Hydrogen Peroxide Alters Splicing of Soluble Guanylyl Cyclase and Selectively Modulates Expression of Splicing Regulators in Human Cancer Cells

Gilbert J. Cote², Wen Zhu², Anthony Thomas¹, Emil Martin¹, Ferid Murad³, Iraida G. Sharina¹*¹

¹ Department of Internal Medicine/Cardiology, University of Texas Medical School, UTHealth, Houston, Texas, United States of America, ² Department of Endocrine Neoplasia and Hormonal Disorders, MD Anderson Cancer Center, Houston, Texas, United States of America, ³ Department of Biochemistry and Molecular Biology, George Washington University, Washington, DC, United States of America

Abstract

Background: Soluble guanylyl cyclase (sGC) plays a central role in nitric oxide (NO)-mediated signal transduction in the cardiovascular, nervous and gastrointestinal systems. Alternative RNA splicing has emerged as a potential mechanism to modulate sGC expression and activity. C-α1 sGC is an alternative splice form that is resistant to oxidation-induced protein degradation and demonstrates preferential subcellular distribution to the oxidized environment of endoplasmic reticulum (ER).

Methodology/Principal Findings: Here we report that splicing of C-α1 sGC can be modulated by H₂O₂ treatment in BE2 neuroblastoma and MDA-MD-468 adenocarcinoma human cells. In addition, we show that the H₂O₂ treatment of MDA-MD-468 cells selectively decreases protein levels of PTBP1 and hnRNP A2/B1 splice factors identified as potential α1 gene splicing regulators by in silico analysis. We further demonstrate that down-regulation of PTBP1 by H₂O₂ occurs at the protein level with variable regulation observed in different breast cancer cells.

Conclusions/Significance: Our data demonstrate that H₂O₂ regulates RNA splicing to induce expression of the oxidation-resistant C-α1 sGC subunit. We also report that H₂O₂ treatment selectively alters the expression of key splicing regulators. This process might play an important role in regulation of cellular adaptation to conditions of oxidative stress.

Introduction

Alternative splicing expands transcriptome diversity [1,2] and allows cells to meet the requirements of an ever-changing extracellular environment. Common stressors such as heat-shock, amino acid starvation or ethanol toxicity have been demonstrated to regulate alternative splicing [3,4]. Oxidative stress often persists in cellular microenvironment when there is an imbalance in the production and elimination of reactive oxygen species (ROS). This imbalance is associated with a plethora of pathologic conditions including carcinogenesis, cardiovascular disorders and neurodegeneration [5,6,7,8,9]. Recent evidence indicates that the alternative splicing can be influenced by changes in oxidative balance. Hypoxic and hypoxia/reoxygenation conditions, which alter ROS homeostasis, have been demonstrated to modify splicing of a number of genes in normal tissues and cancer cell lines [10,11,12,13,14]. Moreover, emerging data indicate that ROS can also alter the abundance of splicing factors or modify their activity. Low concentrations of hydrogen peroxide (H₂O₂), an ubiquitous ROS molecule, have been demonstrated to induce phosphorylation of the splicing factor hnRNP C leading to modulation of its RNA-binding affinity [15]. Increased hnRNP-C expression is also found in intimal hyperplasia and atherosclerosis, which is proposed to be associated with increases in H₂O₂ levels produced by activated vascular endothelium [16].

Soluble guanylyl cyclase (sGC) is a key enzyme of the nitric oxide (NO) signaling pathway and plays an important role in cardiovascular, neuronal and gastrointestinal functions [17]. Active sGC protein is a ferrous heme-containing heterodimer composed of α and β subunits, which is activated in response to the binding of NO to its heme moiety. Heme oxidation is proposed to be one of the mechanisms responsible for attenuation of NO/cGMP signaling in conditions of oxidative stress [18]. The sGC-specific inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ) oxidizes sGC ferrous heme to the ferric form to suppress the binding of NO. Exposure of cells and tissues to ODQ triggers sGC degradation due to destabilization of sGC heterodimer through ubiquitination and targeted proteasomal degradation [18]. We have previously identified a novel alternatively spliced isoform of sGC, C-α1, which is fully functional despite an extensive deletion in N-terminus [19]. Surprisingly, C-α1 splice form was significantly more resistant to protein degradation induced by the treatment with ODQ [19] and had a different
cellular localization in differentiating stem cells than the canonical α1 sGC [20]. Recent studies by Kraehling et al. revealed that, unlike the canonical α1 sGC isoform, the C-α1 isoform tends to localize to the more oxidized environment of the endoplasmic reticulum (ER) inside the cell [21]. These observations have led us to propose that the expression of the alternative C-α1 protein isoform can be induced by oxidative stress as a part of adaptation mechanism to preserve sGC activity in oxidative conditions.

In present study, we examined if splicing of α1 sGC gene can be modulated by ROS, specifically, by the treatment with H2O2. We found that H2O2 induces the expression of C-α1 transcript and C-α1 protein in human cancer cells expressing α1/β1 sGC. In an effort to gain insight into the regulatory mechanism, we examined the expression levels of several RNA binding proteins potentially involved in the splicing of C-α1 splice variant. Our studies show that H2O2 treatment selectively decreases the expression of putative α1 sGC splicing regulators PTBP1 and hnRNP A2/B1. Furthermore, we demonstrate that H2O2-induced degradation of PTBP1 differs in various breast cancer cell lines. To our knowledge, this is a first report demonstrating that H2O2-induced oxidative stress affects alternative splicing of sGC and selectively modulates protein level of major splice factors.

**Results**

**H2O2 induces expression of C-α1 sGC splice variant**

To investigate if alternative splicing of GUCY1A3 (α1 sGC subunit gene) is regulated in response to oxidative stress, we treated human breast carcinoma MDA468 and human neuroblastoma BE2 cell lines with 1 mM H2O2. MDA468 and BE2 cells endogenously express α1 and β1 sGC. The H2O2 concentration was chosen based on previous observations demonstrating that interaction with serum proteins in cell culture media, absorption by cellular membranes and neutralization by enzymatic components of cellular anti-oxidative defense consume a significant portion of exogenously added H2O2 [22,23]. MDA468 and BE2 cells demonstrated a significant increase in C-α1 sGC alternative transcript expression upon H2O2 treatment (Fig. 1 A, B). The relative increase of C-α1 isoform in comparison to α1 transcript was greater in BE2 cells, consistent with our previous studies [19].

The results indicated that GUCY1A3 splicing is altered to allow the recognition of C-α1 specific 3′ splice site within exon 4 in response to H2O2 exposure. Although in some experiments the level of C-α1 was increased by ODQ, neither cell line demonstrated statistically significant changes in C-α1 transcript level suggesting that ODQ treatment alone is not sufficient to affect splicing regulation.

Next, we evaluated the time-course of the changes in abundance of α1 and C-α1 sGC proteins in response to H2O2. Coincident with increases in GUCY1A3 splicing we observed a time-dependent modulation of α1 and C-α1 sGC protein levels. As shown in Fig. 1C and D, H2O2 increased the relative content of C-α1 protein in MDA468 cells. To assess the contribution of proteasome-dependent degradation in regulation of the α1 sGC splice forms, we performed the experiment in presence of MG132, a potent cell-permeable proteasome inhibitor. We found that pre-treatment with MG132 did not affect the rate of accumulation of C-α1 splice variant, or the level of the canonical α1 sGC subunit. These data suggest that the observed increase in C-α1 protein abundance (Fig. 1D) is likely due to an augmented C-α1 expression, and not to a selective degradation of canonical α1 sGC. Of note is that MG132 treatment also elevated levels of an unknown polypeptide recognized by anti-α1 antibodies with molecular weight lower than C-α1 sGC (Fig. 1D). As the identity of this protein remains to be determined, its intensity was not included in the densitometry analysis.

Together our results suggested that H2O2 induces preferential splicing and expression of C-α1 sGC splice form in our cell models.

**In silico analysis identifies potential α1 sGC (GUCY1A3) splicing regulators**

To gain the initial insight into potential mechanisms of α1 sGC splicing regulation, we performed *in silico* analysis using several bioinformatics tools [24]. The C-α1 splice variant is generated by the use of an alternative 3′ splice site 179 base pairs downstream of the constitutive site (Fig. 2A, Fig. S1). Constitutive and alternative splice sites for exon 4 of α1 sGC were defined with the UCSC Genome Bioinformatics tool and their relative consensus value examined using Human Splicing Finder [25,26]. Alternative splicing of exon 4 plays a central role in GUCY1A3 transcript diversity; in addition to the constitutive site, the exon contains 2 alternative donor sites and 2 alternative acceptor sites (see Fig. 2A and Fig. S1). With the exception of the constitutive 5′ splice site donor, the relative consensus strength of all sites proved to be low (Fig. S1). This is a common feature of alternatively processed exons that is thought to facilitate regulation by the serine-arginine rich (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) proteins [2]. The ASD, Alternative Splicing/Splicing Rainbow tool from European Molecular Biology Laboratory [27], was used to analyze the relevant exon and proximal intron sequences for potential SR and hnRNP regulators of exon 4 splicing. We generated a composite overview of regulator binding sites using the derived values for individual SR and hnRNP proteins (Table S1). This analysis identified a relatively even distribution of SR binding sites throughout exon 4 and the flanking regions. At the same time, the constitutive 3′ splice site intron region showed a dense peak of hnRNP binding sites (Fig. 2A). A closer examination identified in this sequence several overlapping binding sites for hnRNP 2A/B1, polypyrimidine-tract-binding protein 1 (hnRNP I, PTBP1), SRp20, SRp40 and Hu antigen R (HuR, ELAVL1) splicing regulators (Fig. S1). The location of these sites suggested that corresponding splice factors are likely to affect the use of both canonical and alternative splice sites, promoting the generation of the C-α1 sGC transcript.

H2O2 selectively alters the expression of splicing factors

It is well established that the expression levels and the relative stoichiometry of splice factors modulate alternative splicing [28]. Therefore, we investigated the effect of H2O2 exposure on protein levels of the splice factors identified by our *in silico* analysis as potential sGC regulators. As shown in Fig. 2B, the exposure of MDA468 cell to H2O2 selectively decreased the protein level of PTBP1 and hnRNP A2/B1 splicing repressors. H2O2 did not affect the level of HuR regulator and only slightly altered levels of SRp40 protein from SR family of splicing enhancers. Both PTBP1 and hnRNP A2/B1 proteins are decreased in a dose-dependent manner in MDA468 cells (Fig. 2 C, D and F). Our data indicate that the observed H2O2-dependent switch in splicing of GUCY1A3 gene coincides with the changes in the level of regulatory splice factors. However, the exact mechanism and the contribution of specific splice factors in the regulation of sGC splicing remains to be determined.
Insights into the mechanism of H₂O₂-induced PTBP1 protein degradation

Next we explored potential mechanisms of hnRNP regulation by H₂O₂. We chose to focus on PTBP1 since this extensively studied RNA-binding protein plays an important role in various steps of cellular mRNA processing, including splicing, regulation of stability, localization and translation [29]. Moreover, PTBP1 has been demonstrated to be essential in the regulation of cell growth and cancer cells survival [30,31,32]. The ability of H₂O₂ to reduce PTBP1 levels suggested that, additionally, it may play a role in the regulation of cellular adaptation to oxidative conditions. However, the effect of elevated ROS levels on PTBP1 expression has never been examined previously.

We first investigated if H₂O₂ alters PTBP1 mRNA steady-state levels. RT-qPCR analysis performed with RNA samples isolated from MDA468 cells treated with different H₂O₂ concentrations found no changes in PTBP1 mRNA levels (Fig. 3C) indicating that PTBP1 is likely to be controlled at the protein level. To examine the role of proteasomal degradation, we monitored the dynamics of changes in PTBP1 protein in response to H₂O₂ treatment over a 16 hours period in the presence or absence of proteasome inhibitor MG132. We found that PTBP1 protein was reduced in a time-dependent manner starting at 8 hours post-exposure (Fig. 3A, B). Interestingly, the protein degradation was not prevented, but rather enhanced, by MG132 treatment. This was particularly evident at the 16-hour and later time points (Fig. 3A and data not shown). Thus, while MG132 treatment showed that the proteasome had no direct effect on PTBP1 degradation, the fact that its inhibition accelerates degradation implies an indirect role in regulation, most likely through stabilization of an unknown protease or protein cofactor. The pre-treatment of MDA468 cells with cycloheximide (CHX, the inhibitor of de novo protein synthesis) also did not prevent PTBP1 decrease, indicating that H₂O₂ was inducing a pre-existing protein degradation pathway (Fig. 3D). It has been previously demonstrated that cellular apoptosis leads to PTBP1 degradation through Caspase-3 activation [33]. Thus, we explored the possibility that Caspase-3 might play a role in H₂O₂-induced PTBP1 decreases. However, we found that the addition of Caspase-3 inhibitor IV (Ac-DMQD-CHO, Calbiochem) did not prevent H₂O₂–induced PTBP1 degradation (results are not shown), indicating that Caspase-3 is not involved in this process. To determine whether other exogenous sources, besides direct addition of H₂O₂ solution, might affect the stability of PTBP1, we applied glucose oxidase (GO) to the cell culture media. Consistent with the direct role of H₂O₂ in the induction of PTBP1 degradation, steady-state production of H₂O₂ by GO treatment reproduced the effect.

H₂O₂ induces post-translational protein modifications such as oxidation of intracellular thiols and thiolate anions. These modifications are an integral part of H₂O₂ signaling affecting a variety of cellular processes [34]. It has been previously demonstrated that in oxidative conditions PTBP1 may form dimers due to the formation of intermolecular disulfide bridges [35]. Indeed, in our experiments we also detected the formation of a high molecular weight protein band recognized by PTBP1...
antibodies following the treatment of MDA468 cells with H2O2 (results are not shown).

To evaluate the contribution of thiol oxidation to PTBP1 degradation, we exposed MDA468 cells to two different compounds altering cellular thiol metabolism. Treatment with 2-hydroxyethyl disulphide (HEDS), an agent that acts as thiol-specific oxidant, significantly decreased PTBP1 levels (Fig. 3D), indicating that direct thiol oxidation might play an important role in PTBP1 degradation response. To further test if PTBP1 degradation is induced by diminished cellular thiol-reducing activity in response to H2O2, we also treated the cells with L-buthionine-S,R-sulfoximine (BSO). BSO is an irreversible inhibitor of glutathione biosynthesis, decreasing intracellular glutathione pool. We observed no significant degradation of PTBP1 in response to BSO, suggesting that a decreased level of GSH is not sufficient to induce PTBP1 (Fig. 3D) degradation. These results may also be related to the intrinsic ability of MDA468 cells to compensate for the GSH loss induced by BSO, as has been previously reported for cell lines with high SOD expression [36]. Thus, oxidation of Cys thiols by H2O2 could initiate the formation of the PTBP1 dimers and contribute to subsequent protein degradation.

H2O2 effect on PTBP1 protein levels varies in different breast cancer cell lines

To determine the generality of H2O2-induced PTBP1 degradation, we tested additional breast cancer lines, including: MDA-MD-453, MDA-MD-231 and MCF7. Western blot analysis of H2O2-treated cells demonstrated various degrees of PTBP1 reduction in MDA468, MCF7 and MDA231 cells, but no change in MDA453 cells (Fig. 4A). The failure to induce PTBP1 degradation in MDA453 cells was observed at H2O2 concentrations that readily elicited significant decreases in MDA468 cells (Fig. 4B to Fig. 2B and C). These data suggested that the extent of H2O2-induced degradation of PTBP1, thought prevalent, is still rather cell line specific. To explore if the resistance to PTBP1 protein degradation is associated with increased ability of cells to survive oxidative stress, we compared H2O2-induced cytotoxicity in MDA468 and MDA453 cells. MDA468 cells were less resistant to H2O2-induced cytotoxicity (IC50 = 450 ± 60 μM) compared with MDA453 cells (IC50 = 660 ± 2 μM) (Fig. 5). In an attempt to directly link a reduction in PTBP1 levels to H2O2-induced cytotoxicity we performed siRNA-mediated PTBP1 knockdown in MDA453 cells. Despite a greater than 50% reduction in PTBP1 mRNA (as detected by RT-qPCR), cell viability measurements revealed only a small insignificant decline in resistance to H2O2 treatment (Fig. S2). These data suggest that the level of PTBP1 expression alone is not critically important to support oxidative resistance in MDA453 cells.

Discussion

In this report we demonstrate that the oxidative stress induced by H2O2 influences splicing of the α1 sGC gene (GUCY1A3) and selectively decreases protein level of PTBP1 and hnRNP A2/B1 splicing factors. We previously established that GUCY1A3 transcripts undergo alternative splicing and that the C-α1 sGC splice isoform encodes a protein that is resistant to ODQ-induced...
mRNA levels. MDA468 cells were treated with indicated concentrations of H2O2 for 24 hours. Relative abundance of PTBP1 mRNA in samples was normalized on β-actin served as a loading control. Biological duplicates for each treatment are shown; blots are representative of three independent experiments with similar results. B: Densitometry analysis of PTBP1 protein levels normalized on β-actin. Averages for representative biological duplicates for each treatment are shown. C: H2O2 exposure does not affect PTBP1 mRNA levels. MDA468 cells were treated with indicated concentrations of H2O2 for 24 hours. Relative abundance of PTBP1 mRNA in samples was analyzed by RT-qPCR analysis. Average ΔCt ± SD for biological triplicates are shown. D: PTBP1 degradation depends on thiol oxidation and is not rescued by inhibition of de novo protein synthesis. Western blot analysis performed on MDA468 cell lysates treated for 24 hours with inhibitor of protein synthesis cycloheximide (2 μg/ml) and different factors inducing oxidative stress: 1 mM BSO (GSH depletion inducer); 1 mM HEDS (thiol oxidation inducer) and 0.01 units/ml of Glucose Oxidase (increases production of ROS). Shown Western blots are representative of three independent experiments with similar results.

Figure 3. Insights into the mechanism of H2O2-induced PTBP1 down-regulation. A: PTBP1 degradation occurs in time-dependent manner and is not prevented by proteasome inhibitor MG132. MDA468 cells were treated as in Fig. 1C with 1 mM H2O2 in the presence or absence of MG132 (10 μM). Western blot analysis was performed to visualize the expression of PTBP1. β-actin served as a loading control. Biological duplicates for each treatment are shown; blots are representative of three independent experiments with similar results. B: Densitometry analysis of PTBP1 protein levels normalized on β-actin. Averages for representative biological duplicates for each treatment are shown. C: H2O2 exposure does not affect PTBP1 mRNA levels. MDA468 cells were treated with indicated concentrations of H2O2 for 24 hours. Relative abundance of PTBP1 mRNA in samples was analyzed by RT-qPCR analysis. Average ΔCt ± SD for biological triplicates are shown. D: PTBP1 degradation depends on thiol oxidation and is not rescued by inhibition of de novo protein synthesis. Western blot analysis performed on MDA468 cell lysates treated for 24 hours with inhibitor of protein synthesis cycloheximide (2 μg/ml) and different factors inducing oxidative stress: 1 mM BSO (GSH depletion inducer); 1 mM HEDS (thiol oxidation inducer) and 0.01 units/ml of Glucose Oxidase (increases production of ROS). Shown Western blots are representative of three independent experiments with similar results.

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Figure 4. **PTBP1 response to H2O2-induced degradation varies in different breast cancer cell lines.**

A: Western blot analysis examining PTBP1 expression in MDA231, MDA453, MDA468 and MCF7 cells treated with 1 mM H2O2 for 18 hours. Representative biological duplicates are shown. B: MDA453 cells are resistant to H2O2-induced PTBP1 degradation. Top panel: MDA453 cell were treated with different concentration of H2O2 and cell lysates were subjected to Western blot analysis with antibodies towards PTBP1 and β-actin. Shown blots are representative of four independent experiments with similar results. Bottom panel: densitometry analysis of PTBP1 protein levels normalized on β-actin levels. Averages for representative biological duplicates for each treatment are shown.

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Figure 5. **H2O2 cytotoxicity analysis in MDA468 and MDA453 cells.** Survival curve was generated in response to H2O2 dosage using trypan exclusion method and expressed as % of survival to untreated controls. Mean ± SD of three independent passages performed in triplicates are shown. *: p<0.05 by Student's t-test in comparison to control.

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H2O2. Indeed, we found that MDA453 cells were significantly less sensitive to cytotoxic concentrations of H2O2 than MDA468 (Fig. 5). This observation is in agreement with previous reports suggesting that preserving PTBP1 expression is beneficial to cancer cells survival in oxidative stress [30,31]. However, we were unable to demonstrate a direct role for PTBP1 in MDA453 cells response to H2O2 as siRNA-mediated knockdown had no effect on cytotoxicity (Fig. S2). Additional studies are necessary to uncover the precise role of PTBP1 in H2O2-mediated cytotoxicity and determine if the lack of PTBP1 degradation in response to a treatment with oxidants may serve as a marker for resistance to oxidative stress in individual cancer cell lines.

In summary, our data demonstrate that the oxidative stress induced by H2O2 promotes splicing of oxidation-resistant Cα1 sGC splicing variant and selectively alters protein levels of major splice factors.

Materials and Methods

Cell Culture and Preparation of Protein Lysates

BE2 human neuroblastoma cell line (American Type Culture Collection) was cultured in DMEM/F12K media supplemented with 10% FBS, 0.1 mM MEM nonessential amino acids, penicillin-streptomycin mixture (50 units/ml and 50 µg/ml), 10 mM Hepes (pH 7.4), 1 mM sodium pyruvate, 2 mM L-glutamine (all from Gibco/Invitrogen) and maintained at 37°C and 5% CO2. Human adenocarcinomas MDA-MD-468, MCF7, MDA-MD-231 and dactal carcinoma MDA-MD-453 cells (generous gift of Dr. Hesham Amin, MD Anderson Cancer Center) were cultured in RPMI supplemented with 10% FBS, 0.1 mM MEM nonessential amino acids and penicillin-streptomycin mixture (50 units/ml and 50 µg/ml) and maintained at 37°C and 5% CO2. For treatments, 70–80% confluent cell cultures were exposed with different agents up to 24 hours. To prepare lysates, the cells were collected by trypsinolysis, washed twice with PBS, re-suspended in 50 mM TEA (pH 7.4) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and disrupted by sonication. The lysates were centrifuged at 15,000×g for 30 min to prepare the cleared supernatant fractions, which were used for Western blotting.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA from cells was isolated using UltraSpec total RNA isolation reagent (Biotex, Houston, TX). cDNA was prepared using a high-capacity cDNA kit (Applied Biosystems, Foster City, CA). The semi-quantitative RT-PCR assay for detection of Cα1 sGC mRNA (Transcript 5, NCBI, Accession No. NM_001130685) was performed as described previously [19]. A set of specific primers flanking the deletion in Cα1 sGC Transcript 5 was used to perform PCR amplification. Primer sequences were as follows: Forward Primer 5'-cagccccgggtgtagctcag-3'; Reverse Primer 5'-gagccgaggtctggtttcagt-3'. PCR products representing canonical Cα1 sGC mRNA (270 bp, encoding full size protein) and Transcript 5 mRNA (94 bp, encoding Cα1 sGC splicing variant), were separated on agarose gel and visualized by Ethidium Bromide staining. Obtained picture was inverted and the band intensity was quantified by densitometry using QuantityOne software (BioRad). We determined PTBP1 mRNA expression levels with the TaqMan assay (Hs00259176-m1), which specifically measures the presence of exon 2. Ribosomal RNA (18S) (4308329) was used as an endogenous control to perform the comparative ΔCT method. Both TaqMan assays were performed according to the manufacturer’s suggested protocol using 10 ng of cDNA.

[30,42,43,44]. Therefore, we chose to further explore the effect of H2O2 treatment on the PTBP1 expression. Our results demonstrated that H2O2 decreases PTBP1 expression in a dose- and time-dependent manner (Fig. 2C, F and Fig. 3A, B). No change in PTBP1 mRNA level was observed, indicating that the down-regulation occurs post-transcriptionally (Fig. 3C). Furthermore, the several hours delay in response to H2O2 treatment suggests an indirect mechanism of PTBP1 down-regulation. This conclusion is supported by the observation that proteasome inhibitor MG132 facilitated, and not inhibited, the H2O2-induced decline in PTBP1 protein (Fig. 3A, B). Because PTBP1 degradation did not require new protein synthesis (it was not blocked by cycloheximide treatment) our findings indicate that the down-regulation of PTBP1 mRNA may potentially enhance proteasomal degradation of some unknown factor responsible for PTBP1 stabilization. Given that H2O2 exposure is known to induce an oxidative response [45], we considered a caspase-mediated degradation of PTBP1. Previous studies have demonstrated that PTBP1 is targeted by Caspase-3 during apoptotic response [33]. However, Inhibitor IV failed to prevent H2O2-mediated reduction in PTBP1 protein levels (data not shown). Thus, we conclude that H2O2 probably induces PTBP1 degradation by a different from previously described mechanisms.

It is also important to point out that PTBP1 degradation was not limited to the direct addition of H2O2 solution to cells. Application of extracellular glucose oxidase (GO), which catalyzes the conversion of glucose into H2O2 and D-glucono-δ-lactone, also induced a significant decline of PTBP1 protein in MDA468 cells (Fig. 3D).

The reaction of H2O2 with protein thiols (R-SH) and thiolate anions (R-S-) is known to generate sulfenic acids (ROSOH) modifications and promote disulfide bond formation. These proteins were implicated in a wide variety of biochemical effects mediating ROS signaling [34,46]. Interestingly, dimerization of PTBP1 molecules via intermolecular Cys bridge formation was previously observed in oxidative conditions [35]. We explored the possibility that down-regulation of PTBP1 protein by H2O2 is mediated by thiol oxidation. Indeed, non-specific thiol oxidizing compound HEDS elicited a significant decline of PTBP1 levels, similar to a direct H2O2 exposure (Fig. 3D). Thus, dimerization through cysteine oxidation might target PTBP1 protein to subsequent degradation. Delineation of an exact mechanism responsible for selective ROS-induced degradation of this important regulator may offer additional important insights into cellular oxidative response.

Previously, PTBP1 expression has been correlated with increases in proliferation and metastatic potential of cancer cells; however, this effect varies in different cell lines [31,32,47,48]. In addition, elevated PTBP1 levels have been shown to support aerobic glycolysis and enhance translation of hypoxia-inducible proteins were implicated in a wide variety of biochemical effects associated with altered ROS homeostasis [42,49,50,51]. Previously, PTBP1 expression has been correlated with increases in proliferation and metastatic potential of cancer cells; however, this effect varies in different cell lines [31,32,47,48].
**Western Blot Analysis**

Western blot analysis was performed as described previously [19]. Cleared supernatant fractions of protein lysates (20 μg) were loaded on 8% polyacrylamide gels, separated by electrophoresis and transferred on PVDF membranes. Membranes were blocked with 5% non-fat milk, incubated with primary antibodies for 1 h and with secondary horseradish peroxidase-conjugated antibodies (Sigma) in DPBS buffer for 45 min at room temperature. The signal was visualized by enhanced chemiluminescence (ECL, Plus, Amersham). Densitometry analysis was performed using QuantityOne software (BioRad). The following primary antibodies were used: custom made rabbit polyclonal anti-si GR antibodies raised against human C-terminal peptide FTPRSREELPPNFP (1:1000 dilution); anti-β-actin (Sigma-Aldrich; 1:7000 dilution); anti-PGK1 (T25H, Roche Applied Science; 1:2000 dilution); anti-β-actin (Sigma-Aldrich; 1:7000 dilution); anti-β2-MG (3A2, Santa Cruz Biotechnology; 1:200 dilution); anti-actin (1H4, Santa Cruz Biotechnology; 1:200 dilution); anti-β-actin (Sigma-Aldrich; 1:7000 dilution); anti-β-actin (Sigma-Aldrich; 1:7000 dilution); anti-β2-MG (3A2, Santa Cruz Biotechnology; 1:200 dilution); anti-actin (1H4, Santa Cruz Biotechnology; 1:200 dilution).

**H₂O₂ Cytotoxicity Assay**

Cells grown on 96-well plates (1 × 10⁵ cells/ml) were treated with different concentrations of H₂O₂. After 24 hours, viable cells were visualized with trypan blue exclusion (Life Technologies/Invitro) to determine the cell number and viability by hemocytometer count. Knock-down of PTBP1 expression was performed using siRNA gene silencing (Santa Cruz Biotechnology) according to manufacturer’s instructions.

**In Silico and Statistical Analysis**

Calculation of relative splice site strengths was performed using the Human Splicing Finder version 2.4.1 online tool (www.umd.be/HSF7) [25-26]. Mapping of hnRNP and SR regulatory sequences was performed using the ASD-Alternative Splicing/Splicing Rainbow tool from European Molecular Biology Laboratory (http://www.ebi.ac.uk/asd-srv/wb.cgi?method=0) [27]. The GUCY1A3 genomic sequences used for this analysis were directed from the human GRCh37/hg19 assembly and included: Exon 2 region (150 bp) chr:156588321–156588670, Exon 4 region (568 bp) chr:156617808–156618375 and Exon 5 region (150 bp) chr:156624983–156625132. All data are presented as mean ± standard deviation. The Splicing Rainbow tabular output file was used to generate a predicted binding score based on the output score for individual nucleotide positions (see Table S1 for an example). Statistical comparisons between groups were performed by Student’s t-test using Excel software with a p<0.05 considered statistically significant. Nonlinear regression and calculations of IC₅₀ were performed using Graph Pad Prism 3.0 software (GraphPad Software).

**Supporting Information**

**Figure S1 Distribution of Predicted Splicing Factor Binding Sites.** Shown is 294 bp of GUCY1A3 genomic sequence (GRCh37/hg19 assembly Chr4:156617821–156618114) spanning the intron 3 (low case)/exon 4 (upper case) junction. The position of alternative splice sites along with their predicted strength is shown. The C→T SS1 splice site is used to generate C→T mRNA isoform. Details regarding the use of other splice sites is reviewed in [Sharin, I.G., et al., RNA splicing in regulation of nitric oxide receptor soluble guanylyl cyclase. Nitric Oxide, 2011]. The location of predicted regulatory sites for splicing factors examined in Figure 2B is shown. This information was derived using the ASD-Alternative Splicing/Splicing Rainbow tool with a detailed output of this analysis provided in Table S1. (TIF)

**Table S1 ASD Splicing Rainbow Output for GUCY1A3 Predicted Splicing Factor Binding Sites.** (DOC)

**Author Contributions**

Conceived and designed the experiments: IGS GJC ESM FM. Performed the experiments: IGS WZ AT ESM. Analyzed the data: GJC IGS. Contributed reagents/materials/analysis tools: GJC ESM FM. Wrote the paper: IGS GJC.
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