ or PGD₂. Annealed double-strand oligonucleotides were cloned into a plasmid (pSilencer 2.1-U6 hygromycin vector, Ambion, #AM5760) and transfected into GBM cells. Cells were further cultured with hygromycin 125 ng/ml. Apoptosis was induced by staurosporine

treatment (150 nM, 12 h). Cell death was assessed by Trypan blue exclusion; proteins were extracted and DEVDase activity was measured as described previously¹⁶.

For Bid and Bax knock-down experiment, commercial lentiviral particles were purchased from Sigma (France). Particles including a shRNA targeting GFP were used as a negative control. Infection was realized with a MOI of 10 and cells were further cultured with puromycin 1 µg/ml. Four different shRNA were tested for each target protein and the most efficient was used for cell death experiments.

PGE₂ /PGD₂ measurements.

The amount of PGE₂ was measured in cells lysates and culture medium using the PGE₂ Biotrak EIA System (Amersham Biosciences) according to the manufacturer instructions. 10⁴ cells were plated in 96 well plates the day before. Apoptotic treatment was adjusted to each cell line as follows: a range was realized to establish the amount of inducer triggering cell death in 24 h, then cells were treated for 18 h to avoid cell disruption (assessed by Trypan blue exclusion) thereby enabling the measurement of intracellular PGE₂. Treatments applied were 20 µM etoposide for HCT116, HeLa, LN18 cells and 5µM etoposide for SH-SY5Y cells, 50 nM staurosporine for all the four cell lines. Plates were read at 450 nm. PGE₂ concentrations were normalized to the protein concentration of the samples. Extracellular PGD₂ was measured in serum-free medium using the Prostaglandin D₂ EIA kit (Cayman Chemical Company #512021) according to the manufacturer.

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Author's contributions

Lisenn Lalier performed the in vitro experiments and drafted the manuscript. Pierre Francois Cartron performed the acellular studies, Christophe Olivier performed some of the microinjection experiments, Gwenola Bougras carried out the phenotyping characterization of the cell lines and Lisa Oliver the immunohistochemical characterization and confocal studies. Cedric Logé and Jean-Michel Robert participated in the molecular modelling of PGE₂/Bax interaction. Francois M. Vallette conceived of the study and wrote the final version of the manuscript.

Conflict of interest:

The authors declare that they have no conflict of interest

FIGURES LEGENDS

Fig. 1: PGE₂-like prostaglandins specifically activate Bax. (A) Prostaglandins or analogs were microinjected into GBM cells (20 μM injected, corresponding to a 2 μM intracellular raise) and cell death in the injected cells was assessed every hour. (B) ³⁵S *in vitro* translated (IVT)-Bax was incubated with indicated prostaglandins and then added to isolated mitochondria. Bax translocation was assessed by the amount of mitochondria-associated Bax. (C) A pepscan experiment was done with biotinylated PGE₂ as described in Methods. The grey bars over the graph show the α helices position of Bax and the black bars show the BH domains. (D) Cytosolic Bax was incubated with the cross-linker alone (con), or EGS plus PGE₂ (5 μM) or 1% TX100. PGE₂ does not induce Bax dimerization. The blot shown is representative from 3 independent experiments. (E) DU-145 Bax-deficient cells were transfected either with Bax α or with the Hα9-deleted form of Bax (BaxΔC). Cell lysates were applied to a 96-well plate coated with an anti-Bax antibody (2D2). PGE₂ was added to the wells with PGE₂-AChE as a tracer.

Fig. 2: Bax C126 residue is necessary to PGE₂-induced activation. (A) The same assay as in Fig. 1D was done with Bax-expressing cell lysates. The assay was performed after lysates incubation with N-acetylcysteine or iodoacetamide (thiol-reagent) when indicated. (B) PGE₂ (20 μM, corresponding to a 2 μM intracellular raise) was microinjected into glioma cells, with or without previous treatment by 10mM NAC. Cell death was assessed as in Fig. 1A. (C) Bax mutants were transfected into a Bax-deficient cell line (DU-145). PGE₂ microinjection was then performed and cell death was assessed every hour. (D) The interaction between PGE₂ and Bax was illustrated in the neighbourhood of C126. The most stable docking model is shown (upper panel). The ionic or hydrogen bonds are indicated as yellow dotted lines. MOLCAD solvent accessible surface area (program Sybyl 7.2) of the NMR structure of protein Bax, with the lipophilic potential mapped onto the surface (lower panel). The colour codes indicate blue areas for hydrophilic, brown for lipophilic, and green for neutral.

Fig. 3: PGD₂ inhibits PGE₂-Bax interaction and subsequent Bax activation. (A) The same enzyme immunoassay as described in Fig. 1D between fixed Bax and AChE-PGE₂ was realized in the presence of raising amount of PGD₂. (B) ³⁵S-IVT-Bax or Bax^{C126S} was incubated with indicated prostaglandins and immunoprecipitated with the 6A7 antibody, which solely recognizes the activated conformation of Bax. IVT Bax

was detected by autoradiography after SDS-PAGE. Equimolar amount of PGD₂ inhibits the activation of Bax induced by PGE₂. The 2D2 antibody was used as a positive control for Bax immunoprecipitation and Triton X100 (TX100) was used as a positive control for Bax activation. (C) ³⁵S IVT-Bax was incubated with indicated prostaglandins and added to isolated mitochondria as described in Fig. 1B. The subsequent mitochondrial release of cytochrome c was assessed by western blot for each condition. Input is the total amount of mitochondrial cytochrome c. (D) Glioma cell death was monitored following PGE₂ microinjection. In microinjection experiments, injected solutions are diluted about 1/10 in the cell. The experiment can thus be considered as representative for a 2 μM raise in PGE₂ intracellular concentration. The indicated amount of PGD₂ was co-injected with PGE₂. (E) Microinjected cells were fixed and stained with the 6A7 antibody raised against the active conformation of Bax. The anti-F₁-ATPase antibody was used as a positive control. Cells were observed by confocal microscopy. (F) A pepscan experiment was realized as described in Fig. 1E with biotinylated PGE₂ and non-labelled PGD₂. ECL revealed bound PGE₂ and the percentage of inhibition versus PGE₂ alone was determined.

Fig. 4: PGE₂ is related to cell death and PGD₂ to cell survival in GBM. (A) GBM cells were transfected with a plasmid encoding for a shRNA sequence targeting the indicated enzyme. Apoptosis was induced by staurosporine (150 nM, 12 h), and cell death was measured by Trypan blue exclusion (upper graph). mPGES-1 and COX-2 expression was assessed by western-blot and normalized to actin. The ratio is shown on the lower graph. (B) GBM cells were treated by staurosporine (150 nM) or by UV-B irradiation (30 s exposure) 1 hour after PGD₂ microinjection (20 μM, representative for a 2 μM raise in PGD₂ intracellular concentration). Cell death was assessed among microinjected cells respectively 12 h and 18 h after treatment. (C) Glioma cells were treated simultaneously by 5 or 50 μM SeCl₄ as indicated on the upper graph. The pH of the SeCl₄ solution was checked before treatment. Secreted PGD₂ was measured as described in *Materials and Methods*. Cells were then co-treated by SeCl₄ and staurosporine. Cell death was measured by Trypan blue exclusion after 16 h (lower graph). The graph shown is representative for 3 independent experiments. (D) 4 cell lines were treated by apoptosis inducers as indicated in the *Materials and methods* section (C: control, ETO: etoposide, STS: staurosporine). PGE₂ was measured in cell lysate and supernatant and normalized by corresponding protein concentration. The graphs represent the increase in PGE₂ concentration in treated versus control conditions.

Fig. 5: PGE₂ and Bid are independent activators of Bax. (A) Glioma cells were injected by sub-lethal amounts of PGE₂ and BH3^{Bid} (respectively 5 μM and 20 nM) and by an equimolar amount of PGD₂ (20 μM) (left graph). Alternatively, 100 nM BH3^{Bid} was injected together with a large excess of PGD₂ (150 μM) (right graph). (B) Bid expression was repressed by 4 different shRNA lentiviral particles in glioma cells and PGE₂ was injected as described earlier. The upper graph shows cell death counting 5 h after injection and the lower graph indicates the respective shRNA efficiency revealed by western-blot analysis of Bid (insert). (C) Glioma cells were transfected by a plasmid encoding for mPGES-1 or L-PGDS cDNA. Cells were treated by TRAIL (100 nM, 24 h) and cell death was assessed as described earlier. (D) Bax or Bid expression was repressed in glioma cells by validated shRNA sequences (residual expression ≤10%) and cells were treated by staurosporine 150 nM +/- SeCl₄ 5 μM for 16 h.

Fig. 6: Bax activation is submitted to a double balance, namely the PGE₂/PGD₂ intracellular balance on the one hand and the intracellular/extracellular PGE₂ balance on the other hand. COX-2 product PGH₂ is further metabolized into PGE₂ and PGD₂. PGE₂ is rapidly exported to the extracellular compartment or transformed into PGA₂ and 15-keto PGE₂. PGE₂, PGA₂ and 15-keto PGE₂ all induce Bax activation, as Bid-like BH3-only proteins do. PGD₂ inhibits this activation, despite its final metabolite 15deoxyΔ¹²⁻¹⁴ PGJ₂ (15d-PGJ₂) is also reported to induce apoptosis. When exported in the extracellular compartment, PGE₂ can bind its membrane receptors EP(1-4) and promote long-term survival of cancer cells.

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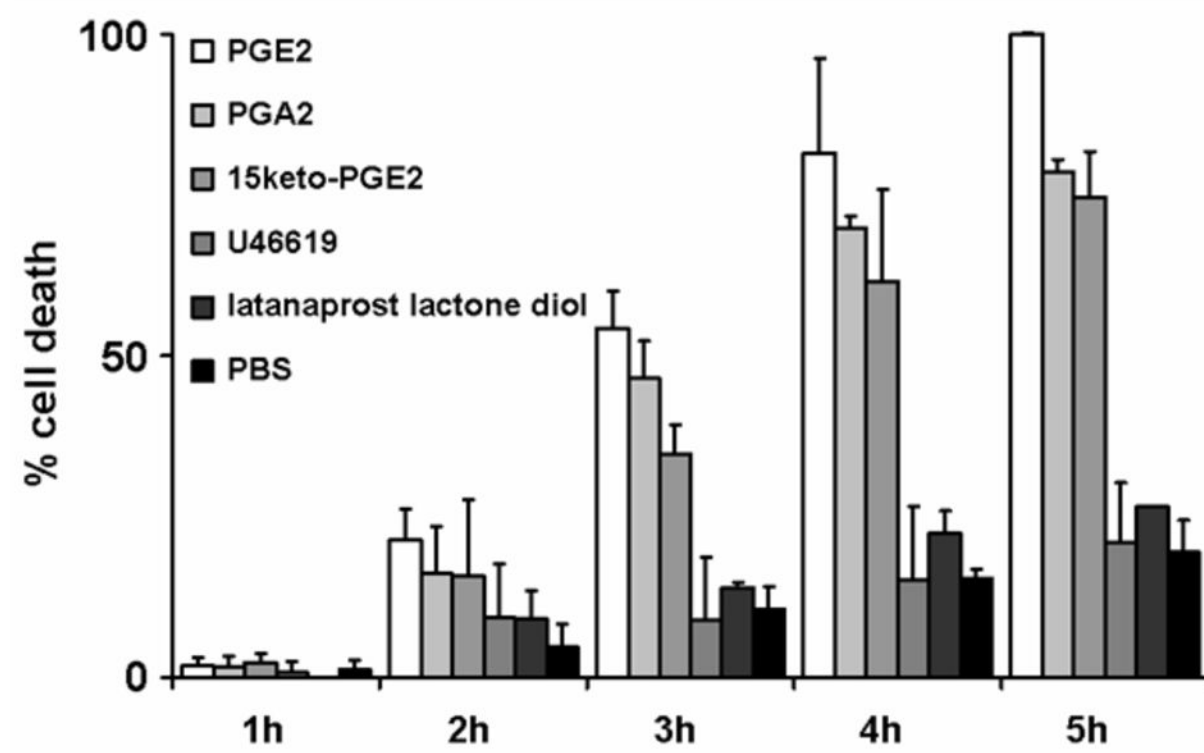
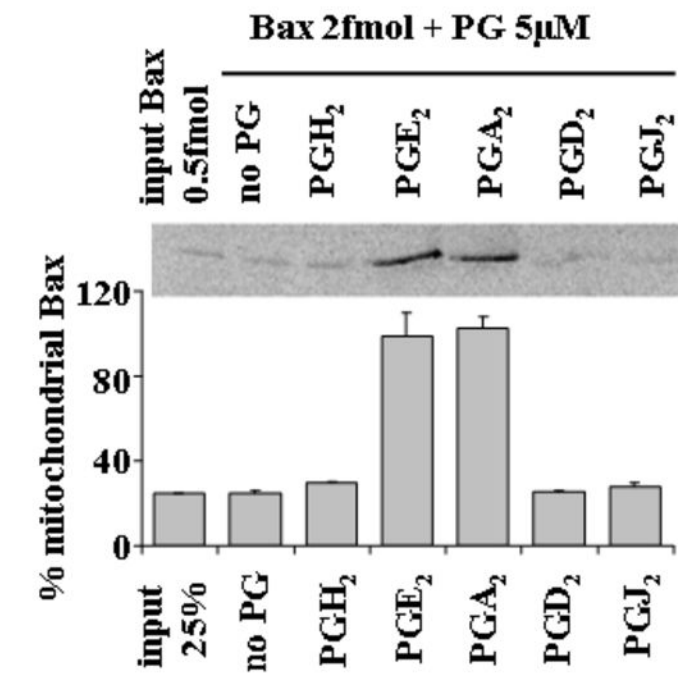
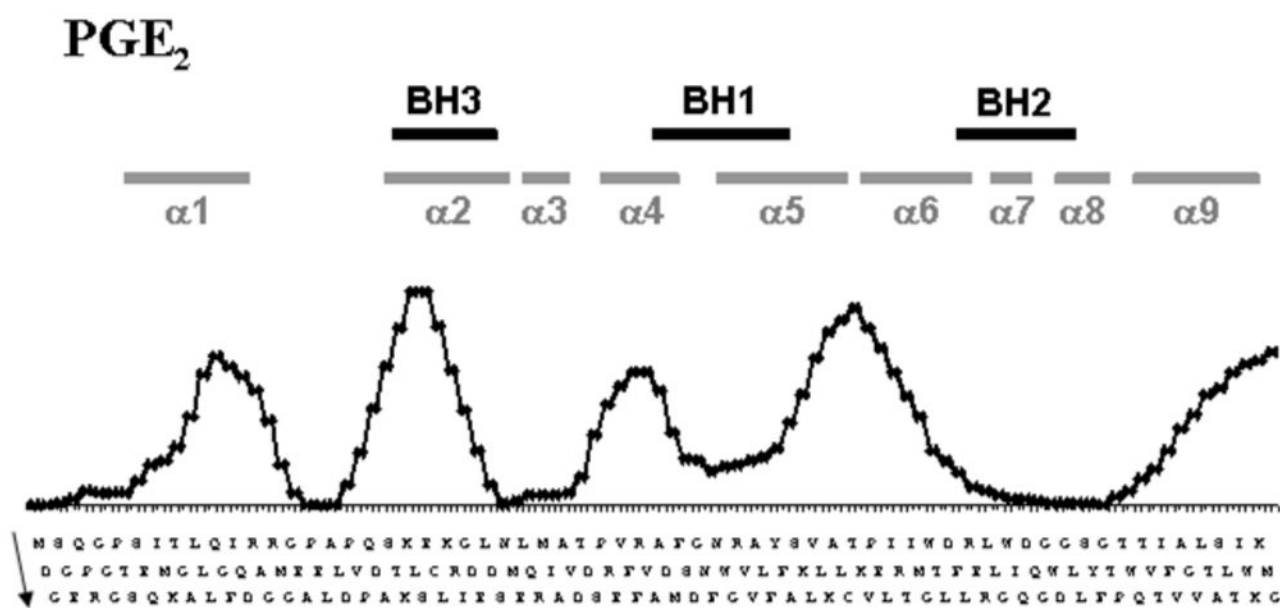
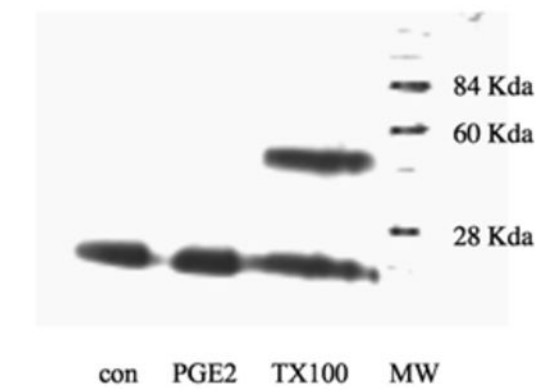
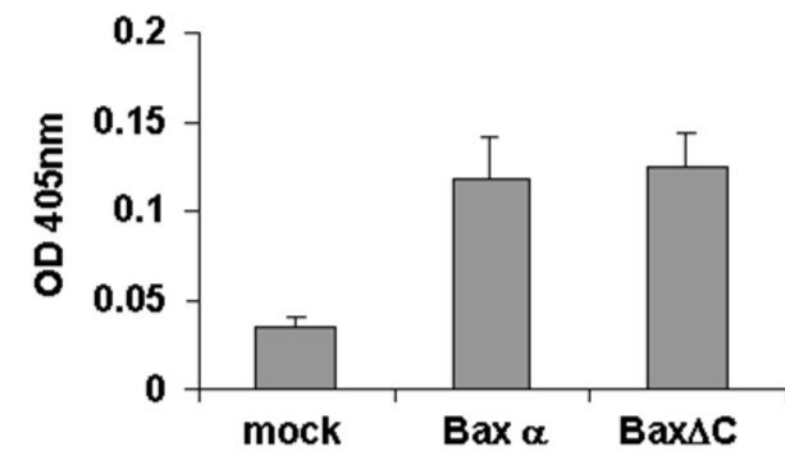
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figure 1

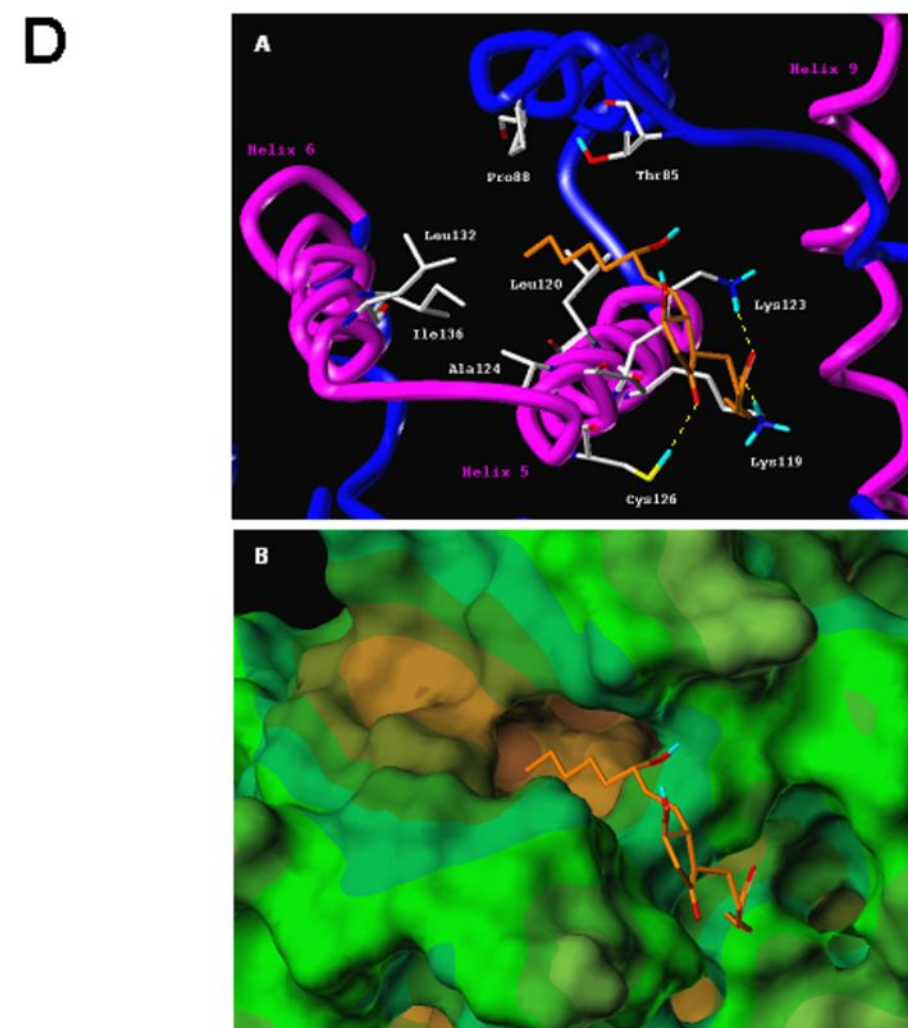
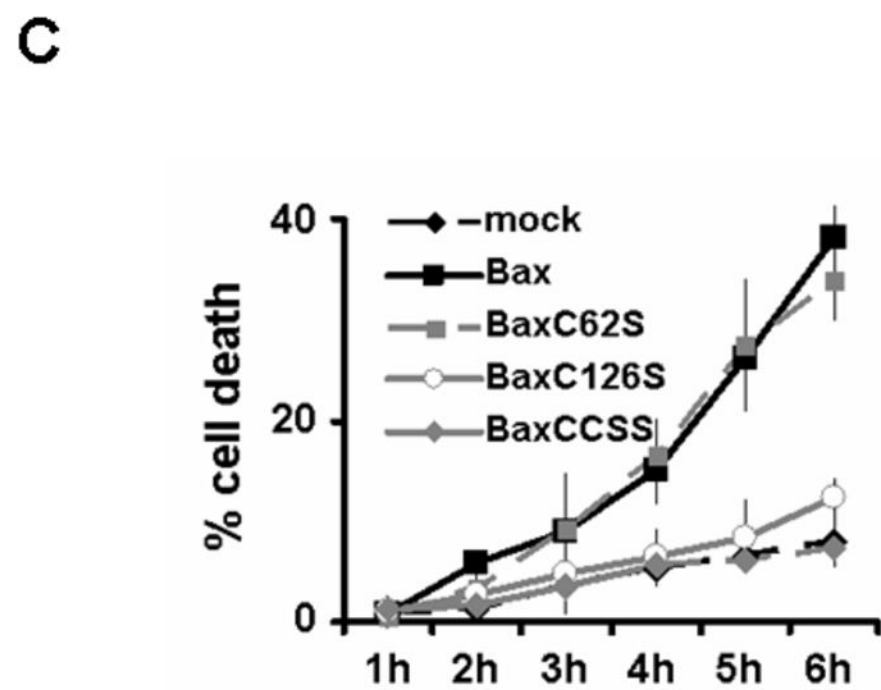
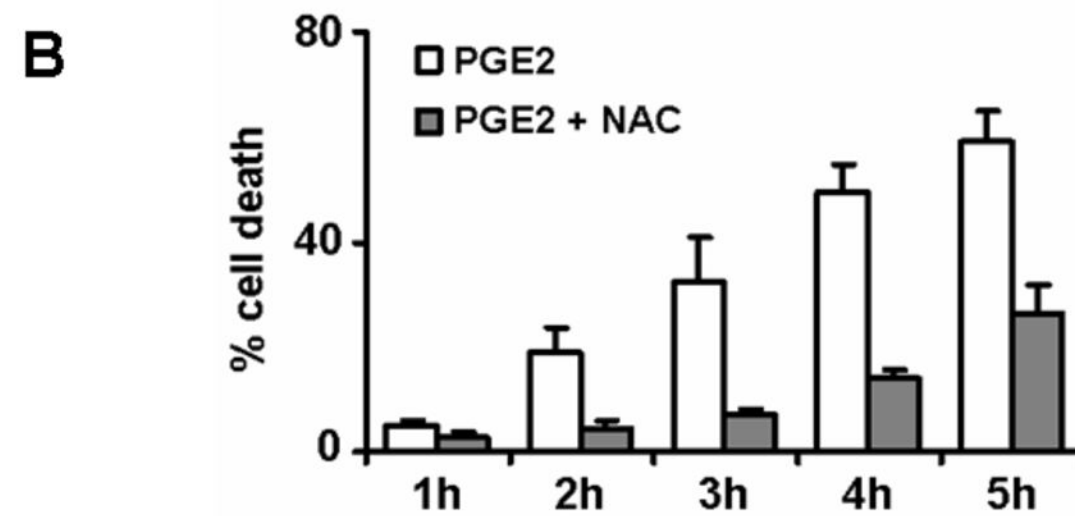
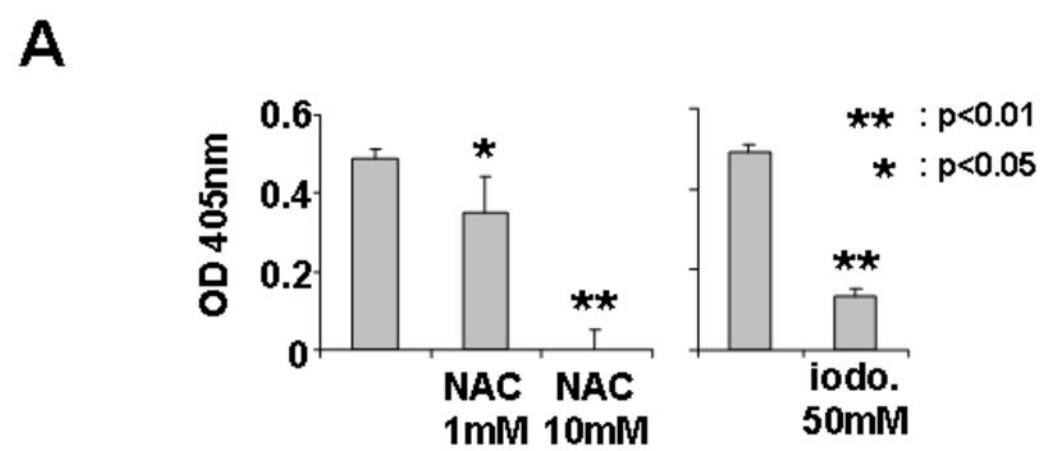


figure 2

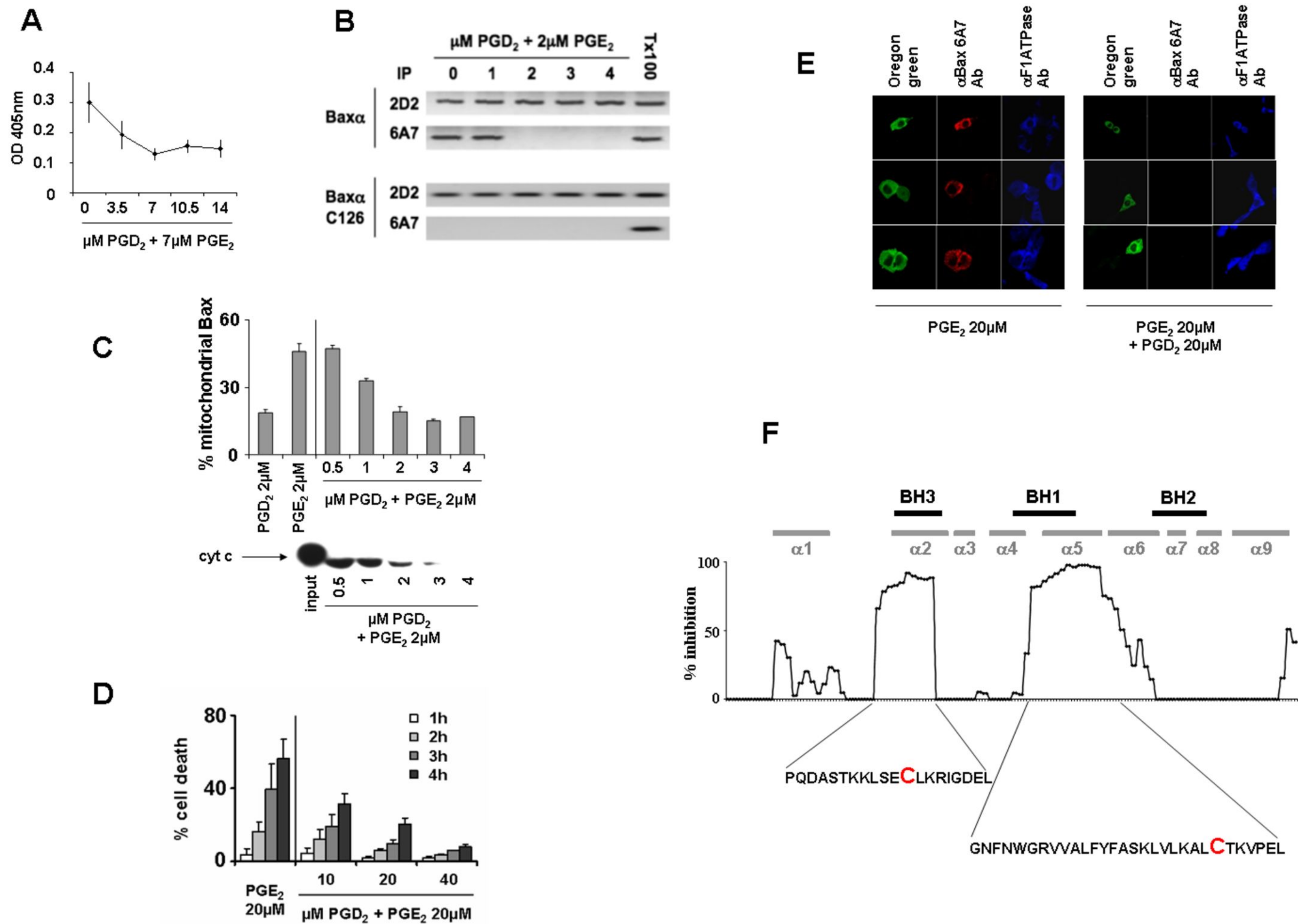


figure 3

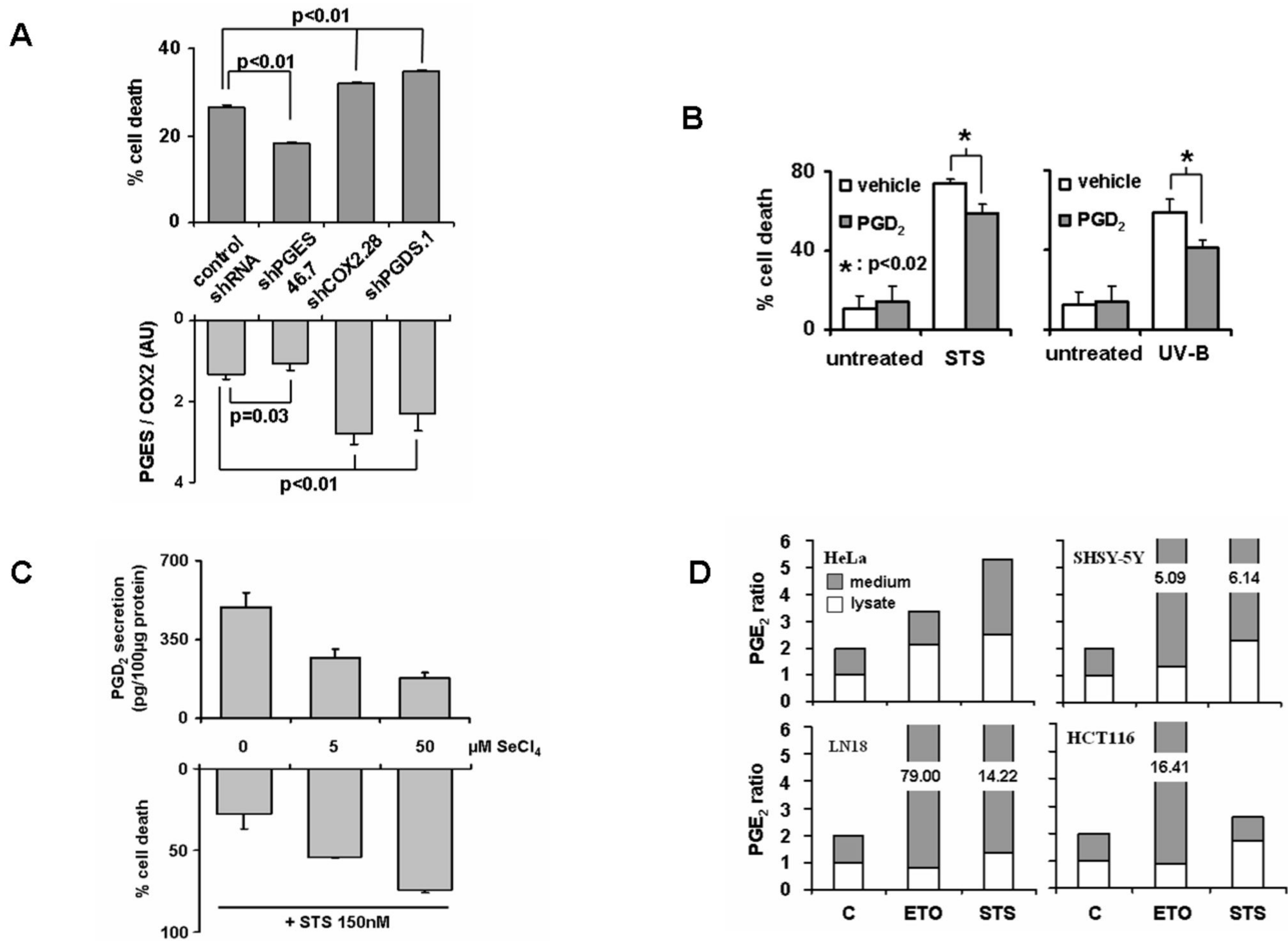


figure 4

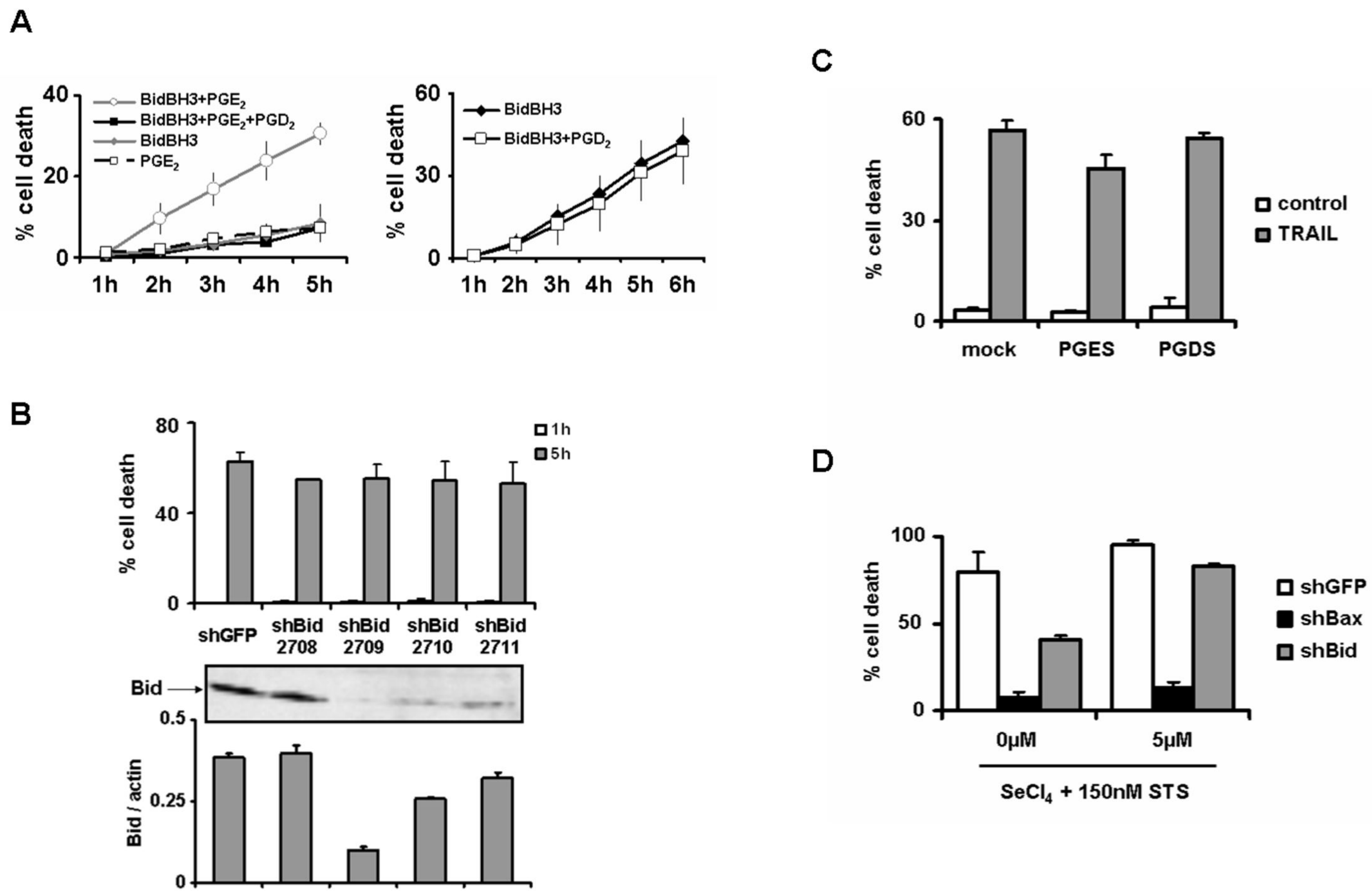


figure 5

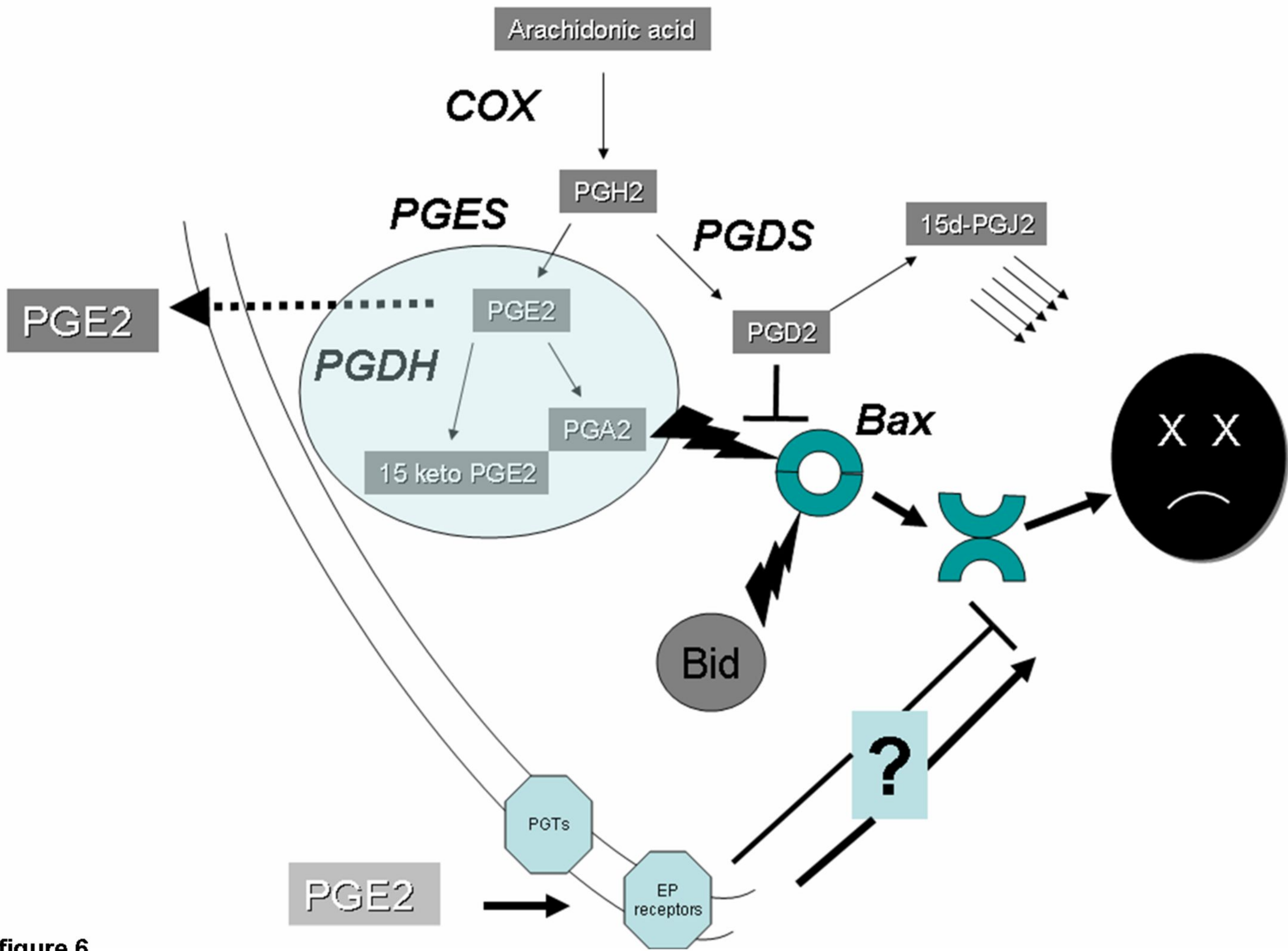


figure 6