

# Regulation of Thromboxane Receptor Signaling at Multiple Levels by Oxidative Stress-Induced Stabilization, Relocation and Enhanced Responsiveness

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## Abstract

**Background:** Thromboxane A<sub>2</sub> (TxA<sub>2</sub>) is a major, unstable arachidonic acid metabolite, and plays a key role in normal physiology and control of vascular tone. The human thromboxane receptor (TPβ), expressed in COS-7 cells, is located predominantly in the endoplasmic reticulum (ER). Brief hydrogen peroxide exposure increases the efficiency of translocation of TPβ from the ER into the Golgi complex, inducing maturation and stabilization of TPβ. However, the ultimate fate of this post-ER TPβ pool is not known, nor is its capacity to initiate signal transduction. Here we specifically assessed if functional TPβ was transported to the plasma membrane following H<sub>2</sub>O<sub>2</sub> exposure.

**Results:** We demonstrate, by biotinylation and confocal microscopy, that exposure to H<sub>2</sub>O<sub>2</sub> results in rapid delivery of a cohort of TPβ to the cell surface, which is stable for at least eight hours. Surface delivery is brefeldin A-sensitive, indicating that translocation of this receptor cohort is from internal pools and via the Golgi complex. H<sub>2</sub>O<sub>2</sub> treatment results in potentiation of the increase to intracellular calcium concentrations in response to TPβ agonists U46619 and 8-iso PGF<sub>2α</sub> and also in the loss of ligand-dependent receptor internalization. Further there is increased responsiveness to a second application of the agonist. Finally we demonstrate that the effect of H<sub>2</sub>O<sub>2</sub> on stimulating surface delivery is shared with the FP prostanoid receptor but not the EP3 or EP4 receptors.

**Conclusions/Significance:** In summary, brief exposure to H<sub>2</sub>O<sub>2</sub> results in an immediate and sustained increase in the surface pool of thromboxane receptor that is capable of mediating a persistent hyper-responsiveness of the cell and suggests a highly sophisticated mechanism for rapidly regulating thromboxane signaling.

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## Introduction

Oxidative stress is a common factor in many aspects of cardiovascular disease [1]. Specifically, reactive oxygen species (ROS) impair vascular relaxation and promote apoptosis of endothelial cells, augment expression of adhesion molecules and also lead to the proliferation, hypertrophy and migration of smooth muscle cells, which contribute to development of hypertension and atherosclerosis [2].

Thromboxane A<sub>2</sub> (TxA<sub>2</sub>) is a major, unstable arachidonic acid metabolite, and plays a key role in normal physiology [3] but is additionally implicated in many pathological states such as unstable coronary artery disease and severe unstable angina [4]. TxA<sub>2</sub> is an agonist for the G protein-coupled thromboxane receptor, of which two variants, TPα (343 amino acids) and TPβ (407 amino acids), arise by alternate splicing of transcripts derived from a single gene [5] and exhibit distinct tissue expression profiles. There are clear differences between the mechanisms of downstream signaling for these two isoforms. Both TPα and TPβ

are coupled to downstream signaling pathways via interaction with predominantly Gq11, and exhibit complex interactions, including activation of protein kinase C, RhoA [6] and AMP-activated protein kinase [7] and can stimulate release of intracellular calcium stores. Regulation of TP signaling itself is similarly complex, with multiple kinase-mediated pathways implicated in receptor desensitization, while oligomerization of the distinct TP splice variant products has also been implicated in modulating function [8,9].

Here we examined the effects of oxidative stress on the thromboxane receptor to understand the molecular and cellular consequences of exposure to ROS on thromboxane receptor activity. Previously, using COS-7 cells that naturally express TP receptor, we demonstrated that brief exposure to hydrogen peroxide, as a model for acute oxidative stress, significantly increased translocation of TPβ from the ER to the Golgi complex [10]. Translocation of TPα from the ER to the Golgi complex as a consequence of TP activation via a reactive oxygen species-dependent mechanism has also recently been reported [11]. TPβ

is rapidly turned over in the ER, while TP $\beta$  degradation is modulated following exposure to hydrogen peroxide and was most likely due to activation of the unfolded protein response (UPR), resulting in increased ER folding efficiency of TP $\beta$ , and subsequent exit from the ER, effectively removing the polypeptide from the ER-associated degradation system [10]. The TP $\alpha$  splice variant, despite differential tissue expression and signalling properties, undergoes a similar translocation and stabilization essentially indistinguishable from the  $\beta$ -splice variant. Further, four arginine residues between the C-terminal-most transmembrane region and the C-terminus of TP $\beta$  are required for responsiveness to hydrogen peroxide [12]. From these data it appeared that only a very limited population of thromboxane receptors existed on the plasma membrane of COS-7 and human coronary artery smooth muscle cells in the resting state. However, the role of the surface pool, and its capacity to initiate signal transduction were not investigated. The thromboxane receptor signals from the plasma membrane and therefore an understanding of the effects of oxidative stress on the cell surface population and its ability to mediate intracellular events is important.

The present study aimed specifically to determine if TP $\beta$  was efficiently transported to the plasma membrane following exposure to hydrogen peroxide. Further, we wished to determine the rate of translocation of TP $\beta$  to the cell surface, the stability, fate and functional competence of this pool and, most importantly, if the surface pool was capable of transducing a signal delivered by TP receptor agonists. Finally we also aimed to establish if hydrogen peroxide-stimulated trafficking of TP was specific or mirrored in the behaviour of additional prostanoid receptors. We demonstrate that oxidative stress results in rapid delivery of a large cohort of TP $\beta$  to the cell surface, that this cohort of TP $\beta$  is both highly stable and capable of mediating enhanced prostanoid responses and that not all prostanoid receptors respond to hydrogen peroxide by increased presence at the plasma membrane.

## Materials and Methods

### Materials

HEK293 and PC3 cells were kind gifts from David Guiliano and Charlotte Bevan (Imperial College, London, UK). The mammalian expression vector pcDNA3.1, Dulbecco's-modified Eagle's medium (DMEM), foetal calf serum, penicillin/streptomycin solution, Fungizone and trypsin-EDTA were all purchased from Invitrogen. TP receptor polyclonal antibody, which recognizes both TP $\alpha$  and TP $\beta$  isoforms, was purchased from Cayman Chemical, Estonia. Mouse anti-HA monoclonal antibody and anti-ribosomal S6 goat polyclonal antibody were purchased from Santa Cruz Chemicals. Mouse monoclonal anti-Na<sup>+</sup>-K<sup>+</sup> ATPase was from AbCam, Cambridge, UK. Fluorescein-conjugated goat anti-mouse polyclonal antibody and goat anti-mouse horseradish peroxidase conjugate were from Sigma. Cell permeant Fura-Red acetoxymethyl ester was obtained from Invitrogen and the amine reactive EZ-Link Sulfo-NHS-LC-Biotin from Pierce. U44619 and 8-iso PGF<sub>2 $\alpha$</sub>  were obtained from Cayman Chemicals. Unless otherwise stated, all other reagents were from Sigma.

### Generation of epitope-tagged thromboxane receptor

Human TP $\beta$  cDNA was amplified by PCR with HindIII and EcoRI restriction sites using the following primers CGAAGCT-TATGTGGCCCAACGGCAGT and CGCAGTGAATTCCG-CCTGTAATCCC AG (restriction sites underlined, 5' to 3'). The product of the PCR reaction was subcloned into HA-tagged pcDNA3.1 using HindIII and EcoRI restriction sites. Correct orientation of the insert was confirmed by sequencing.

Stable expression of HA-TP $\beta$  in HEK293 cells and PC3 culture. To create a stable HEK293 cell line over-expressing HA-TP $\beta$ , cultures were transfected with ScaI linearized pcDNA3.1-HA-TP $\beta$  using Escort V reagent, according to the manufacturer's guidelines (Sigma). Following a 24 hour incubation period the cells were re-seeded onto 48 well plates containing DMEM supplemented with G418 (800  $\mu$ g/ml). The cultures were incubated for three weeks during which time the G418-supplemented medium was replaced every three days. G418 resistant colonies were then expanded into 48 well plates. Routinely, HEK293 cells stably expressing HA-TP $\beta$  were maintained in DMEM supplemented with 800  $\mu$ g/ml G418, 10% serum, 1% penicillin/streptomycin and 25  $\mu$ g/ml Fungizone in a humidified atmosphere 95% air and 5% CO<sub>2</sub> at 37°C. Upon reaching ~80% confluence, the cells were recovered with 0.05% trypsin-EDTA solution and reseeded into 25 cm<sup>2</sup> flasks at a dilution of 1:10. This reliably yielded cultures of 60–70% confluence after a 24 hour incubation period. PC3 cells were cultured in phenol red-free DMEM supplemented with 10% FCS at 37°C in 5% CO<sub>2</sub>.

### Confocal microscopy

HEK293 cells stably expressing HA-TP $\beta$  or PC3 cells were grown on glass coverslips and fixed with 3.7% paraformaldehyde for 30 minutes at room temperature. Non-specific labelling was blocked by incubation for one hour in blocking buffer (1% (w/v) bovine serum albumin in PBS) at room temperature. All subsequent labelling was carried out in blocking buffer (primary antibody 1:500 and secondary 1:250). Cells were viewed under a Leica SP2 upright microscope and the images processed in LCS Light (Leica).

### Cell surface biotinylation

Cell surface expression of TP $\beta$  was assayed by the biotinylation of surface proteins using the membrane impermeable, amine-reactive biotin analogue SS-biotin (Pierce). Cultures were cooled on ice and washed three times with ice-cold biotinylation buffer (phosphate buffered saline, 15 mM glucose pH 8.0). Biotinylation was carried out using 0.5 mg/ml biotin in biotinylation buffer for 1 hour on ice. Following one wash with ice-cold biotinylation buffer the biotin was quenched with two washes of quenching buffer (phosphate buffered saline supplemented with 10 mM Tris, 100 mM glycine, pH 8.0). Cell lysis was achieved by addition of RIPA buffer to the monolayers (1% Triton X-100 (v/v), 1% sodium deoxycholate (w/v), 0.1% SDS (w/v), 150 mM NaCl, 5 mM EDTA, in PBS, pH 7.5). Following a 15 minute incubation on ice, the cell lysate was cleared by centrifugation at 14 000 rpm for 10 minutes in an Eppendorf 4417C microcentrifuge. The cleared lysates were then incubated for 1 hour with 20  $\mu$ l streptavidin beads (Sigma) with end-over-end rolling. The streptavidin beads and the supernatant were separated by brief centrifugation and the beads washed three times in lysis buffer. Proteins in the supernatant were precipitated with 15% (w/v) trichloroacetic acid followed by incubation on ice for an hour. The precipitate was recovered by centrifugation at 14 000 g for an hour in a 4417C microcentrifuge (Eppendorf). The resulting pellets were washed with ice-cold 90% acetone to remove excess trichloroacetic acid. The precipitated protein and the streptavidin beads were heated to 95°C for 5 minutes in the presence of SDS-PAGE loading buffer and loaded onto discontinuous 12% SDS-PAGE gels.

### Western blotting

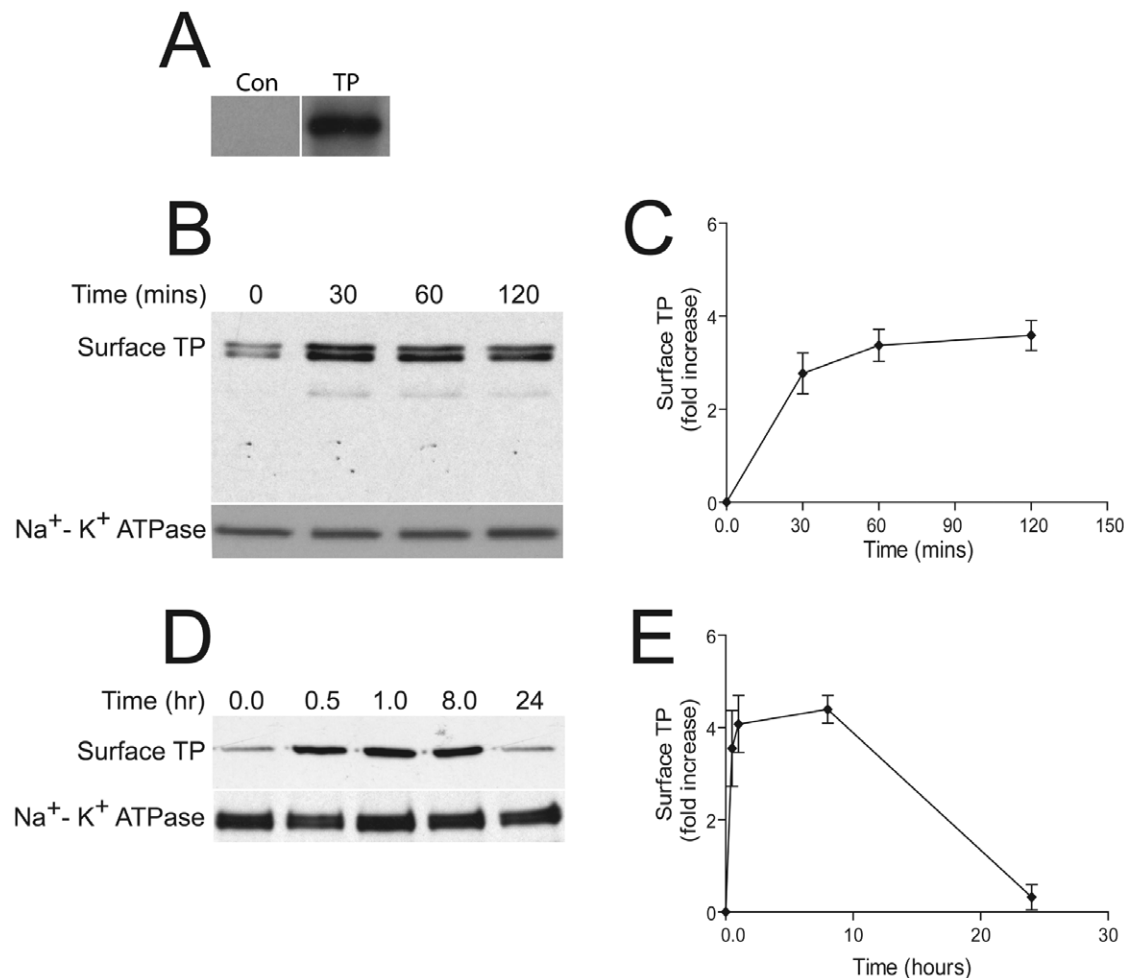
Proteins were transferred onto polyvinylidene fluoride membrane (BioRad, Hercules, California) in transfer buffer (25 mM Tris, 192 mM glycine and 20% (v/v) methanol) at 10 volts overnight. The membrane was subsequently blocked for 1 hour with 5% (w/v) non-fat milk in Tris-buffered saline supplemented

with 0.05% (v/v) Tween. Both primary and secondary antibodies were used at a concentration of 1:5000 for one hour with gentle rocking. Detection was carried out by enhanced chemiluminescence followed by exposure to Amersham Hyperfilm. Blots were quantified in ImageJ 1.34s (NIH, USA). HA-TP $\beta$  expression was compared by one-way ANOVA with the Bonferroni post test. A P value <0.05 was taken to be statistically significant.

### Calcium mobilization studies

HEK293 cells stably expressing HA-TP $\beta$  were plated onto ibidi 8 well  $\mu$ -Slides (Thistle Scientific, Glasgow). Measurement of [Ca<sup>2+</sup>]<sub>i</sub> was performed using a ratiometric method with Fura-Red, a dual excitation probe [13]. Loading was carried out for 30 minutes in loading buffer (20  $\mu$ M Fura-Red, 0.1% (w/v) pluronic F127, 10 mM glucose, 1% (v/v) foetal calf serum in PBS) followed

by two washes. All incubations were carried out at 37°C. In experiments testing the effect of hydrogen peroxide, 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> was added with the Fura-Red. At the end of the incubation period agonist (U46619 or 8-iso PGF<sub>2 $\alpha$</sub> ) was added, calcium mobilization was visualized under a Zeiss Axiovert 200 widefield microscope (excitation 460 and 490 nm emission 590 nm) and the data processed using Volocity 4 software (Improvision, UK). In some experiments a second administration of agonist was given. In these experiments cells were washed three times with loading buffer and re-incubated in the presence of 10  $\mu$ M H<sub>2</sub>O<sub>2</sub>. In each experiment the second U46619 concentration was the same as the first, i.e. either 1, 30 or 100 nM. Maximal response (R<sub>max</sub>) was measured by the addition of 0.2% (v/v) Triton X-100 and minimal response (R<sub>min</sub>) by the addition of 2 mM EGTA. The ratio of the fluorescence at 460 and 490 nm is a measure of [Ca<sup>2+</sup>]<sub>i</sub> assuming



**Figure 1. Effects of hydrogen peroxide exposure on cell-surface expression of TP $\beta$ .** Panel A: HA-TP $\beta$  is detected only in transfected HEK293 cells. Cell-surface TP $\beta$  was labeled using a water-soluble biotin analogue and recovered with streptavidin beads. Streptavidin-bound proteins were analysed by immunoblotting using anti-HA antibodies. Lane 1 (Con) is the nontransfected negative control and lane 2 (TP) is transfected cells. Note the blot is from the same membrane but lanes have been rearranged for presentation only, and the greater density of the band here compared to panels B and C is due to longer exposure to enhance the negative control. Panels B-E: HEK293 cells stably expressing TP $\beta$  were incubated in DMEM, stimulated with 10  $\mu$ M (final) hydrogen peroxide and then incubated for the times indicated. Cell-surface TP $\beta$  was detected as for panel A. Equal loading was confirmed by re-probing of membranes with antibodies against the Na<sup>+</sup>-K<sup>+</sup> ATPase. Cell-surface levels were monitored over two hours (Panel B) or up to 24 hours (Panel D). Note doublet on panel B is due to a loading artifact from high volume samples and is seen only in some blots and does not indicate the presence of an additional form of TP $\beta$ . Panels C and E represent quantified data for panels B and D respectively where the values are means  $\pm$  SEM of three independent experiments. Note that these graphs report 'fold increase' in surface TP expression; 0 represents no change from resting state expression. These data show that cell-surface HA-TP $\beta$  increases following H<sub>2</sub>O<sub>2</sub> exposure ~3.5 fold-increase after 2 hours. The extended time course in panels D and E demonstrates the H<sub>2</sub>O<sub>2</sub>-induced augmentation of cell-surface is sustained for at least 8 hours declining to resting values by 24 hours.

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the  $K_d$  of Fura-Red is 222 nM. Sigmoidal concentration-response curves were fitted using Prism 4 (GraphPad Software, Inc). Curves obtained in the presence and absence of hydrogen peroxide were compared by two-way ANOVA with the Bonferroni post test. A P value  $<0.05$  was taken to be statistically significant.

## Results

### Exposure to hydrogen peroxide leads to a rapid and persistent increase in TP $\beta$ receptor expression at the cell-surface

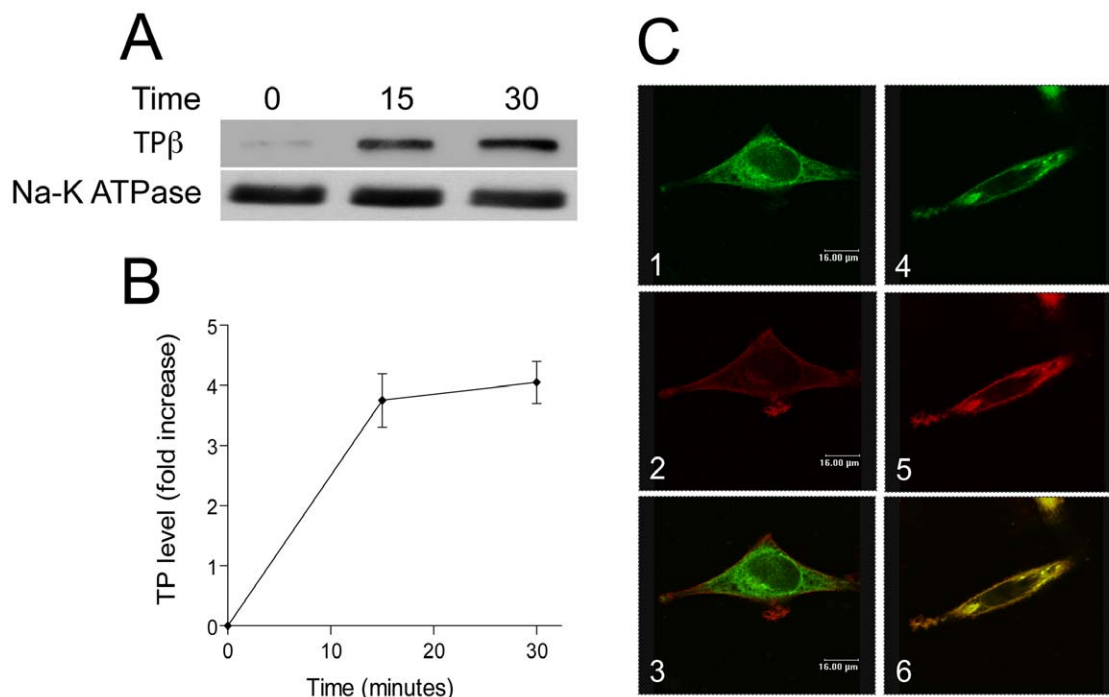
HEK293 cells express no detectable amounts of endogenous TPs but have been used as a relevant cellular model for study of TP $\beta$  behavior previously [14,15,16]; hence we selected this cell line for much of our analysis.

Cell surface expression of HA-tagged TP $\beta$  (HA-TP $\beta$ ) was quantitated in HEK293 cells stably expressing HA-TP $\beta$  by surface biotinylation using a membrane impermeable biotin reagent followed by Western blotting to quantitate levels of biotin-derivatized receptor (Figure 1). In untransfected cells no HA-reactive material was detected by Western analysis of biotinylated material, but in contrast a clear band corresponding to the predicted molecular weight at  $\sim 65$  kDa of biotinyl-HA-TP $\beta$  was detected in transiently-transfected HEK293 cells (Figure 1A). This is consistent with the reported apparent molecular weight of TP $\beta$ , and also with our previous studies in COS cells [10]. When HEK293 cells were challenged with a single low level dose of H<sub>2</sub>O<sub>2</sub> (10  $\mu$ M final), initially comparatively low cell-surface expression of HA-TP $\beta$  was detected (Figure 1B and C). Strikingly, HA-TP $\beta$  levels increased at the cell-surface during the following

60 minutes ( $3.4 \pm 0.34$  fold increase,  $n = 3$ ) and remained elevated at two hours post-H<sub>2</sub>O<sub>2</sub> treatment ( $3.6 \pm 0.32$  fold increase,  $n = 3$ ). To determine for how long increased surface expression of TP $\beta$  persisted an extended kinetic analysis of these cells, up to 24 hours post H<sub>2</sub>O<sub>2</sub> challenge, was performed. These data indicated that the increase in cell-surface HA-TP $\beta$  was sustained for at least eight hours ( $4.3 \pm 0.3$  fold increase,  $n = 3$ ) following H<sub>2</sub>O<sub>2</sub> treatment and declined to steady state levels within 24 hours (Figure 1D and E).

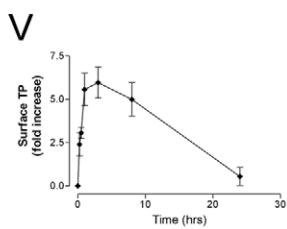
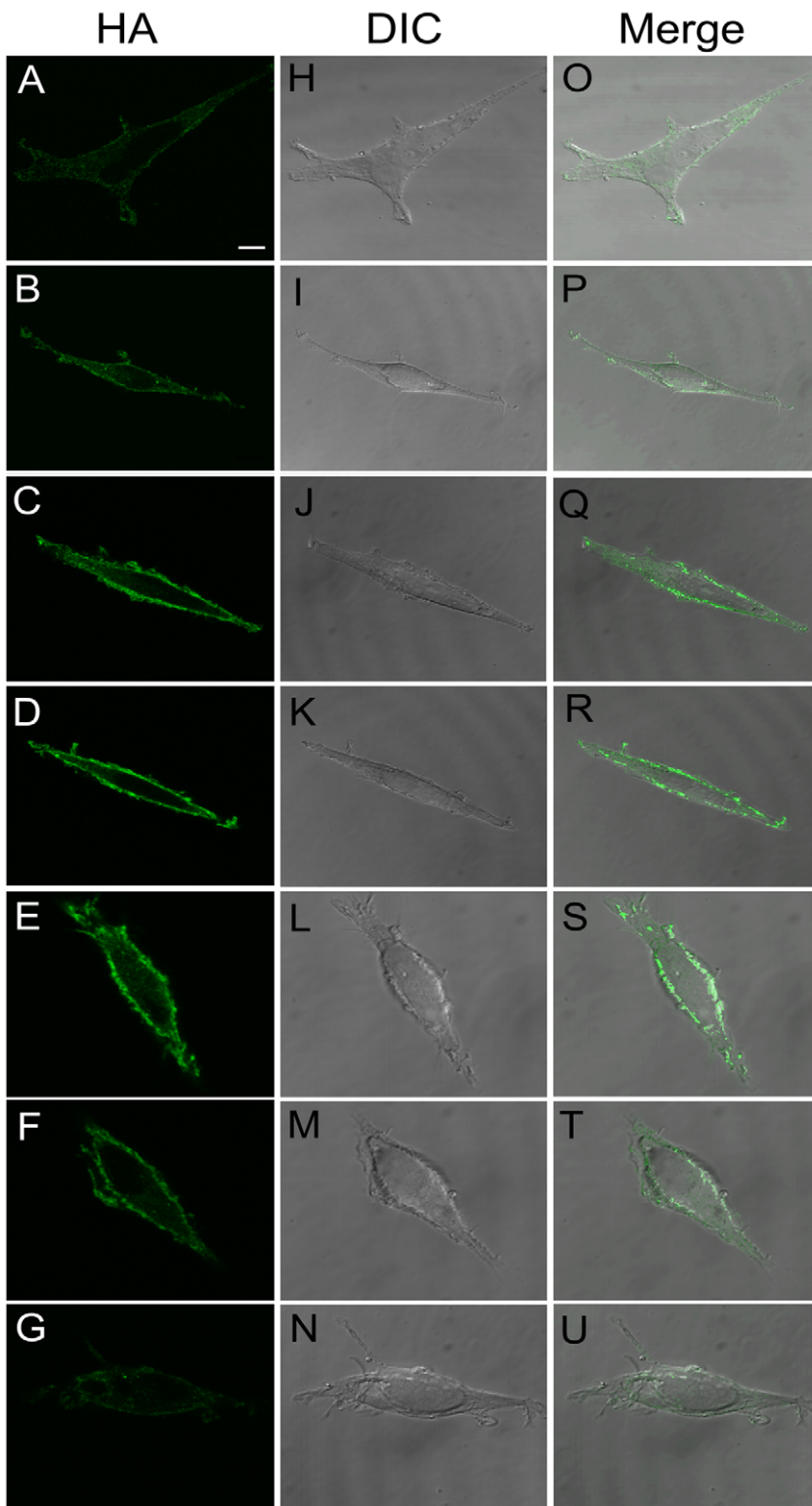
PC3 cells express a large intracellular pool of thromboxane receptor and both the TP $\alpha$  and TP $\beta$  isoforms are endogenously expressed, allowing examination of the effects of H<sub>2</sub>O<sub>2</sub> on endogenous receptor and comparisons to HA-tagged TP $\beta$  expressed in HEK293 cells [6]. Exposure of PC3 cells to H<sub>2</sub>O<sub>2</sub> resulted in an increase of endogenous TP $\beta$  at the cell-surface over 30 minutes, with strikingly similar kinetics to the behavior of HA-tagged TP $\beta$  expressed in HEK293 cells (Figure 2). These observations were confirmed by quantitation of the levels of biotin-captured TP $\beta$  and also by demonstration of translocation to the surface of TP $\beta$ , using confocal microscopy (Figure 2).

Using confocal microscopy and indirect immunofluorescence of nonpermeabilized cells, a kinetic analysis of surface fluorescence demonstrated rapid and persistent increased detection of the HA-epitope in HEK293 cells expressing HA-TP $\beta$  and following exposure to H<sub>2</sub>O<sub>2</sub> (Figure 3A–U). The HA-epitope, which is located at the N-terminus of the TP $\beta$  construct is exposed to the surface and hence accessible to antibody added to nonpermeabilized HEK293 cells. Quantification of the FITC-fluorescence intensity associated with the increased surface expression of TP $\beta$  (Fig. 3 panel V) confirmed that HA-TP $\beta$  increased at the plasma membrane within 30 minutes of exposure to oxidative stress ( $3.1 \pm 0.7$  fold increase,  $n = 5$ ). Peak



**Figure 2. Effects of hydrogen peroxide on surface expression of endogenously expressed TP in PC3 prostate carcinoma cells.** Panel A: TP $\beta$  detection on the surface of PC3 cells. Endogenous TP $\beta$  was labeled using a water-soluble biotin analogue and recovered with streptavidin beads at the cell surface following challenge with 10  $\mu$ M hydrogen peroxide. Streptavidin-bound proteins were analysed by immunoblotting. Panel B: Quantitation of delivery of TP $\beta$  to the cell surface from two separate experiments, demonstrating rapid increase of streptavidin-accessible molecules. Panel C: Confocal imaging of TP translocation to the cell surface. Panels 1 to 3 are resting cells and 4 to 6 following hydrogen peroxide challenge at 40 minutes. Panels 1 and 4 are stained for TP, 2 and 5 for TRITC-phalloidin to visualize cortical actin, and 3 and 6, are merged images. Scale bar is 16  $\mu$ m.

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**Figure 3. Confocal microscopy demonstrates increased cell-surface expression of HA-TP $\beta$  after hydrogen peroxide stimulation.** HEK293 cells stably expressing HA-TP $\beta$  were visualized on glass-coverslips by staining with an anti-HA murine monoclonal and FITC-labelled goat anti-mouse antibodies under non-permeabilizing conditions. Indirect immunofluorescence images were collected using a Leica SP2 upright confocal microscope at times 0, 15, 30, 60 minutes and 3, 8 and 24 hours post a single challenge with hydrogen peroxide (10  $\mu$ M) treatment (panels A–G). DIC images were also collected (H–N) and processed in Leica confocal software Lite to generate overlay images (O–U). Panel V represents the quantified confocal data (means  $\pm$  SEM,  $n = 5$ ). Fluorescence was quantitated in ImageJ 1.34s (NIH, USA) and normalized to  $t = 0$ . These data demonstrate cell-surface HA-TP $\beta$  increases  $\sim 5$ -fold following hydrogen peroxide stimulation and predominates for approximately 8 hours, returning to non-stimulated levels 24 hours post-stimulation. Scale bar = 5  $\mu$ m.  
doi:10.1371/journal.pone.0012798.g003

surface expression was observed within 60 minutes ( $5.5 \pm 0.9$  fold increase  $n = 5$ ), which slowly declined to resting state levels within 24 hours. These data correlate very well with the biotinylation studies and provided an independent method for determination of cell surface expression of HA-TP $\beta$ .

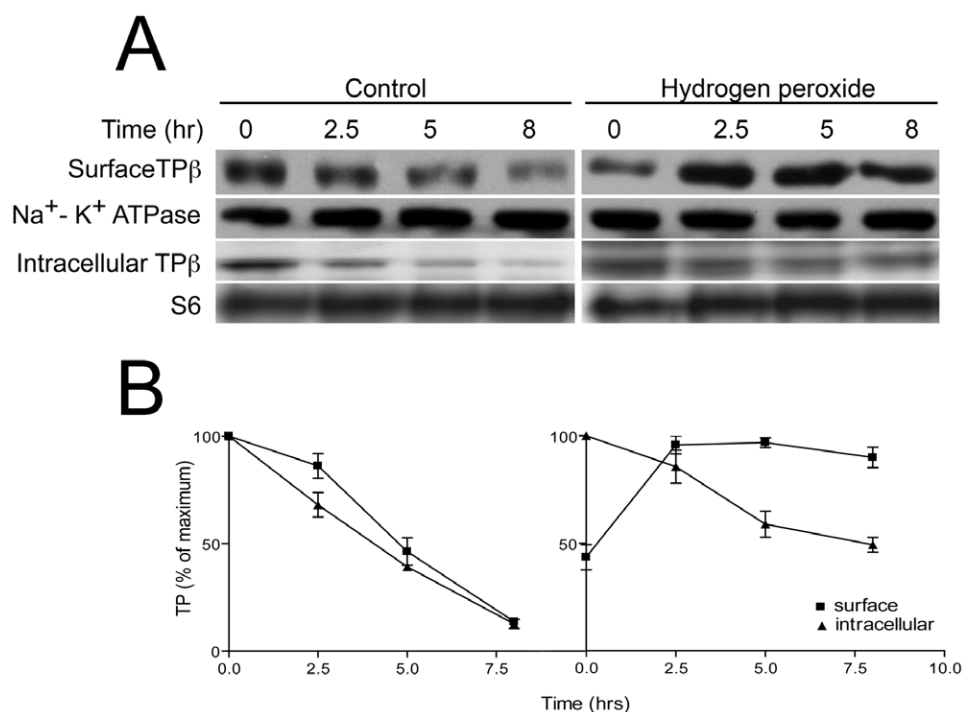
#### Increased TP $\beta$ expression at the cell surface is accompanied by concomitant loss from internal pools

HEK293 cells stably expressing HA-TP $\beta$  were pre-dosed for 30 minutes with cycloheximide, or vehicle alone (final concentration 0.0004% (v/v) ethanol) and cell-surface HA-TP $\beta$  was labelled using a water-soluble biotin analogue and captured with streptavidin beads as before. The remaining intracellular fraction was precipitated using trichloroacetic acid (Fig. 4A and B). Na<sup>+</sup>-K<sup>+</sup> ATPase and ribosomal S6 protein were used as loading controls for biotin accessible and inaccessible fractions respectively. In

addition the biotinylated fractions were re-probed with the anti-S6 antibody; no reactivity was detected, ruling out non-specific cell lysis during the procedure (data not shown). In the absence of H<sub>2</sub>O<sub>2</sub>, cell-surface HA-TP $\beta$  decreased to  $\sim 20\%$  ( $n = 3$ ) of resting levels after 8 hours (Figure 4A). This observation contrasts to the substantial H<sub>2</sub>O<sub>2</sub>-induced cell-surface HA-TP $\beta$  increase, which, in the presence of cycloheximide, was sustained over 8 hours (Figure 4B). Similarly, intracellular HA-TP $\beta$  was more rapidly degraded in the absence of H<sub>2</sub>O<sub>2</sub>, decreasing to  $\sim 20\%$  ( $n = 3$ ) after eight hours, compared to nearly 50% ( $n = 3$ ) in the H<sub>2</sub>O<sub>2</sub> treated cells.

#### Increased TP $\beta$ surface expression is sensitive to brefeldin A

HEK293 cells expressing HA-TP $\beta$  were treated with cycloheximide and H<sub>2</sub>O<sub>2</sub>, and the effect of brefeldin A on surface delivery



**Figure 4. Kinetic analysis of intracellular and cell-surface HA-TP $\beta$  levels following hydrogen peroxide exposure.** Cycloheximide (30  $\mu$ M) was added to HEK293 cells stably expressing HA-TP $\beta$  and kinetic studies performed in the absence and presence (Panel A) of hydrogen peroxide (10  $\mu$ M). At times 0, 2.5, 5 and 8 hours following addition of cycloheximide and hydrogen peroxide the cultures were placed on ice and cell-surface proteins labelled with a water-soluble biotin analogue. Labelled cell-surface HA-TP $\beta$  was recovered by incubating with streptavidin beads and the remaining intracellular fraction precipitated using trichloroacetic acid. Both fractions were analyzed using anti-HA murine monoclonal and HRP-conjugated secondary antibodies. Equal loading was confirmed by re-probing membranes with anti-Na<sup>+</sup>-K<sup>+</sup> ATPase antibody (surface) and anti-ribosomal S6 antibodies (total lysate). Re-probing the membrane fraction with S6 antibodies indicated the absence of any contamination by intracellular proteins, i.e. from cell lysis (data not shown). Panel B represents cell-surface HA-TP $\beta$  expression in the absence and presence of hydrogen peroxide, with data normalized to  $t = 0$ . In the absence of hydrogen peroxide, both cell-surface and intracellular HA-TP $\beta$  decline over 8 hours, but the hydrogen peroxide treated cultures demonstrate sustained HA-TP $\beta$  levels. The data are representative of the means  $\pm$  SE of three independent experiments.  
doi:10.1371/journal.pone.0012798.g004

assessed by biotinylation and Western analysis (Figure 5). In the absence of brefeldin A, and consistent with our previous studies, H<sub>2</sub>O<sub>2</sub> induced increased expression of HA-TP $\beta$  at the cell surface. By contrast, pretreatment of cells with brefeldin A abolished this cell-surface HA-TP $\beta$  delivery. This suggests that transit through the Golgi complex is required for surface delivery, and is consistent with our earlier data that demonstrated translocation of the ER pool to the Golgi complex rapidly following H<sub>2</sub>O<sub>2</sub> stimulation. Taken together with the data above, these observations indicate that H<sub>2</sub>O<sub>2</sub> leads to translocation of TP $\beta$  into the Golgi apparatus, and that this pool is then routed to the cell surface.

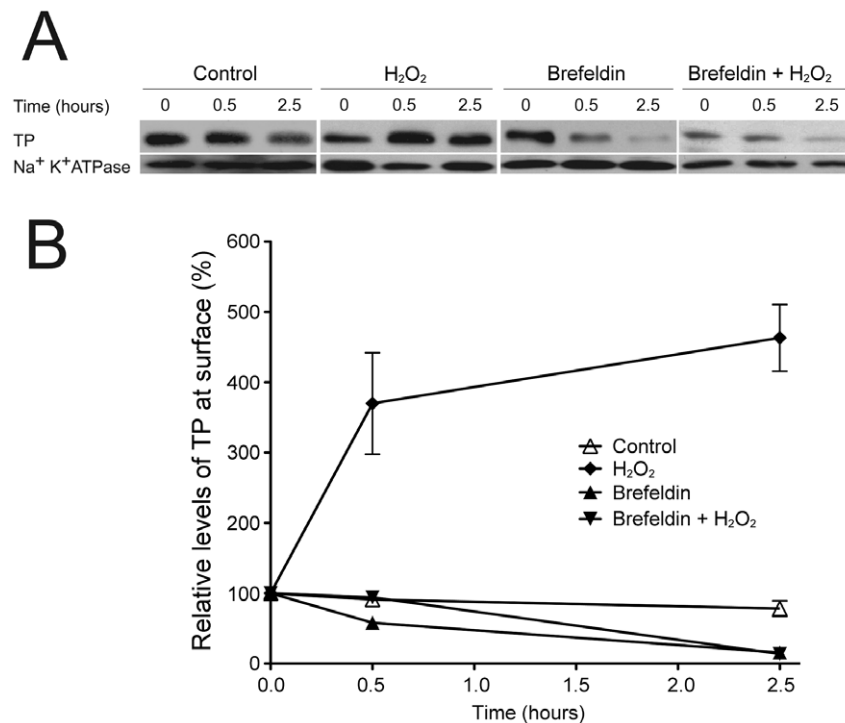
### Involvement of the proteasome in cell-surface expression of TP $\beta$

HEK293 cells stably expressing HA-TP $\beta$  were treated with lactacystin, an irreversible inhibitor of the proteasome, in the presence of cycloheximide. Cell-surface HA-TP $\beta$  was assayed at different time periods with a membrane impermeable biotin analogue both in the absence and presence of H<sub>2</sub>O<sub>2</sub> and recovered with streptavidin beads (Figure 6). The duration of the experiment was limited to 2.5 hours (more prolonged periods manifested toxic effects from the combined drug treatment). TP $\beta$  recovery was normalised using Na<sup>+</sup>-K<sup>+</sup> ATPase antibodies as loading control. Interestingly, after 2.5 hours, lactacystin treatment alone produced a modest ~2-fold increase in HA-TP $\beta$  at the cell-surface, reflecting considerable proteasomal-dependent degradation of the receptor even in the resting state, but consistent with turnover of intracellular pools by ER-associated degradation (ERAD). When cells were challenged with H<sub>2</sub>O<sub>2</sub>

together with lactacystin, a dramatically increased level of TP $\beta$  was detected at the cell surface, approaching eight-fold at 2.5 hours. As this increase is significantly greater than that observed in the absence of lactacystin these data suggest that the proteasome contributes to turnover of TP $\beta$ , and potentially turnover from the surface pool.

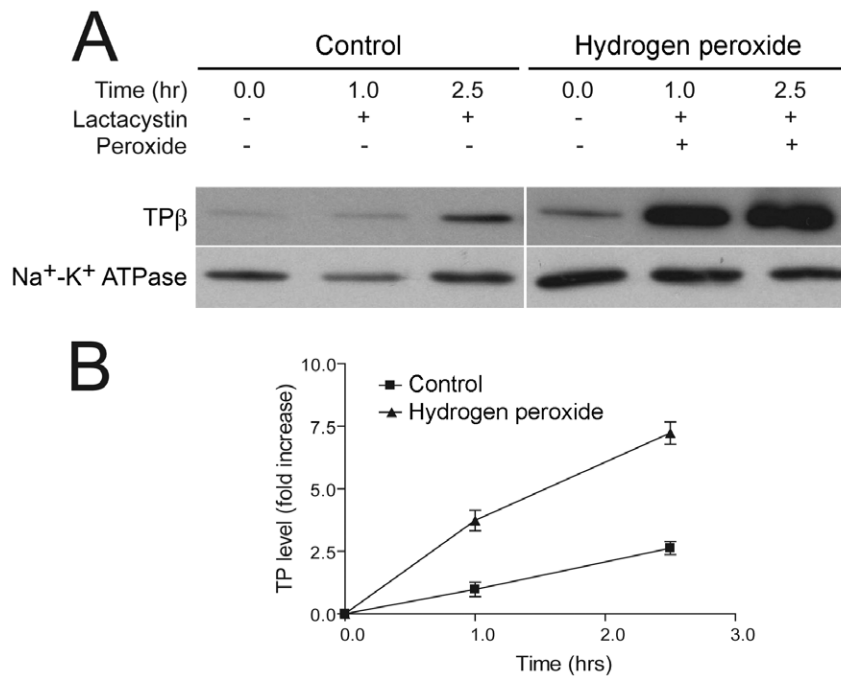
### H<sub>2</sub>O<sub>2</sub> exposure induces translocation of functional thromboxane receptor to the plasma membrane

To determine if the increased pool of TP $\beta$  was functional and capable of signal transduction, we analysed calcium mobilization in HEK293 cells stably expressing HA-TP $\beta$ . We have previously shown that in COS-7 cells H<sub>2</sub>O<sub>2</sub> results in an increase in the number of thromboxane-binding sites in whole cell extracts, but did not demonstrate the ability to engage in downstream signaling [10]. Cells were preloaded with FuraRed in the presence of the mild detergent pluronic F127. Preincubation of non-transfected HEK293 cells with the TP $\beta$  antagonist SQ29548 or the TP $\beta$  agonist U46619 confirmed there was no significant background Ca<sup>2+</sup> flux (data not shown), consistent with a low level of expression of TP $\beta$  in these cells. The effects of U46619, a thromboxane A<sub>2</sub> mimetic, and 8-iso PGF<sub>2 $\alpha$</sub>  on calcium mobilization at agonist concentrations between 1 nM and 1  $\mu$ M were examined in the absence and presence of H<sub>2</sub>O<sub>2</sub> in the HEK293 cells stably expressing HA-TP $\beta$  (Fig. 7). Following addition of agonist a significant and transient rise (~75 seconds) in intracellular calcium was observed (Fig. 7 inset). The increase in intracellular calcium was far greater than in control cells in the absence of hydrogen



**Figure 5. Increased surface expression of TP $\beta$  is sensitive to brefeldin A.** Kinetic analysis of HEK293 cells stably expressing HA-TP $\beta$  were performed in the absence and presence of brefeldin A. Cultures were treated with cycloheximide (30  $\mu$ M) and brefeldin A (10  $\mu$ M) prior to the addition of hydrogen peroxide (10  $\mu$ M). At times 0, 0.5 and 2.5 hours post hydrogen peroxide exposure, cell-surface proteins were labeled with a water-soluble biotin analogue for 30 minutes on ice and recovered on streptavidin beads. TP $\beta$  was analysed by immunoblotting with anti-HA monoclonal antibodies. Panels are representative data from three independent experiments where loading was controlled by reprobing membranes with anti-Na<sup>+</sup>-K<sup>+</sup> ATPase antibodies.

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**Figure 6. Turnover of TPβ and control of cell-surface expression requires proteasomal function.** Inhibition of the proteasome in HEK293 cells stably expressing HA-TPβ was achieved by incubating the cultures with lactacystin (1 μM) and cycloheximide (30 μM) both in the absence and presence of H<sub>2</sub>O<sub>2</sub> (Panel A). At times 0, 1 and 2.5 hours, cell-surface HA-TPβ was labelled with a water-soluble biotin analogue and bound to streptavidin beads. Recovered HA-TPβ was probed by Western blotting with anti-HA antibodies. Quantified data (Panel B), normalized to control cells suggest that the proteasome plays a role in the negative regulation of cell-surface HA-TPβ during oxidative stress. Data are representative of the means ± SEM of three independent experiments where the presence of hydrogen peroxide caused a significant increase in HA-TPβ (P<0.05) at 2.5 hours (\*) compared to the non-H<sub>2</sub>O<sub>2</sub> group. doi:10.1371/journal.pone.0012798.g006

peroxide (Supplementary Figure S1). Both U46619 and 8-iso PGF<sub>2α</sub> at concentrations between 10 nM and 1 μM produced an increase in intracellular calcium concentration in non-H<sub>2</sub>O<sub>2</sub>-treated cells. However, in the presence of H<sub>2</sub>O<sub>2</sub>, this effect was markedly potentiated, suggesting that the increase in TPβ levels facilitated greater Ca<sup>2+</sup> mobilization (Fig. 7A and B).

Further experiments were carried out to investigate the effects of a second U46619 challenge 30 minutes following exposure to the initial stimulus. In non-H<sub>2</sub>O<sub>2</sub> treated cells, exposure to a second U46619 challenge failed to elicit an observable calcium response consistent with normal downregulation of responsiveness (Fig. 8A). Unexpectedly, in the presence of H<sub>2</sub>O<sub>2</sub> a second administration of U46619 produced a highly unusual and marked potentiation of calcium mobilization (Fig. 8A and B). It should be noted that the repeat stimulation experiment was performed after translocation of TPβ to the cell surface is complete, i.e. 60 minutes (Figures 1 and 3), and therefore the increased second response cannot be ascribed alone to a further increase in the surface pool. These observations suggest that H<sub>2</sub>O<sub>2</sub> exposure results in an alteration to the TPβ microenvironment, such that the normal downregulation in Ca<sup>2+</sup> mobilization has been avoided, and which could potentially be due to a change to the turnover kinetics of TPβ. While it is possible that hydrogen peroxide affects calcium mobilization through a mechanism independent of the TPβ receptor, and that hydrogen peroxide alone is capable of inducing calcium mobilization [17,18,19], these earlier investigations used hydrogen peroxide concentrations very much higher than the current study and we observed that treatment with 10 μM hydrogen peroxide had no observable effect on calcium mobilization in the absence of TPβ agonist.

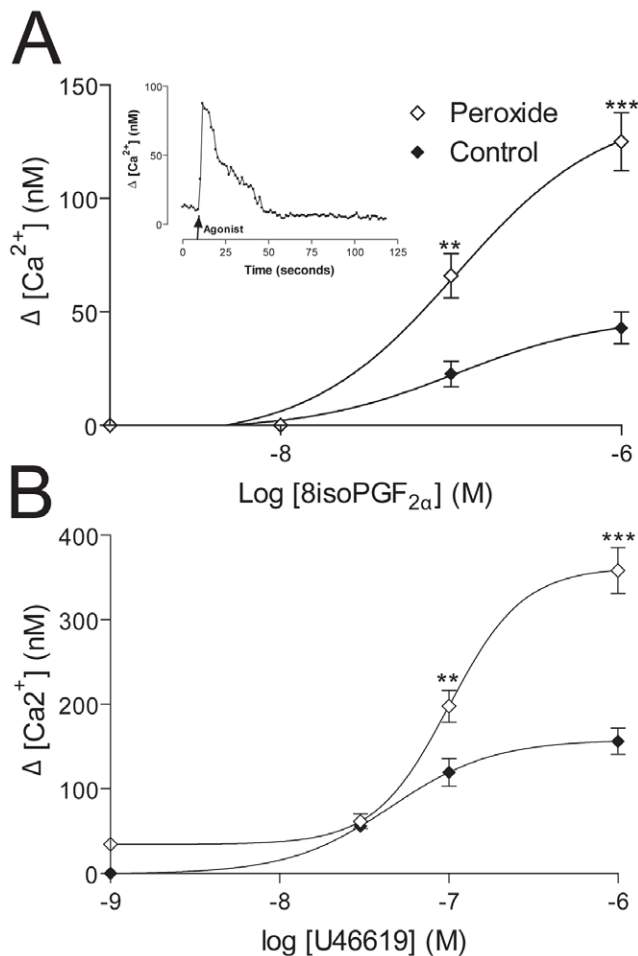
### H<sub>2</sub>O<sub>2</sub> exposure attenuates ligand-induced internalization of the thromboxane receptor

Ligand-induced internalization studies were performed both in the presence and absence of hydrogen peroxide in HEK293 cells stably expressing HA-TPβ. Determination of the surface expression of TPβ was once again performed by surface biotinylation and Western blotting, where protein synthesis was blocked by cycloheximide. Addition of H<sub>2</sub>O<sub>2</sub> was found to lead to an increase in detectable surface TPβ after two hours as before (Figure 9). By contrast, addition of U46619 was found to decrease the detectable surface TPβ, suggesting that in the resting state the receptor is internalized following ligand stimulation. More interestingly, this ligand-dependent internalization was abolished in the presence of hydrogen peroxide. This is consistent with the increased Ca<sup>2+</sup> mobilization observed on repeat ligand challenge and also with the long half-life of receptor at the cell surface following H<sub>2</sub>O<sub>2</sub> challenge (Figures 1, 2 and 3).

### Specificity in response to H<sub>2</sub>O<sub>2</sub> challenge amongst prostanoid receptors

To examine if the alterations in stability and location described here and previously [10] for both isoforms of TP following H<sub>2</sub>O<sub>2</sub> exposure were restricted to TP or extend to additional members of the prostanoid receptor family, we investigated the stability of EP isoforms 3 and 4 and also FP. We constructed HA-tagged expression vectors for these receptors and expressed them in HEK293 cells as before, and detected HA-reactive bands with migrations consistent with the predicted molecular weights in whole cell lysates (Figure 10 and data not shown). Analysis of the influence of H<sub>2</sub>O<sub>2</sub> on membrane-impermeable biotin-accessible levels of each receptor revealed a differential response to oxidative stimulus. Specifically,



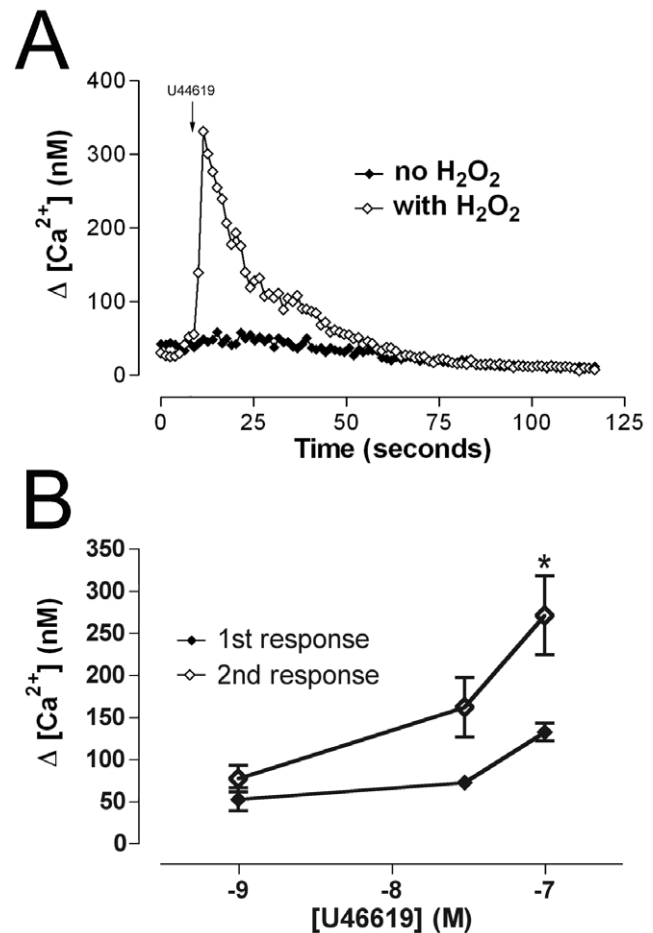


**Figure 7. Concentration-response effect of thromboxane mimetic U46619 and 8-iso PGF<sub>2α</sub> on calcium flux in HEK293 cells stably expressing HA-TPβ.** HEK293 cells were loaded with FuraRed (20 μM) for 30 minutes at 37°C. Cell visualization was performed using a Zeiss Axiovert 200 microscope and data processed using Volocity software (Improvision). Cytosolic calcium levels in response to stimulation were determined in the absence or presence of hydrogen peroxide (10 μM) and calculated as described under methods. Figure A inset represents a single trace following the addition of 8-iso PGF<sub>2α</sub> (nM). Following addition of agonist at the point indicated in the figure, a rapid increase in calcium concentration is observed (~100 nM) which is sustained for ~60 seconds. Similar response curves were generated for 8-iso PGF<sub>2α</sub> (Panel A) and U46619 (Panel B). Data represent the mean ± SEM (n = 7), \*\* P<0.01, \*\*\* P<0.001. doi:10.1371/journal.pone.0012798.g007

surface levels of neither EP3 nor EP4 were affected by exposure to H<sub>2</sub>O<sub>2</sub>, while by contrast levels of FP accessible to the surface probe were significantly and rapidly increased (Figure 10). We did not investigate the mechanism underpinning this increase or the persistence of the response further, but we consider it likely that the UPR mediates the increase in FP levels. Regardless of mechanism, these data clearly indicate that the expression levels of members of the prostanoid receptor family display differential sensitivity to cellular hydrogen peroxide exposure, providing potentially complex responses to oxidative stresses in vivo.

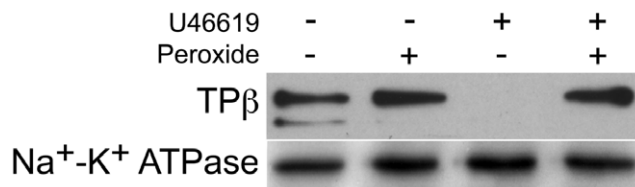
## Discussion

Thromboxane (TXA<sub>2</sub>) is a potent signaling molecule under normal physiological conditions, and is the product of a distinct



**Figure 8. Hydrogen peroxide exposure relieves receptor desensitization and leads to potentiation of calcium mobilization following repeat challenge by U46619.** HEK293 cells stably expressing HA-TPβ were loaded with FuraRed (20 μM) for 30 minutes at 37°C in the presence of pluronic F127. Cultures were given an initial challenge of U46619 (1, 30 and 100 nM) and visualized under a Zeiss Axiovert 200 microscope. Cytosolic calcium concentration was calculated as described in methods. Following the first challenge, cells were washed three times in recording buffer and incubated for a further 30 minutes at 37°C. A second challenge of U46619 was carried out as previously. Panel A is representative single traces demonstrating the effect of receptor desensitization in the presence and absence of hydrogen peroxide. Following an initial challenge of U46619 the second dose of ligand (100 nM) was added at the point indicated in the figure. In the absence of hydrogen peroxide an additional calcium transient was not detected, but by contrast, a calcium transient was observed in H<sub>2</sub>O<sub>2</sub>-treated cells. Panel B shows concentration-response data for the repeated U46619 challenge in the presence of hydrogen peroxide (10 μM). These observations were found to be significant (P<0.05) at 100 nM (\*), the data representing the mean ± SEM, n = 7. doi:10.1371/journal.pone.0012798.g008

range of cell types [20]. Tight regulatory mechanisms exist to attenuate signaling via TXA<sub>2</sub>, and most notably TXA<sub>2</sub> is rendered biologically inactive within 30 seconds of production [21]. In certain pathophysiological states, however, the effects of TXA<sub>2</sub> may become detrimental. Over-production of TXA<sub>2</sub> in the vasculature results in local vasoconstriction and platelet aggregation; the resulting impedance to blood flow can lead to life-threatening conditions such as myocardial infarction [22]. Concomitant oxidative stress also stimulates synthesis of another thromboxane receptor agonist, 8-iso prostaglandin F<sub>2α</sub>, one of the



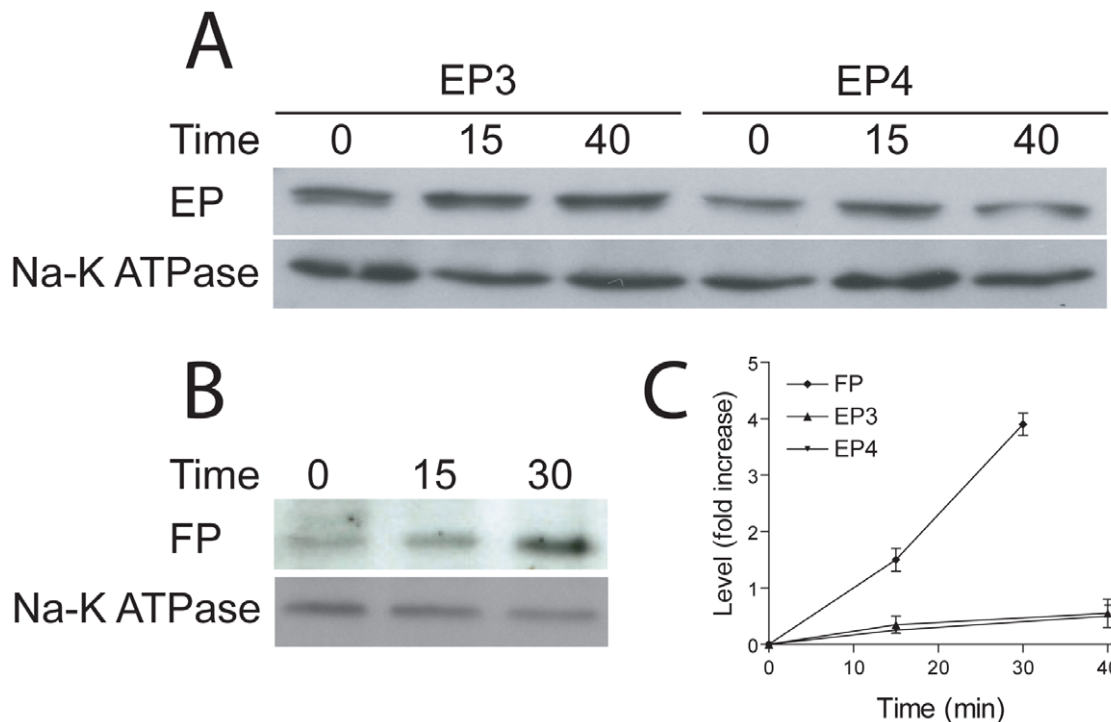
**Figure 9. Effect of hydrogen peroxide on ligand-induced internalization of TP $\beta$ .** HA-TP $\beta$  internalization in response to U46619 was studied in HEK293 cells stably expressing TP $\beta$ , both in the absence and presence of H<sub>2</sub>O<sub>2</sub>. Cultures were treated with H<sub>2</sub>O<sub>2</sub> (10  $\mu$ M) for 40 minutes prior to the addition of U46619 (600 nM) and incubated for a further 90 minutes at 37°C. Cell-surface proteins were labelled with a water-soluble biotin analogue followed by adsorption onto streptavidin beads. HA-TP $\beta$  was detected with anti-HA antibodies following electrophoresis and transfer to membrane and loading equivalence assessed by reprobing membranes with Na<sup>+</sup>-K<sup>+</sup> ATPase antibodies. Data are representative of three independent experiments. doi:10.1371/journal.pone.0012798.g009

more abundant isoprostanes formed in vivo [23] and present in atherosclerotic coronary arteries [24].

We previously demonstrated that the vasoconstrictive action of 8-iso prostaglandin F<sub>2 $\alpha$</sub> , is mediated via the TP receptor [25] and that the response to this isoprostane is enhanced by oxidative stress [26]. We hypothesized that this additional responsiveness to the isoprostane was due to an increased TP $\beta$  receptor reserve. More recently we provided empirical evidence to support this hypothesis by demonstrating that oxidative stress promoted TP $\beta$  receptor translocation from the ER to the Golgi complex, which was accompanied by stabilization [10]. The present study extends that

work by demonstrating that simulating oxidative stress in isolated cells by treatment with hydrogen peroxide causes an enhanced and prolonged increase in the response of TP $\beta$  receptor to ligand binding, and that this is most likely a result of an increase in cell surface TP $\beta$  receptor population mediated via cellular alterations of the TP $\beta$  receptor at multiple levels. We also provide evidence that a similar mechanism may exist for the FP prostanoid receptor, but that this does not extend to the EP3 and EP4 receptors, suggesting differential regulatory mechanisms within the overall prostanoid receptor family.

Initially we considered the surface pool of the TP $\beta$  receptor. Biotinylation and immunofluorescence microscopy demonstrated rapid and prolonged relocation of TP $\beta$  from the ER to the cell surface, maximal within 1 hour and persisting for 8 hours. Resting levels were restored after 24 hours. Therefore oxidative stress induced a rapid sustained upregulation of HA-TP $\beta$  at the cell-surface. Hydrogen peroxide has a short half-life in cell culture medium (up to 20 minutes) [27,28,29], hence we conclude that a single, brief challenge with hydrogen peroxide is able to trigger a prolonged change in cell surface expression of HA-TP $\beta$ . Secondly, by examining the effects of H<sub>2</sub>O<sub>2</sub> on PC3 cells, which endogenously express a large intracellular pool of both the TP $\alpha$  and TP $\beta$  isoforms [6], it was found that the endogenous TP receptor in PC3 cells accumulated at the cell surface upon exposure to H<sub>2</sub>O<sub>2</sub> with strikingly similar kinetics to HA-TP $\beta$  in transiently-transfected HEK293 cells. While expression of Na<sup>+</sup>-K<sup>+</sup> ATPase, used as the loading control in these experiments, can itself be modulated by ROS [30], there was no evidence for this in our experiments, and the concentrations of H<sub>2</sub>O<sub>2</sub> used here are very much lower than those required to elicit such an effect on the Na<sup>+</sup>-K<sup>+</sup> ATPase.



**Figure 10. Hydrogen peroxide treatment augments surface levels of some, but not all, prostanoid receptors.** Panels A and B: HA-tagged EP3, EP4 and FP transiently transfected into HEK293 cells, and surface levels quantitated by derivatizing with a water-soluble biotin analogue and recovered with streptavidin beads following challenge with 10  $\mu$ M hydrogen peroxide. The Na<sup>+</sup>-K<sup>+</sup> ATPase is used in all cases as a loading control. Panel C: Quantitation from two similar experiments for surface levels of EP2, EP3 and FP. There is a clear increase in FP levels, but not for EP2 or EP3.

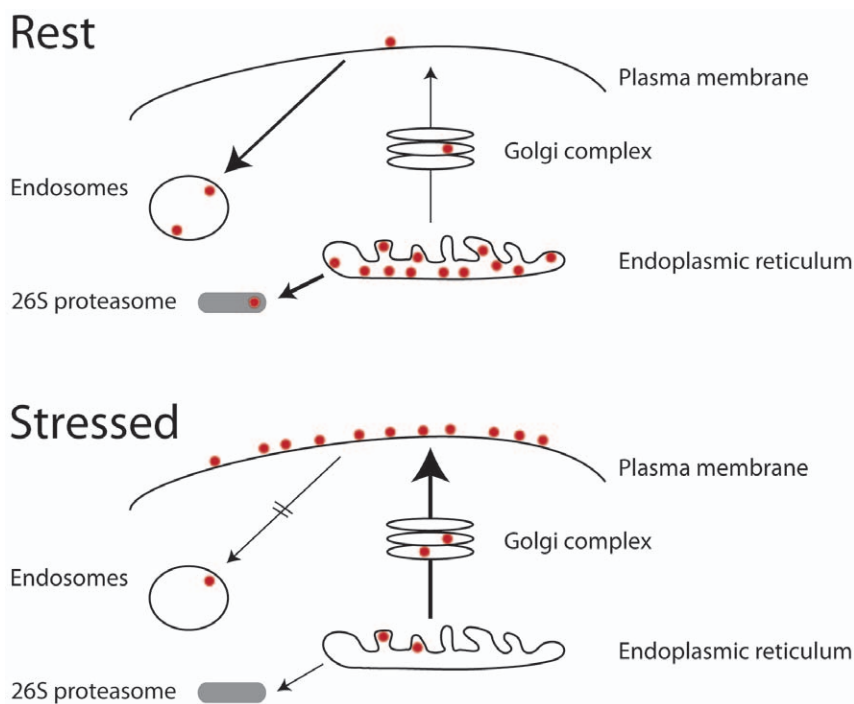
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Next we considered intracellular transport and turnover of TP $\beta$  at several levels. Firstly, both internal and surface pools of TP $\beta$  normally turn over with a half life of  $\sim 4$  hours in HEK293 cells, similar to  $\sim 2.5$  hours in COS-7 cells [10]. In cells exposed to H<sub>2</sub>O<sub>2</sub> there is significant stabilization of TP $\beta$  receptor and the intracellular half-life increases to  $>8$  hours. Again, the presence of increased receptor at the cell surface is detected with a concomitant decrease in the intracellular fraction. Secondly, translocation of TP $\beta$  to the surface was prevented by brefeldin A, an antibiotic with specific effects at the Golgi complex. [31,32]. As TP $\beta$  is translocated from endoplasmic reticulum-localized pools to structures colocalizing with Golgin-97 in COS-7 cells [10], together these data indicate that the ER pool of TP $\beta$  receptor is stabilized and delivered to the cell surface following H<sub>2</sub>O<sub>2</sub> stimulation by anterograde vesicular transport. Thirdly, in the presence of lactacystin, a modest  $\sim$ two-fold increase in TP $\beta$  receptor at the cell-surface suggests proteasomal involvement in ER-derived TP $\beta$  degradation [33]. While oxidative stress inactivates the proteasome a very prolonged incubation with hydrogen peroxide is required [34], and hence the single pulse used here is not expected to disrupt proteasome function. As quality control mechanisms prevent ER exit these data may indicate that additional receptor molecules can achieve the native state if degradation is prevented and the period available for folding extended. The almost eight-fold increase in TP $\beta$  receptor at the surface in cells treated with both H<sub>2</sub>O<sub>2</sub> and lactacystin suggests an important role for the proteasome in TP $\beta$  translocation, and possibly in turnover of surface pools. Therefore the proteasome degrades a significant population of TP $\beta$  and this accounts for ER retention and failure to reach the cell surface. Our

investigations also show that, not only is there slower turnover of cell-surface TP $\beta$  following exposure to hydrogen peroxide, but also that this population escapes ERAD. Finally, expression of the FP receptor also rapidly increases at the cell surface following H<sub>2</sub>O<sub>2</sub> exposure, most likely by a similar mechanism to TP, but that this does not extend to EP3 and EP4. Importantly, these data indicate both sequence-specific responses to H<sub>2</sub>O<sub>2</sub>, and also suggest that the physiological response of prostanoid receptors to oxidative stress exhibits unexpected specificity.

A model for these changes to TP $\beta$  trafficking behavior via exposure to ROS is shown in Figure 11. It is important to note that TP $\alpha$  and TP $\beta$  have distinct internalization characteristics and that these differences are imparted by their unique cytoplasmic tails. Agonist-induced internalization of TP $\beta$  is dynamin- and  $\beta$ -arrestin-dependent, suggesting clathrin-mediated endocytosis [35,36]. The Rab11 GTPase, which mediates endosomal recycling pathways, is also involved in control of cell-surface TP $\beta$  expression [37]. How H<sub>2</sub>O<sub>2</sub> interacts with any of these mechanisms to effect TP $\beta$  stabilization at the cell-surface remains to be understood, and while clearly an important aspect of TP $\beta$  receptor function, is beyond the scope of this study.

Finally, we sought to investigate directly the capability of the surface TP $\beta$  pool as a signaling platform. TP $\beta$  receptor agonists U46619 and 8-iso PGF<sub>2 $\alpha$</sub>  produced an increase in intracellular calcium concentration in resting cells, and the effect markedly potentiated following H<sub>2</sub>O<sub>2</sub> exposure. A second application of TP $\beta$  receptor agonist in the presence of H<sub>2</sub>O<sub>2</sub> potentiated the first response, in contrast to control cells where a second response to agonist was absent. Taken together, these data clearly demonstrate that the H<sub>2</sub>O<sub>2</sub>-augmented cell-surface TP $\beta$  pool is



**Figure 11. A model for intracellular transport of TP in resting and hydrogen peroxide-challenged cells.** At rest (upper panel), most TP is retained in the ER and degraded by the 26S proteasome following dislocation to the cytosol. The small quantities of receptor that do exit the ER and enter into the secretory system are degraded and removed from the cell surface efficiently, presumably by endocytosis. Following exposure to hydrogen peroxide (lower panel), the steady state distribution is altered so that TP levels at the surface are greatly increased. This results from the combination of more efficient ER exit, due to increased chaperone concentrations, and an increased surface half-life. It is presumed that the latter is in part the result of a decrease in endocytosis of TP. TP molecules are shown as maroon dots, and the proteasome as a gray lozenge. Arrow thickness is proportional to flux between destinations.

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functional and exquisitely capable of evoking increased ligand-responsiveness. Further, exposure to  $H_2O_2$  abolished agonist-dependent TP $\beta$  receptor internalization, which could be achieved by decreased endocytosis, more efficient recycling or a combination of both. TP $\beta$  is down-regulated from the cell-surface following exposure to agonist [36] and persistence at the surface during oxidative stress could explain the calcium mobilization following the second agonist challenge. Clearly these data indicate altered engagement with the endocytic system, possibly through changes to the membrane microenvironment or expression of factors within the endocytic apparatus itself. Regardless of the precise mechanism, these data indicate a significant change to receptor fate and a further contribution to enhanced cellular prostanoid hypersensitivity.

In summary, a single, brief oxidative insult is sufficient to induce rapid and persistent translocation of TP $\beta$  receptor to the surface; the surface cohort is functional and hyper-responsive to agonist. Together these processes contribute to a sophisticated and dramatic mechanism for augmentation of prostanoid responsiveness following exposure to ROS. We believe this to be the first report of such complex effects on a G protein-coupled receptor and these findings have a significant implication for our understanding of receptor regulation. Specifically, an increase to the cell-surface TP $\beta$  population could dramatically alter physiological responsiveness to thromboxane receptor agonists.

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## Supporting Information

**Figure S1** Calcium flux in HEK293 cells stably expressing HA-TP $\beta$  in the absence of hydrogen peroxide. Figure represents a single trace following the addition of 8-iso PGF<sub>2</sub> $\alpha$  (nM). HEK293 cells were loaded with FuraRed (20  $\mu$ M) for 30 minutes at 37°C. Cell visualization was performed using a Zeiss Axiovert 200 microscope and data processed using Volocity software (Improvision). Cytosolic calcium levels in response to stimulation were determined in the absence of hydrogen peroxide. Inset is the same data as in Figure 7.

Found at: doi:10.1371/journal.pone.0012798.s001 (3.61 MB TIF)

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## Author Contributions

Conceived and designed the experiments: SB JT MF. Performed the experiments: SB. Analyzed the data: SB JT MF. Wrote the paper: SB JT MF.

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