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► To cite this version:

Georges Kalouche, Céline Boucher, Annick Coste, Laurent Debussche, Cécile Orsini, et al.. Prostaglandin EP2 receptor signaling protects human trabecular meshwork cells from apoptosis induced by ER stress through down-regulation of p53. *Biochimica et Biophysica Acta - Molecular Cell Research*, 2016, 10.1016/j.bbamcr.2016.06.008 . hal-01338443

HAL Id: hal-01338443

<https://hal.sorbonne-universite.fr/hal-01338443v1>

Submitted on 28 Jun 2016

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Title Page

Prostaglandin EP2 receptor signaling protects human trabecular meshwork cells from apoptosis induced by ER stress through down-regulation of p53

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Abstract

E-prostanoid receptor subtype 2 (EP2) agonists are currently under clinical development as hypotensive agents for the treatment of ocular hypertension. However, the effects of EP2 receptor agonists on trabecular meshwork (TM) alterations leading to primary open-angle glaucoma (POAG) are still unknown. Here, we evaluated whether EP2 receptor activation exhibits protective functions on TM cell death induced by endoplasmic reticulum (ER) stress. We show that the EP2 receptor agonist butaprost protects TM cell death mediated by the ER stress inducer tunicamycin through a cyclic AMP (cAMP)-dependent mechanism, but independent of the classical cAMP sensors, protein kinase A and exchange proteins activated by cAMP. The ER stress-induced intrinsic apoptosis inhibited by the EP2 receptor agonist was correlated with a decreased accumulation of the cellular stress sensor p53. In addition, p53 down-regulation was associated with inhibition of its transcriptional activity, which led to decreased expression of the pro-apoptotic p53-upregulated modulator of apoptosis (PUMA). The stabilization of p53 by nutlin-3a abolished butaprost-mediated cell death protection. In conclusion, we showed that EP2 receptor activation protects against ER stress-dependent mitochondrial apoptosis through down-regulation of p53. The specific inhibition of this pathway could reduce TM alterations observed in POAG patients.

Abbreviations: cAMP, cyclic adenosine monophosphate; EP2, E-prostanoid type 2 receptor; Epac, exchange proteins activated by cAMP; IOP, intraocular pressure; PKA, protein kinase A; PUMA, p53-upregulated mediator of apoptosis; TM, trabecular meshwork

Keywords: EP2 receptor, ER stress, cAMP, p53, PUMA, trabecular meshwork cells

1. Introduction

Primary open-angle glaucoma (POAG) is the leading cause of irreversible blindness and affects more than 44 million people worldwide [1]. Elevation of intraocular pressure (IOP), or ocular hypertension (OHT), is the main risk factor for glaucoma and is associated with a dysfunction of the trabecular meshwork (TM), a tissue which regulates IOP by opposing resistance to the aqueous humor outflow. The TM of POAG patients is characterized by a decreased TM cell number [2,3], an increased cellular senescence [4] and an accumulation of extracellular matrix (ECM) [5]. Accelerated TM cell loss might contribute to ECM remodeling and the collapse of the trabecular structure, therefore emphasizing the importance of TM cell survival in the pathophysiology of glaucoma.

Recent evidence suggests that endoplasmic reticulum (ER) stress could be a causal mechanism leading to the development of glaucoma by triggering TM cell death and a subsequent rise in IOP [6]. Indeed, mutation of the gene coding for myocilin, the most common genetic cause of POAG, leads to its accumulation into the ER resulting in chronic and persistent ER stress, TM cell death and increased IOP in mice [7,8]. Furthermore, ER stress has been implicated in the elevation of IOP observed in many susceptible individuals treated with glucocorticoids [9]. Members of the Bcl-2 family are key mediators of ER stress-induced apoptosis involving the mitochondrial pathway [10]. Particularly, the pro-apoptotic Bcl-2 homology domain 3 (BH3)-only p53-upregulated mediator of apoptosis (PUMA) is induced in various cell types by ER stress [11–13] and efficiently activates apoptosis by promoting the mitochondrial outer membrane permeabilization (MOMP) and the release of apoptogenic proteins including cytochrome c [14].

The tumor suppressor p53 plays a pivotal role in coordinating the cellular response to various stresses by regulating cell cycle arrest, senescence, apoptosis, cell metabolism or

autophagy [15]. As a master regulator of apoptosis, the transcription factor p53 trans-activates pro-apoptotic like the BH3-only protein PUMA. However, the role of p53 in PUMA-mediated ER stress-dependent apoptosis is still being debated [12,16–19]. Nonetheless, p53 polymorphisms have been associated with POAG [20], thereby highlighting the potential importance of this tumor suppressor in TM cell death.

Prostaglandins (PG) have long been investigated for their effect on IOP. Latanoprost, a PGF₂ α analog, is the first F prostanoid (FP) receptor agonist approved for POAG treatment and is currently used as first line therapy [21]. The potent hypotensive effect of agonists of the prostanoid EP2 receptor [22,23] recently led to their clinical development as a potential new therapeutic class for POAG [24]. As FP and EP2 receptor agonists involve separate signaling pathways and outcomes in TM cells [25], they could differently modulate pathophysiological events in POAG.

The EP2 receptor is one of the four G-protein coupled receptor (GPCR) subtypes binding the natural ligand PGE₂. Its activation leads to elevation of intracellular cyclic AMP (cAMP) through G α s protein coupling and stimulation of adenylate cyclase. More recently, it has been reported that the EP2 receptor could also activate several signaling pathways independently of cAMP through β -arrestin recruitment [26]. Depending on the cell type, EP2 receptor exerts both beneficial and detrimental effects, especially in the central nervous system where its neuronal activation promotes neuroprotective functions while microglial activation induces neuroinflammation [27]. In addition, EP2 receptor plays a pivotal role in tumorigenesis. In particular, recent studies on lymphoblastic leukemia cells showed that the cAMP pathway promoted cell survival by inhibiting DNA damage-induced apoptosis through attenuation of p53 accumulation [28,29].

The aim of the present work was to evaluate the hypothesis that PG analogs may influence cell death induced by ER stress on human TM cells and to describe the possible

mechanisms involved. We show that an EP2 receptor agonist protects TM cells from ER-induced cell apoptosis through generation of cAMP and decreased PUMA expression in a p53-dependent and PKA-independent manner.

2. Materials and Methods

2.1. Reagents

Butaprost and latanoprost free acid forms were purchased from Cayman (Ann Harbor, MI, USA). The pharmacological inhibitors tunicamycin from *Streptomyces* sp., brefeldin A, thapsigargin, KT5720, 8-(4-chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP), N6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (dbcAMP), Q-VD-OPh hydrate, nutlin-3a, were all from Sigma-Aldrich (St. Louis, MO, USA). 8-(4-chlorophenylthio) adenosine-3',5'-cyclic monophosphorothioate Rp-isomer (Rp-8-CPT-cAMPS) was from Biolog Life Science Institute (Bremen, Germany), PF-04418948 from Tocris Bioscience (Bristol, UK) and PKI [14-22] myristoylated from Life Technologies (Carlsbad, CA, USA). Forskolin from *Coleus forskohlii* was purchased from EMD Millipore (Billerica, MA, USA). Antibodies anti-human cytochrome c, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), p53, PUMA, α -tubulin and phospho-vasodilator-stimulated phosphoprotein (VASP) (Ser157) were from Cell Signaling Technologies (Danvers, MA, USA) and anti-human C/EBP homologous protein (CHOP), glucose-regulated protein of 78 kDa (GRP78) and caspase-4 were from Abcam (Cambridge, UK).

2.2. Cell culture and treatments

Cryopreserved primary human TM cells, isolated from the juxtacanalicular region of a 25-year old Caucasian male, were obtained from Sciencell Research Laboratories (Carlsbad, CA, USA). The TM origin of the cells was confirmed by the increased secretion of myocilin in response to dexamethasone [30]. The cells were plated in poly-L-lysine-coated surface and cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum, sodium pyruvate and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). Cells were maintained in a humidified 5% CO₂ and 95% air atmosphere at 37°C, and used for experiments from passages 2 to 5.

TM cells were serum-starved for 24 h before pre-treatment with inhibitor compounds, or dimethyl sulfoxide (DMSO) 0.1% as control, for 30 min unless otherwise stated. The cells were then incubated with either DMSO 0.1%, or butaprost, or latanoprost 1 µM, unless otherwise stated, for 1 h, alone or in the presence of various compounds. Tunicamycin was added to the cell medium, in the presence of PG analogs and the inhibitor compounds, at the concentration of 1 µg/ml unless otherwise stated.

2.3. Analysis of cell viability and apoptosis

Total amount of ATP of metabolically active cells was assessed by CellTiter-Glo (Promega, Madison, WI, USA) following the manufactures' instructions. Luminescence was measured using a Tecan M1000 plate reader (Männedorf, Switzerland). The total cell number was determined by DAPI staining after 4%-paraformaldehyde cell fixation and automatic counting of cell nuclei by an ArrayScan HCS reader (Thermo Fischer Scientific, Waltham, MA, USA). Activation of caspase-3/7 and caspase-9 was measured by addition of specific luminogenic substrates containing the DEVD or LEHD sequence, respectively, using Caspase-Glo 3/7 and Caspase-Glo 9 assays (Promega). The assays were conducted following the manufacturers' instructions.

2.4. Immunocytochemistry

TM cells were grown on poly-L-lysine-coated 96-well μ -plates (Ibidi, Planegg / Martinsried, Germany). After treatments, TM cell monolayers were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 supplemented with 3% bovine serum albumin (BSA). Cells were then incubated with cytochrome c antibody in a 3% BSA solution for one hour at room temperature. Wells were washed three times and incubated with Alexa Fluor 488-conjugated antibody and 4',6-diamidino-2-phenylindole (DAPI) (both from Life Technologies) for one hour. Wells were washed four times and kept in Dulbecco's phosphate buffered saline (DPBS) for fluorescence detection using IN Cell Analyzer 2200 imaging system (GE Healthcare, Little Chalfont, UK).

2.5. Immunoblotting

For Western blot analysis, cells were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer (Sigma-Aldrich) supplemented with proteases and phosphatases cocktail inhibitors (Sigma-Aldrich). After centrifugation for 10 min, equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis (NuPage, Life Technologies) and transferred to nitrocellulose membranes (Life Technologies). To prevent non-specific binding, membranes were blocked by incubation in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% nonfat dry milk for 1 h. Primary antibodies were incubated overnight at +4°C in a TBS-T with 5% BSA solution under gentle agitation. Membranes were washed 3 times in TBS-T each for 10 min, and incubated with appropriate HRP-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA). After 3 additional washes in TBS-T, membranes were developed with enhanced chemiluminescence reagents (Pierce, Bonn, Germany) and imaged by the Fusion FX7 acquisition system (Vilber

Lourmat, Torcy, France). The optical densities of individual bands were quantified using the ImageJ software (NIH, Bethesda, MD, USA) and normalized to the optical density of GAPDH.

2.6. Real-time quantitative reverse transcription PCR

Total RNA was isolated with RNeasy kit using an automated QIAcube system with a DNase digestion step (all from Qiagen, Valencia, CA, USA). cDNA was synthesized with a reverse transcription kit using random primers for amplification (Applied Biosystems, Foster City, CA, USA) in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). For real-time qPCR, cDNA, TaqMan Universal PCR Master Mix and Taqman gene expression assays were mixed in a 96-well optical reaction plate (all from Applied Biosystems). The following Taqman gene expression assays were used: p53 (Hs01034249-m1), PUMA (Hs00248075-m1), p21 (Hs00355782-m1), MDM2 (Hs01066930-m1), PAI-1 (Hs01126606-m1) and GAPDH (Hs99999905-m1). GAPDH mRNA was used as a housekeeping gene, and amplification of its cDNA was done in parallel with the genes of interest. Threshold cycle (Ct) values were determined using the Applied Biosystems software.

2.7. cAMP level

cAMP content was measured using an homogeneous time-resolved fluorescence (HTRF)-based competitive immunoassay between native cAMP and cAMP labeled with the dye d2. Serum-starved TM cells were pre-incubated with 3-isobutyl-1-methylxanthine (100 μ M) for 30 min, and then butaprost (1 μ M) was added for 15 min, before being lysed in the presence of the FRET acceptor cAMP-d2 and a monoclonal anti-cAMP-cryptate. The TR-FRET signal was detected by microplate reader RUBYstar (BMG labtech, Ortenberg, Germany).

2.8. Statistical analysis

All values have been expressed as mean \pm SEM of at least 3 independent experiments. Data were analyzed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Significance was tested by one-way ANOVA with Dunnet's correction or two-way ANOVA with Sidak's or Tukey's correction for multiple comparisons when appropriate. Significance was denoted as followed: $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) .

3. Results

3.1. Butaprost prevents TM cell death induced by tunicamycin

To elucidate whether PG analogs can protect primary human TM cells against ER stress, the inhibitor of N-glycosylation, tunicamycin, was used as an ER stress inducer. Cell viability, as measured by total ATP content of serum-starved TM cells, was reduced in a concentration-dependent manner by tunicamycin at 24 h (Fig. 1A). Neither the EP2 receptor agonist butaprost nor the FP agonist latanoprost alone were able to modified total ATP amount at 24 h (data not shown). However, at 1 μ M, butaprost significantly protected against tunicamycin-induced cell death whereas latanoprost did not. In correlation, tunicamycin-dependent decrease of the number of TM cell nuclei was prevented by butaprost but not by latanoprost (Fig. 1B and C). Butaprost-mediated protection was concentration-dependent with a calculated EC_{50} of 190 nM (Fig. 1D), in correlation with the values obtained from radioligand competition assays [31]. In comparison, latanoprost was less protective, starting only from 3 μ M.

3.2. EP2 receptor-dependent protection against ER stress-induced cell death is mediated through cAMP generation

As latanoprost only weakly protected TM cells and at high concentrations, we focused our study on unraveling the mechanisms of action of butaprost-mediated prevention of cell death. For this purpose, a butaprost concentration of 1 μM was used based on the maximum effect obtained before reaching a plateau (Fig. 1D). To confirm the protective effect of butaprost, TM cells were treated with two other classical ER stress inducers, brefeldin A which inhibits protein transport from the ER to the Golgi, and thapsigargin, a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase. As observed for tunicamycin, both stressors induced TM cell death at 24 h which was significantly inhibited by butaprost (Fig. 2A). Furthermore, as shown in Figure 2B, the highly specific EP2 receptor antagonist, PF-04418948 [32], fully blocked butaprost-mediated protection against tunicamycin-induced cell death, confirming that this effect was EP2 receptor-dependent. Butaprost led to a concentration-dependent increase in intracellular cAMP level (Fig. 2C). Indeed, cAMP was sufficient for preventing cell death, as observed by the concentration-dependent protective effect of the stable cAMP analog dibutyryl cAMP (dbcAMP), and of the adenylate cyclase activator forskolin (Fig. 2D).

In order to determine the pathway by which cAMP induced protection, we used an inhibitor of the regulatory subunit of PKA, Rp-8-CPT-cAMPS. Surprisingly, this inhibitor did not block the EP2-dependent protection (Fig. 2E) even if it abolished the butaprost-mediated phosphorylation of VASP, a direct substrate of PKA (Supplemental Fig. 1A). In accordance, the EP2-dependent protection was also observed in the presence of two other structurally unrelated PKA inhibitors, KT5720 and PKI [14-22] (Supplemental Fig. 1B), further confirming that butaprost prevents TM cell death through a PKA-independent mechanism. cAMP can also transduce signals through the exchange proteins activated by cAMP (Epac)

[33]. However, the specific agonist of Epac, 8-pCPT-2'-O-Me-cAMP, failed to protect cells against tunicamycin (Fig. 2F). Moreover, brefeldin A, which is also known to inhibit Epac function, did not prevent the butaprost-mediated protection (Fig. 2A), further suggesting an Epac-independent pathway.

3.3. EP2 receptor activation inhibits ER stress-induced mitochondrial-dependent apoptosis

To explore the mechanism of action of EP2-mediated TM cell survival, we investigated the effect of butaprost on the apoptotic pathways. Tunicamycin activated both the initiator caspase-9 and the effector caspase-3/7 following 6 h of incubation (Fig. 3A and B). Butaprost significantly decreased tunicamycin-induced caspase-9 and -3/7 activation at 6 h, and these effects were sustained at 24 h. In contrast, tunicamycin-dependent decrease of cell viability and butaprost-mediated protection were only observed at 24 h (Fig. 3C). To confirm that the mechanism of cell death relied on caspase activation, the broad-spectrum caspase inhibitor Q-VD-OPh was used. Treating TM cells with Q-VD-OPh blocked caspase-3/7 activation at 6 h (Fig. 3D) and resulted in a significant protection against TM cell death at 24 h (Fig. 3E).

ER stress-induced cell death can activate initiator caspase-9 *via* the classical mitochondrial apoptotic pathway, through the release of cytochrome c, or by activating the ER stress-specific caspase-4 [34]. Our data showed that cytochrome c is released from mitochondria in response to tunicamycin at 6 and 24 h (Fig. 3F) as observed by its diffuse localization into the cytosol (Fig. 3G). At both time-points, cytochrome c release was inhibited by butaprost. This mechanism was not due to downstream caspase activation as the pan caspase inhibitor Q-VD-OPh did not block cytochrome c release (Supplemental Fig. 2A and B). On the contrary, the pan caspase inhibitor strongly increased the release of cytochrome c at 24 h, probably through an amplification loop as previously described [35]. To

elucidate if caspase-4 is also involved in the butaprost-mediated protection, caspase-4 proteolytic cleavage was assessed by western blot (Fig. 4A). Cleaved caspase-4 was detected at 24 h in response to tunicamycin, but not at 6 h, and this effect was not modulated by butaprost suggesting a caspase-4 independent protective mechanism.

ER stress initiates the adaptive unfolded protein response (UPR) to restore homeostasis and ultimately leads to cell death if ER stress is persistent. The expression of the ER chaperone GRP78 and the pro-apoptotic transcription factor CHOP, two prototypical UPR targets, was increased by tunicamycin (Fig. 4B). However, butaprost did not down-regulate GRP78 and CHOP expression at both 6 and 24 h. On the contrary, butaprost slightly increased CHOP expression which could reflect a negative feed-back mechanism that aims to limit the protective effect of butaprost. Altogether, these results suggest that butaprost inhibits mitochondrial intrinsic apoptosis independently of caspase-4 activation and key hallmarks of the UPR.

3.4. EP2 receptor-mediated protection involves down-regulation of the p53/PUMA axis

Previous data have shown that elevation of cAMP could protect cells from DNA damage-induced apoptosis through a decrease of p53 accumulation [36]. Consequently, we analyzed the expressions of p53 and the BH3-only PUMA which accounts for most of the p53-dependent proapoptotic activity [37]. As observed in Figure 5A, both p53 and PUMA were up-regulated by tunicamycin and down-regulated by co-incubation with butaprost. Modulation of PUMA expression correlated with its mRNA level (Fig. 5B). In contrast, p53 mRNA remained stable suggesting that butaprost decreases p53 accumulation rather than inhibiting its synthesis. To substantiate the potential regulation of p53 in tunicamycin-induced ER-stress, we measured the mRNA expression of p53-target genes [38] coding for p21, mouse double minute 2 homolog (MDM2) and plasminogen activator inhibitor (PAI)-1 (Fig.

5C). All three genes were down-regulated by butaprost, but only p21 and PAI-1 mRNA, as well as PUMA, were significantly up-regulated by tunicamycin, reflecting the different regulation mode of these p53-response elements [39]. Furthermore, in correlation with our previous findings, the EP2-induced down-regulation of p53 and PUMA was mediated by cAMP as observed by the similar effects obtained with the stable analog of cAMP (dbcAMP) (Fig. 5D), and was PKA-independent as shown by the lack of effect of the specific PKA inhibitor Rp-8-CPT-cAMPS (Fig. 5E).

To determine whether butaprost action is only preventive or could also protect cells from ongoing apoptosis, TM cells were treated with butaprost at 6 h after tunicamycin challenge, a time-point previously validated for caspase activation (Fig. 3B). Both caspase activation and the expression of p53 and PUMA were analyzed at 7 h and 9 h after challenge, corresponding to 1 h and 3 h after butaprost addition, respectively. The EP2 receptor agonist inhibited caspase activation at 1 h after addition, which correlated with p53 and PUMA down-regulation (Fig. 6A and C). Furthermore, both caspase activation and p53/PUMA expressions were further inhibited after 3 hours of butaprost treatment underlying the close interaction between the two effects. The level of cell protection obtained at 24 h (Fig. 6B) was similar to the one obtained in the above pre-incubation protocol (Fig. 3C). These results demonstrate that butaprost is able to rescue TM cell from death in a model of established apoptosis through the rapid down-regulation of p53.

Finally, to further evaluate the involvement of p53 and PUMA in the protective mechanism induced by butaprost, nutlin-3a was used to stabilize p53. Nutlin-3a is an inhibitor of the interaction between p53 and the ubiquitin ligase MDM2 which inhibits p53 degradation [39]. A 24 h pre-incubation of nutlin-3a efficiently stabilized p53 (Fig. 7A) as observed by a higher level of p53 in presence nutlin-3a. p53 stabilization correlated with an enhanced expression of PUMA. Under these conditions, butaprost no longer induced a down-regulation

of p53 and PUMA. In agreement, the butaprost-mediated protection against ER stress-induced TM cell death was blunted by increasing concentrations of nutlin-3a (Fig. 7B).

4. Discussion

A decrease in TM cell number has long been associated with the pathophysiology of glaucoma [2,3]. Here, we demonstrate that an EP2 receptor agonist could protect TM cells from death induced by ER stress through cAMP generation. Furthermore, we show that an EP2 receptor agonist inhibits intrinsic mitochondrial apoptosis induced by ER stress *via* a mechanism inducing the down-regulation of p53 and of the p53-target gene PUMA.

We initially investigated the effects of the hypotensive PGF2 α analog latanoprost and the EP2 receptor agonist butaprost on TM cells. Our results show that the FP agonist is not as potent as the EP2 receptor agonist in preventing ER stress-induced TM cell death. Latanoprost weakly protects TM cells and only at concentrations far above those found in the human aqueous humor after topical application [40].

In our model, cAMP-mediated protection from cell death seems to be independent of the known cAMP sensors, PKA and Epac, as demonstrated by the use of structurally different inhibitors and of a specific agonist. In agreement with the present results, several studies demonstrated that unidentified mediators of cAMP regulate apoptosis [41–44] hormone secretion [45], neuritogenesis [46], or IL-6 production [47]. Interestingly, it has been demonstrated in lymphoid leukemia cells that the second messenger, cAMP, protects against apoptosis induced by DNA damage through decreased stability of p53. This mechanism was initially described as PKA- and Epac-independent [44], in agreement with the present results. Conversely, other studies have pointed out the involvement of PKA through the use of cAMP

analogs [28,29]. However, the interpretation of these effects should take into account the potential non-specific phosphodiesterase inhibition leading to an indirect cAMP generation [48]. Downstream signalings of PKA- and Epac-independent cAMP effects involve activation of extracellular signal-regulated kinase [46], p38 [47], their combination [42], Ca²⁺/calmodulin-dependent protein kinases [45], or the inhibition of phosphoinositide 3-kinase/Akt signaling [43,49].

Recent works reported that, in the context of ER stress, cAMP modulates inflammatory response in glial cells, a mechanism which potentially contributes to neuroprotection [50]. Furthermore, increased expression of EP2 receptor may prevent against mesangial cell injuries by inhibiting ER stress-mediated apoptosis [51]. Future works would clarify the potential protective function of the EP2 receptor-cAMP signaling in other ER stress-mediated pathophysiological contexts.

While the UPR-related proteins GRP78 and CHOP were, as expected, up-regulated in ER stress conditions, we did not observe a decreased expression of these proteins by the EP2 receptor agonist treatment. These data suggest that butaprost-mediated protection does not rely on alleviating ER stress by elimination of unfolded or misfolded proteins but rather on downstream inhibition of pro-apoptotic events. Moreover, the EP2 receptor agonist did not modulate the ER stress-specific caspase-4 proteolytic cleavage but inhibited the cytochrome c release, a hallmark of the mitochondrial apoptotic pathway. These findings are consistent with the down-regulation of the BH3-only protein PUMA by the EP2 receptor agonist and suggest that this mechanism could be extended to other stress-induced cell death involving MOMP.

In conditions of ER stress, PUMA has been extensively linked to the induction of cell death [12,16–19] but the role of p53 is still controversial. Studies have shown that p53 is stabilized by ER stress [52] and such stabilization induced the p53-dependent expression of cell death mediators [16]. In contrast, others have demonstrated in p53 deficient cells, that the

transcription factor is dispensable for ER stress-induced PUMA expression [12,18,19]. However, p53 deficiency could lead to compensatory mechanisms through p73 inducing PUMA expression as both proteins share similar regulation mechanisms [53]. Here, we provide evidence that ER stress-mediated apoptosis in TM cells involves the up-regulation of p53 and PUMA. Moreover, the evidence that cAMP elevation down-regulates both p53 and PUMA and inhibits apoptosis further highlights the role of p53 in modulating ER stress-mediated TM cell death (Fig. 8).

Consistent with the inhibitory effect of cAMP on p53 activity, we showed that p53 target genes were inversely modulated by ER stress and the EP2 receptor agonist. However, each gene revealed a specific pattern of regulation in terms of time and amplitude. Gene-dependent p53 regulation has been extensively explored and was associated with differences in p53 response elements, binding partners and post-translational modification status [38]. Even if we did not observe ER stress-induced p53 phosphorylation in the N-terminal region (Ser15, Thr18 and Ser20; data not shown), it is likely that p53 is modified by ER stress on other sites through phosphorylation, acetylation, or other post-translational events. In particular, it has been demonstrated that p53 is activated and stabilized in ER stress conditions through phosphorylation and decreased MDM2-dependent ubiquitination [52]. Hence, EP2 receptor activation, through cAMP generation, would promote p53 degradation by counteracting its post-translational modifications whether directly or via a p53-targeted ubiquitin ligase. In correlation, we showed that increased p53 stabilization by nutlin-3a blocked the protective effect of the EP2 receptor agonist. Furthermore, previous works reported that cAMP favors p53 proteasomal degradation thereby inhibiting p53 accumulation due to DNA-damaging agents [28,29]. The precise identification of the ER stress-induced p53 post-translational modifications, and the proteins involved in the process, would be of great

interest to gain insight into ER stress-induced degenerative pathologies, and to decipher the role of cAMP in these p53 modifications.

Besides its role in apoptosis, p53 is also a critical modulator of cellular senescence, a process intriguingly associated with tissue aging [54]. Particularly, senescence has been observed in pathological TM [4] and thus potentially contributes to the lack of TM cell repopulation. Interestingly, both p21 and PAI-1, two relevant p53 target genes in the induction of senescence [55], are down-regulated by an EP2 receptor agonist, suggesting that EP2 activation could be an interesting mechanism in preventing TM senescence.

The common polymorphism of the p53 gene at codon 72, either encoding a proline or an arginine, has been largely investigated as a potential risk factor for POAG. If the association between this polymorphism and POAG is still a matter of debate, a meta-analysis of nine independent studies from different populations suggested that the Arg72Pro single nucleotide polymorphism is indeed a significant risk factor for POAG [20]. The proline and the arginine variants affect differently the biological activity of p53 in terms of apoptotic potential [56,57] and senescence predisposition [58]. Unfortunately, the inconsistency of current reports does not allow to associate POAG with an allelic predominance. Nonetheless, these studies reveal an important link between POAG and p53 and suggest that p53 down-regulation would be beneficial for TM protection.

Recent evidence showing that POAG may result from late adult onset protein aggregation in the TM [6] further highlights the need to protect TM cells from ER stress. Importantly in the pathological context, we provide evidence in this work that EP2 receptor activation can both protect and rescue TM cells against ER stress-induced cell death.

In conclusion, the present study demonstrates that EP2 receptor activation efficiently prevents TM cell apoptosis induced by ER stress. The mechanism of TM cell protection involves generation of cAMP and is independent of PKA and Epac. We also present evidence

that cAMP elevation down-regulates the p53/PUMA axis. Despite the close correlation between p53/PUMA down-regulation and TM cell survival, other protective mechanisms could not be excluded. Overall, the present data provide new perspectives for EP2 receptor activation in the management of POAG. EP2-mediated protection of TM dysfunction could offer an interesting add-on mechanism to their potential use as hypotensive drugs for the treatment of glaucoma.

Acknowledgments

We are grateful to Drs Nelson Cortéz, Christophe Lechauve and Yvrick Zagar (Institut de la Vision, France) for their comments, scientific discussions and technical advice. We thank Dr Terence Appelqvist (Sanofi, France) and Charlotte Vernhes (Sanofi Pasteur, Florida, USA) for critical reading and editing of the manuscript. The authors also wish to acknowledge Drs Patrick Avenet and Caroline Leriche (Sanofi, France) to make this work possible.

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Footnotes

This work was supported by Sanofi Research & Development and by the Vision institute (Paris). G.K., A.C., L.D., C.O., T.D. and X.V. are employed by Sanofi Research & Development.

Figure Legends

Figure 1: Butaprost protects against tunicamycin-induced TM cell death. TM cells were pre-treated for 1 h with either butaprost (buta) or latanoprost (latano) and then with the ER stress inducer, tunicamycin, for 24 h. (A) Concentration-effect of tunicamycin on TM cell viability pre-treated with either butaprost or latanoprost (1 μ M). Cell viability was measured by CellTiter-Glo and expressed as a percentage of the control DMSO condition (mean \pm SEM, n=5). *P<0.05, ***P<0.001 when comparing DMSO with PG analogs for each concentration of tunicamycin (two-way ANOVA). (B) Pictures of TM cell monolayer obtained at 24 h post-tunicamycin treatment (1 μ g/ml) and (C) TM cell nuclei were counted after DAPI staining. Nuclei number was expressed as a percentage of the control condition (mean \pm SEM, n=5). ***P<0.001 (two-way ANOVA). (D) Concentration-dependent effect of PG analogs on TM cell death induced by tunicamycin. TM cells were pre-treated with indicated concentrations of PG analogs before tunicamycin treatment (1 μ g/ml) for 24 h. Cell viability was measured and expressed as in (A) (mean \pm SEM, n=6). *P<0.05, ***P<0.001 when comparing DMSO with each PG analog concentration (one-way ANOVA).

Figure 2: EP2 receptor-dependent protection against ER stress-induced cell death is mediated by cAMP but independent of the canonical PKA/Epac pathways. (A) TM cells were pre-incubated with butaprost (1 μ M) before ER stress inducers tunicamycin (1 μ g/ml), brefeldin A (10 μ M) or thapsigargin (1 μ M). Cell viability was measured at 24 h by CellTiter-Glo and expressed as a percentage of the control condition (mean \pm SEM, n=6). *P<0.05, **P<0.01, ***P<0.001 (two-way ANOVA). (B) TM cells were incubated with an EP2 antagonist, PF-04418948 (10 μ M), treated with butaprost (1 μ M) and then tunicamycin (tuni, 1 μ g/ml). Cell viability was measured and expressed as in (A) (mean \pm SEM, n=5).

***P<0.001 (two-way ANOVA). (C) TM cells were treated with indicated concentrations of butaprost for 15 min and cAMP levels were measured using HTRF competitive immunoassay (mean \pm SEM, n=3). *P<0.05, ***P<0.001 when comparing DMSO with each butaprost concentration (one-way ANOVA). (D) TM cells were pre-incubated with butaprost (1 μ M) or forskolin (10 μ M) or the analog of cAMP (dbcAMP). Cell viability was measured and expressed as in (A) (mean \pm SEM, n=3). ***P<0.001 when comparing DMSO with butaprost or forskolin or dbcAMP (two-way ANOVA). (E) TM cells were pre-incubated with PKA inhibitor Rp-8-CPT-cAMPS (100 μ M) before butaprost and tunicamycin. Cell viability was measured and expressed as in (A) (mean \pm SEM, n=3). ***P<0.001 (two-way ANOVA for each inhibitor). (F) TM cells were pre-treated with either butaprost or the specific Epac agonist 8-pCPT-2'-O-Me-cAMP (8-pCPT-2'-O-Me, 100 μ M). Cell viability was measured and expressed as in (A) (mean \pm SEM, n=3). ***P<0.001 when comparing DMSO with either butaprost or the Epac agonist (two-way ANOVA).

Figure 3: EP2 receptor agonist inhibits tunicamycin-dependent caspase activation and cytochrome c release. TM cells were pre-treated with butaprost (1 μ M) and then tunicamycin (tuni, 1 μ g/ml). (A, B) Caspase activation was analyzed at 6 and 24 h by using Caspase-Glo 9 or Caspase-Glo 3/7. Caspase activation was expressed as fold over the control condition (mean \pm SEM, n=5). *P<0.05, **P<0.01, ***P<0.001 (two-way ANOVA for each time-point). (C) Cell viability was measured by CellTiter-Glo at 6 and 24 h and expressed as a percentage of the control condition (mean \pm SEM, n=5). ***P<0.001 (two-way ANOVA for each time-point). (D, E) TM cells were pre-incubated with the broad spectrum caspase inhibitor Q-VD-OPh (20 μ M) before butaprost and then tunicamycin treatments. Caspase 3/7 activation at 6 h (D) and cell viability at 24 h (E) were analyzed as in (B) and (C), respectively. (F) Cytochrome c release was quantified by immunocytochemistry at 6 and 24 h.

Cells with released cytochrome c were measured as those with diffuse cytochrome c staining, in contrast to reticular staining indicating mitochondrial localization of cytochrome c, and expressed as a percentage of DAPI positive cells. At least 300 cells per condition were analyzed at the 60x magnification (mean \pm SEM, n=3). **P<0.01, ***P<0.001 (two-way ANOVA for each time-point). (G) Representative pictures of cytochrome c (green) and DAPI-stained nuclei (blue) were obtained after butaprost and then tunicamycin treatments for 6 h. Diffuse cytochrome c staining is indicated by arrowheads.

Figure 4: Caspase-4 processing and UPR markers, GRP78 and CHOP, are not down-regulated by EP2 receptor activation. TM cells were pre-treated with butaprost (buta, 1 μ M) and then tunicamycin (tuni, 1 μ g/ml). Cell lysates obtained at 6 h or 24 h were analyzed by western blot to study the expression of GRP78 and CHOP (A) and the proteolytic cleavage of caspase-4 (B). The bands corresponding to the pro-caspase-4 and the cleaved caspase-4 are indicated by arrow heads. The band marked by an asterisk is presumably an intermediate cleavage product of caspase-4. Results are representative of 3 independent experiments.

Figure 5: EP2 receptor agonist inhibits p53 accumulation and down-regulates p53-target genes. TM cells were pre-treated with butaprost (buta, 1 μ M) and then tunicamycin (tuni, 1 μ g/ml). (A) Cell lysates obtained at 3 h or 6 h were analyzed by western blot to study the expression of p53 and PUMA. Values were obtained from the densitometric analyses of at least 4 independent experiments and expressed as fold over the control condition (mean \pm SEM). (B,C) qPCR analyses of the mRNA expression of p53, PUMA and the p53 target genes coding for p21, MDM2, and PAI-1. mRNA level was expressed in fold change over the control condition (mean \pm SEM, n=3). *P<0.05, **P<0.01, ***P<0.001 (two-way ANOVA for each time-point). (D) TM cells were pre-treated with the analog of cAMP (dbcAMP, 1

mM) and then tunicamycin for 6 h. Cell lysates were analyzed as in (A) (mean \pm SEM, n=3). (E) TM cells were pre-incubated with the PKA inhibitor Rp-8-CPT-cAMPS (100 μ M) before butaprost and then tunicamycin treatment for 6 h. Cell lysates were analyzed as in (A) (mean \pm SEM, n=3).

Figure 6: EP2 receptor-mediated rescue of TM cell apoptosis correlates in time with p53/PUMA down-regulation. TM cells were first treated with tunicamycin (tuni) for 6 h and then incubated with butaprost (buta). (A) Caspase activation was analyzed at 7 h and 9 h by using either Caspase-Glo 9 or Caspase-Glo 3/7. Caspase activation was expressed as fold over the control condition (mean \pm SEM, n=5). *P<0.05, ***P<0.001 (two-way ANOVA for each time-point). (B) Cell viability was measured by CellTiter-Glo and expressed as a percentage of the control condition (mean \pm SEM, n=5). ***P<0.001 (two-way ANOVA). (C) Cell lysates were analyzed at 7 h and 9 h after tunicamycin treatment to study the expression of p53 and PUMA. Values were obtained from the densitometric analyses of at least 4 independent experiments and expressed as fold over the control condition (mean \pm SEM).

Figure 7: Stabilization of p53 abolishes EP2 receptor-mediated protection against cell death. TM cells were pre-incubated for 24 h with indicated concentration of the MDM2-p53 inhibitor nutlin-3a. TM cells were then treated with butaprost (buta, 1 μ M) and tunicamycin (tuni, 1 μ g/ml). (A) Cell lysates were analyzed at 6 h after tunicamycin treatment to study the expression of p53 and PUMA. Values were obtained from the densitometric analyses of at least 4 independent experiments and expressed as fold over the control condition (mean \pm SEM). (B) Cell viability was measured by CellTiter-Glo and expressed as a percentage of the control condition (mean \pm SEM, n=4). **P<0.01, ***P<0.001 (two-way ANOVA for each nutlin-3a concentration).

Figure 8: Proposed model for EP2-dependent protection against ER stress-induced TM cell death via p53/PUMA axis. ER stress induces accumulation of p53 and the transcription of the BH3-only PUMA which sensitizes TM cells to mitochondrial-dependent apoptosis. EP2 receptor agonist activates adenylate cyclase through stimulation of GPCR and promotes cAMP production which inhibits p53 accumulation and PUMA expression thereby protecting TM cells against ER stress-induced apoptosis.

Supplemental Figure 1: Butaprost-dependent protection is insensitive to PKA inhibition.

(A) TM cells were pre-treated with or without the PKA inhibitor Rp-8-CPT-cAMPS (100 μ M) for 30 min before butaprost (buta, 1 μ M) and then tunicamycin (tuni, 1 μ g/ml). Cell lysates were obtained at 6 h and analyzed by western blot to study the phosphorylation of VASP. Results are representative of 3 independent experiments. (B) TM cells were pre-incubated with PKA inhibitors PKI [14-22] myristoylated (1 μ M) or KT5720 (1 μ M) before butaprost and tunicamycin. Cell viability was measured and expressed as in (A) (mean \pm SEM, n=3). ***P<0.001 (two-way ANOVA for each inhibitor).

Supplemental Figure 2: Cytochrome c release in the presence of Q-VD-OPh. TM cells

were pre-treated with Q-VD-OPh (20 μ M) for 30 min before butaprost (buta, 1 μ M) and then tunicamycin (tuni, 1 μ g/ml). (A) Cytochrome c release was quantified by immunocytochemistry at 6 and 24 h. Cells with released cytochrome c were measured as those with diffuse cytochrome c staining, in contrast to reticular staining indicating mitochondrial localization of cytochrome c, and expressed as a percentage of DAPI positive cells. At least 300 cells per condition were analyzed at the 60x magnification (mean \pm SEM, n=3). **P<0.01, ***P<0.001 (two-way ANOVA for each time-point). (B) Representative

pictures of cytochrome c (green) and DAPI-stained nuclei (blue) were obtained after butaprost and then tunicamycin treatments for 6 h. Diffuse cytochrome c staining is indicated by arrowheads.

ACCEPTED MANUSCRIPT

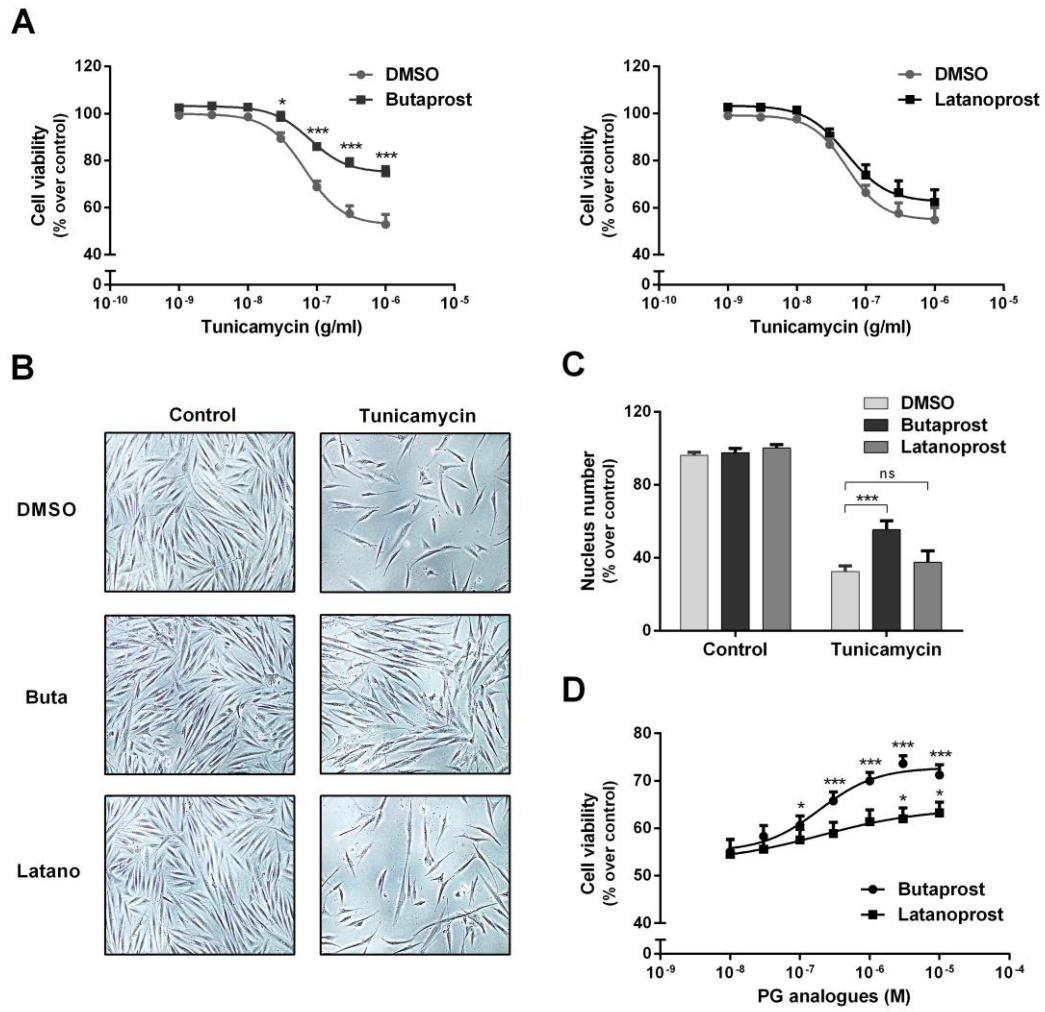


Figure 1

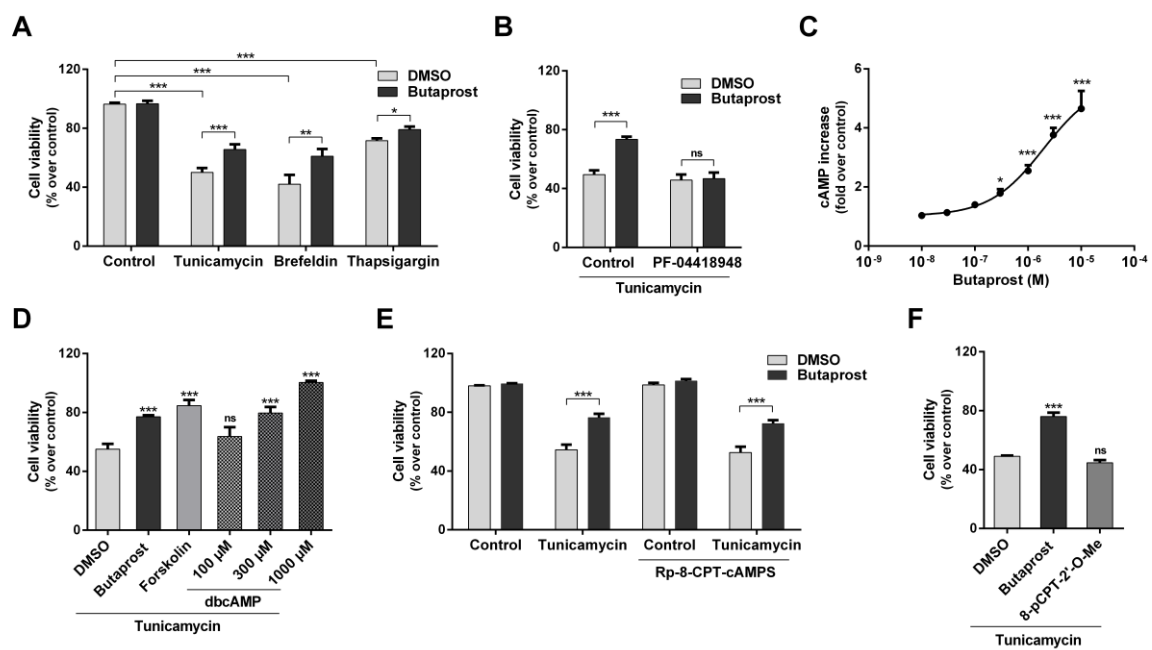


Figure 2

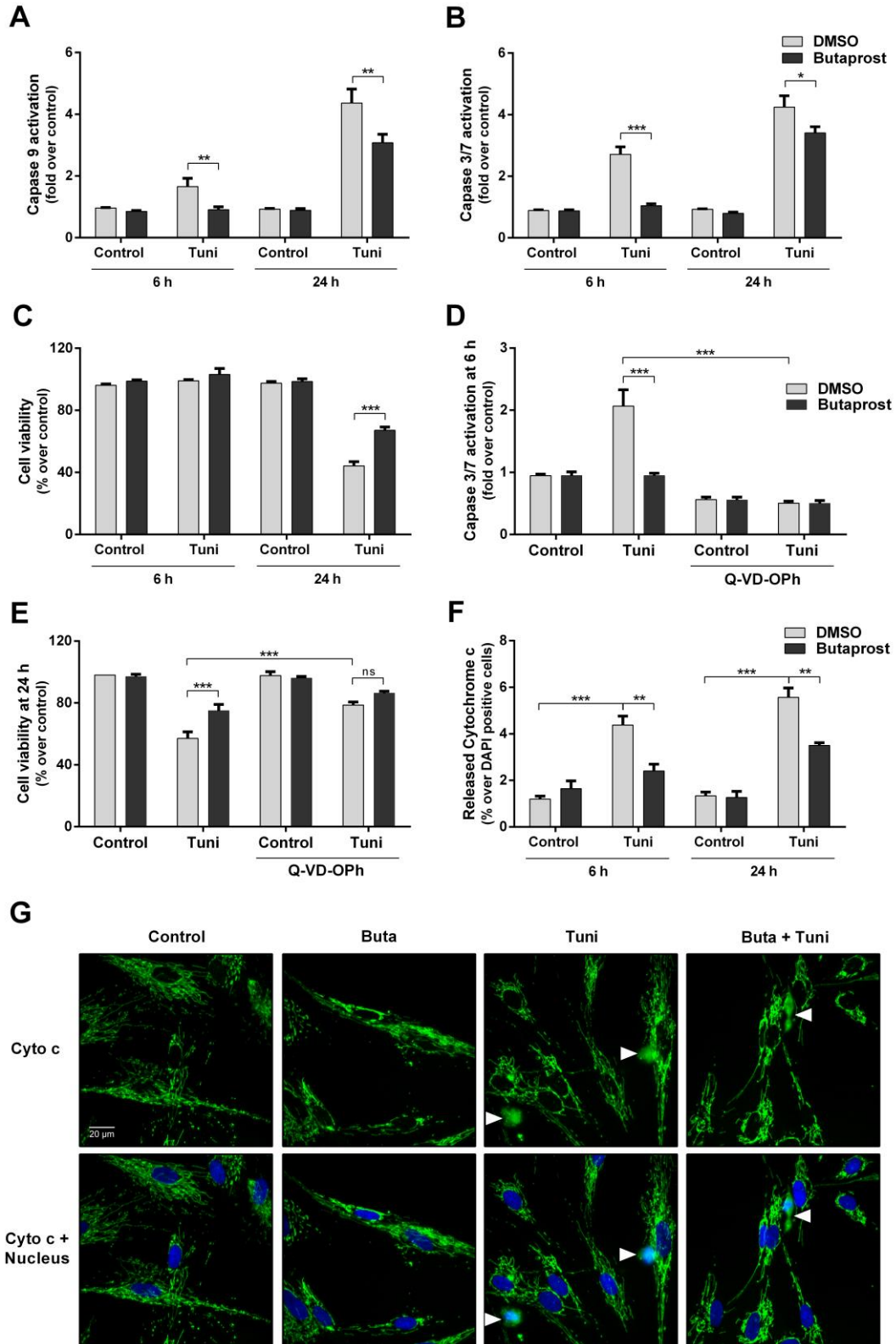


Figure 3

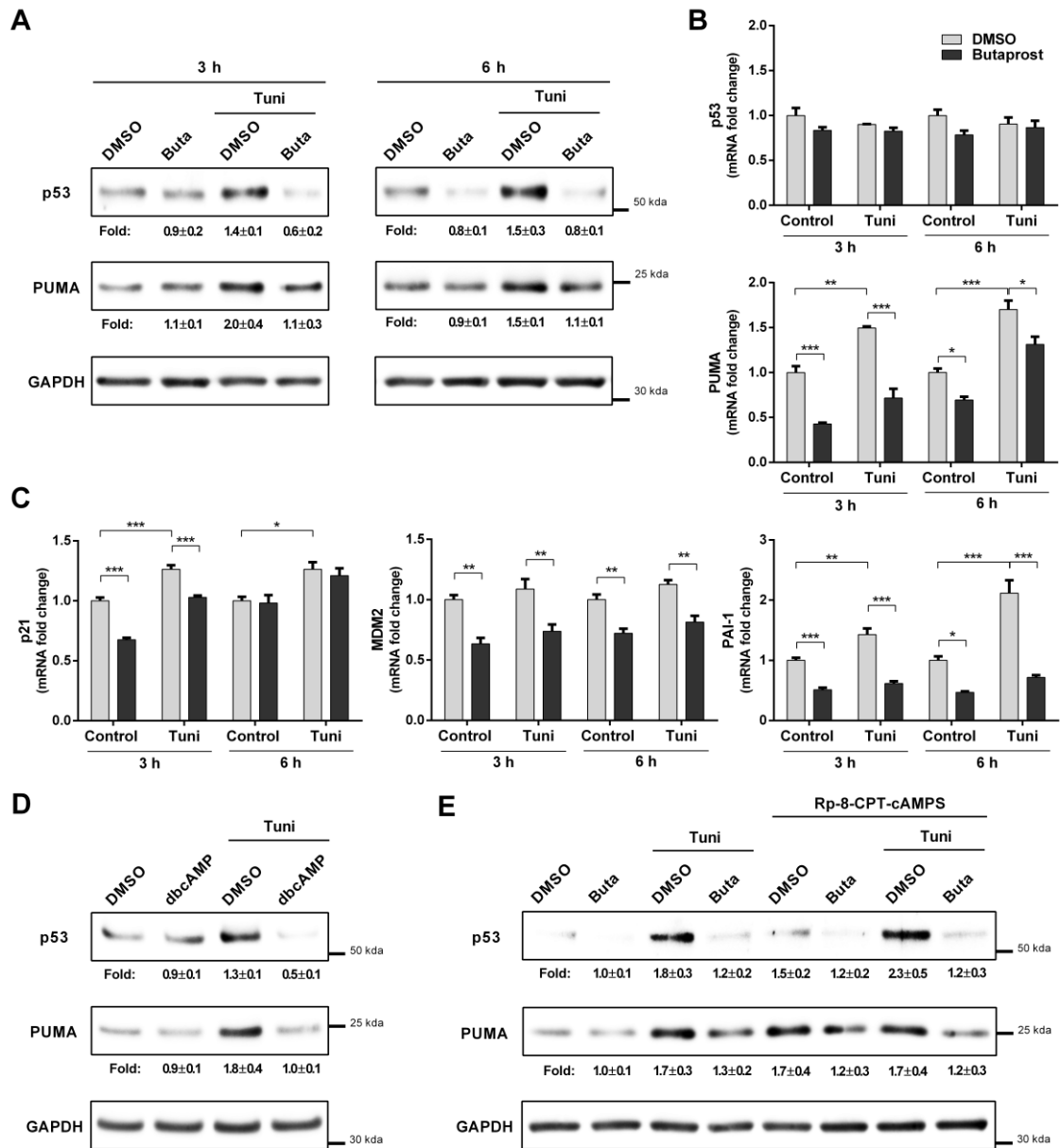


Figure 5

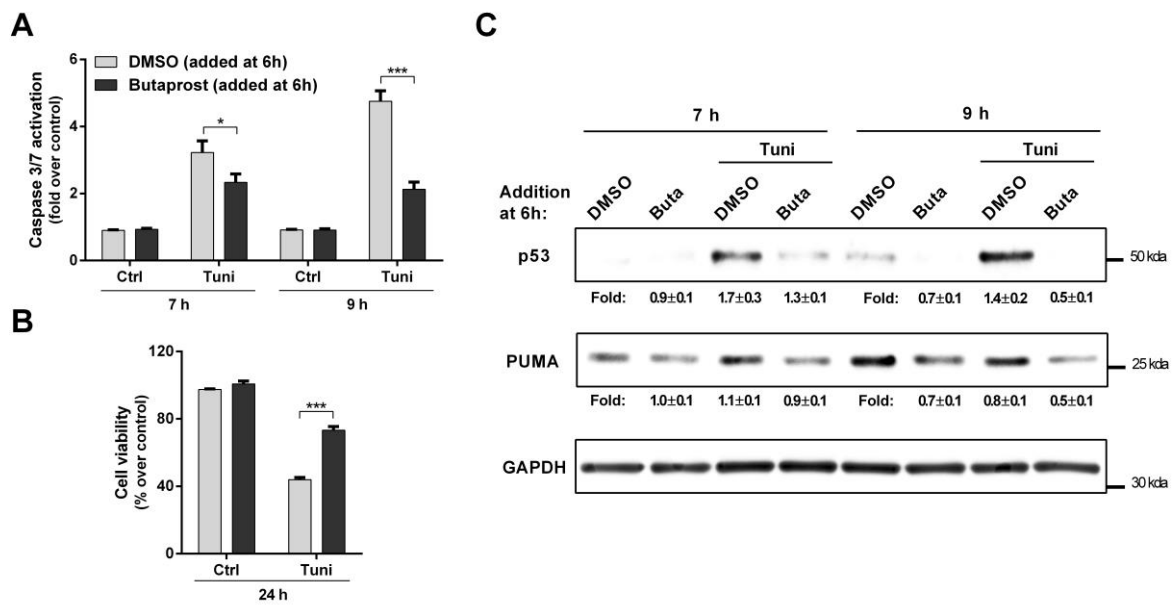


Figure 6

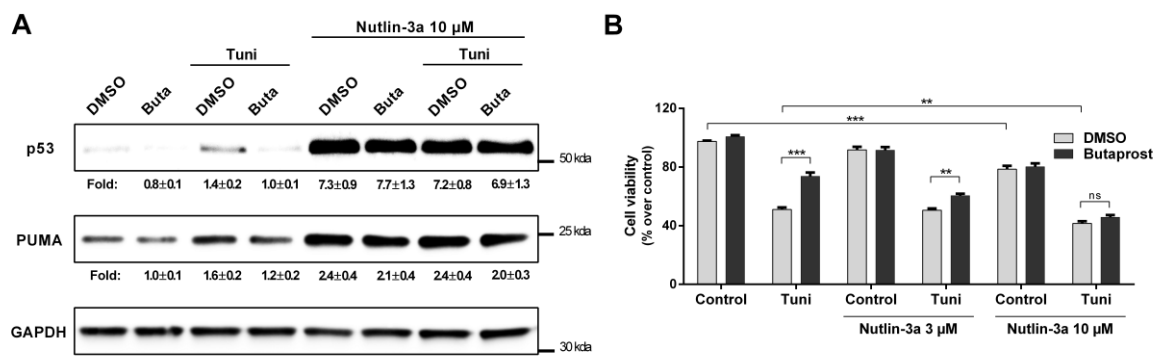


Figure 7

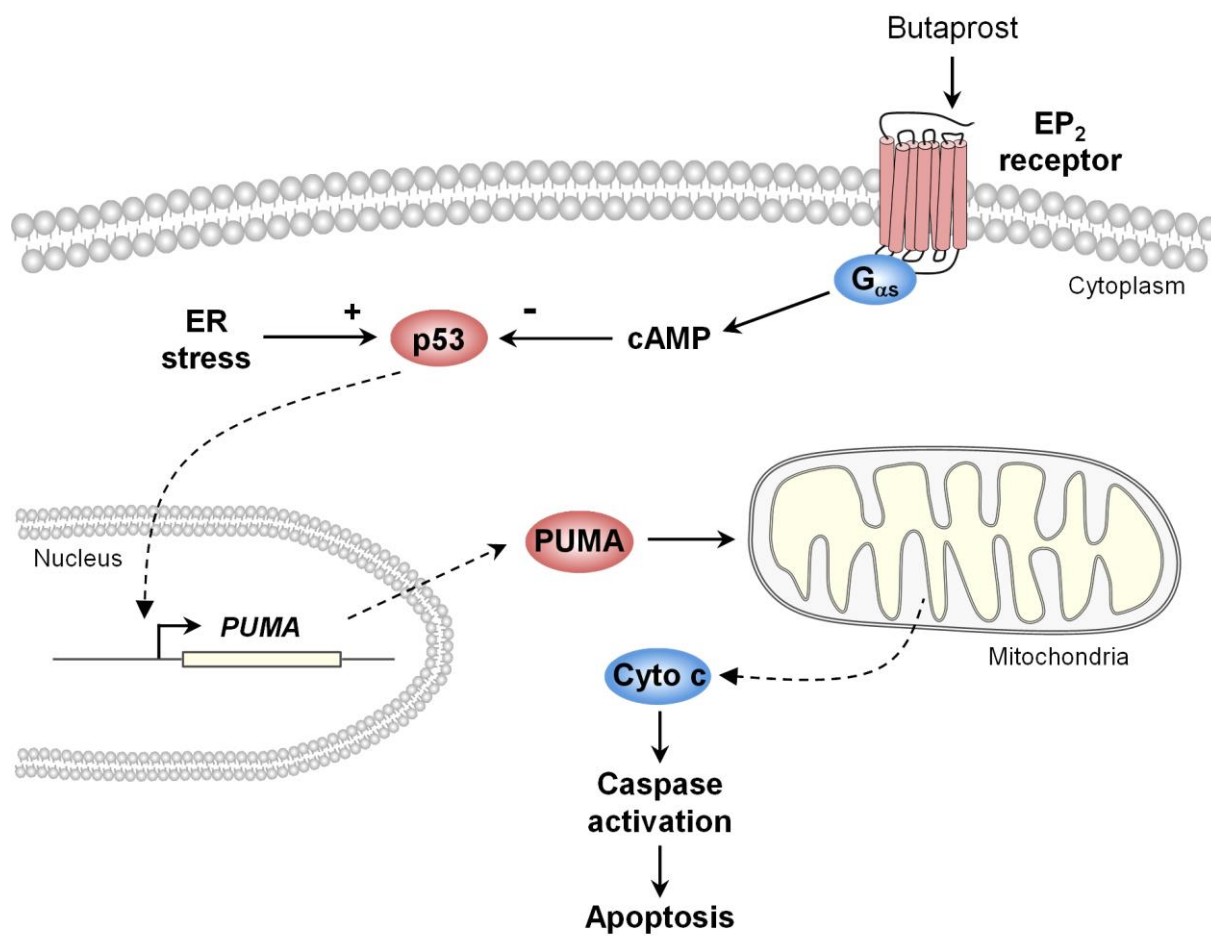


Figure 8

Highlights

- EP2 receptor protects trabecular meshwork cells from ER stress-induced cell death
- EP2 receptor-dependent protection is mediated through cAMP production
- EP2 receptor inhibits mitochondrial intrinsic apoptosis
- The mechanism involves down-regulation of the p53/PUMA axis

ACCEPTED MANUSCRIPT