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ORIGINAL ARTICLE

Inhibitors of hydroperoxide metabolism enhance ascorbate-induced cytotoxicity

K. E. Olney[1], J. Du[1], T. J. van 't Erve[2], J. R. Witmer[1], Z. A. Sibenaller[1], B. A. Wagner[1], G. R. Buettner[3] & J. J. Cullen[4]

[1]*Department of Radiation Oncology, University of Iowa, Iowa City, IA, USA,* [2]*Department of Toxicology, University of Iowa, Iowa City, IA, USA,* [3]*Department of Radiation Oncology, University of Iowa, Holden Comprehensive Cancer Center, Iowa City, IA, USA, and* [4]*Department of Radiation Oncology and Surgery, University of Iowa, Veterans Affairs Medical Center, Iowa City, IA, USA*

Abstract

Pharmacological ascorbate, *via* its oxidation, has been proposed as a pro-drug for the delivery of H_2O_2 to tumors. Pharmacological ascorbate decreases clonogenic survival of pancreatic cancer cells, which can be reversed by treatment with scavengers of H_2O_2. The goal of this study was to determine if inhibitors of intracellular hydroperoxide detoxification could enhance the cytotoxic effects of ascorbate. Human pancreatic cancer cells were treated with ascorbate alone or in combination with inhibitors of hydroperoxide removal including the glutathione disulfide reductase inhibitor 1,3 *bis* (2-chloroethyl)-1-nitrosurea (BCNU), siRNA targeted to glutathione disulfide reductase (siGR), and 2-deoxy-D-glucose (2DG), which inhibits glucose metabolism. Changes in the intracellular concentration of H_2O_2 were determined by analysis of the rate of aminotriazole-mediated inactivation of endogenous catalase activity. Pharmacological ascorbate increased intracellular H_2O_2 and depleted intracellular glutathione. When inhibitors of H_2O_2 metabolism were combined with pharmacological ascorbate the increase in intracellular H_2O_2 was amplified and cytotoxicity was enhanced. We conclude that inclusion of agents that inhibit cellular peroxide removal produced by pharmacological ascorbate leads to changes in the intracellular redox state resulting in enhanced cytotoxicity.

Keywords: hydrogen peroxide, glutathione disulfide reductase, catalase, oxidative stress, pancreatic cancer

Introduction

Pharmacological ascorbate is high-dose ascorbate (ascorbic acid, vitamin C) delivered intravenously to achieve plasma levels that are 100–1000 times that of normal nutritional levels. These high levels are only possible with intravenously delivered ascorbate, not oral ascorbate [1–3]. These levels are in the range that can be cytotoxic to tumor cells [4]. Recent studies from our group have demonstrated that ascorbate induces cytotoxicity and oxidative stress in pancreatic cancer cells and this cytotoxicity appears to be greater in tumor *vs*. normal cells [5]. Pharmacological ascorbate has been hypothesized to be a pro-drug for formation of hydrogen peroxide (H_2O_2) [4]; ascorbate is oxidized to ascorbate radical by redox-active metals associated with protein; the reduced metal then donates this electron to O_2 forming superoxide radical that leads to formation of H_2O_2 [6]. Previous studies have shown that the oxidation of ascorbate occurs preferentially in the extracellular fluid [7]. Therefore, we propose that the H_2O_2 formed in the extracellular fluid diffuses into cells, leading to an increase in intracellular H_2O_2 and subsequent cytotoxicity. These previous studies have clearly demonstrated the increase in extracellular H_2O_2 with pharmacological ascorbate; however, direct evidence for ascorbate-induced changes in the intracellular level/flux of H_2O_2 has been difficult to obtain. For example, the use of the oxidation sensitive 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$) in assays that presumably measure intracellular H_2O_2 has many artifacts [8–10]. In addition ascorbate will reduce the free radical intermediate of the oxidation cascade of $DCFH_2$ blunting the formation of DCF, the fluorescent oxidation product, thereby interfering with the detection of intracellular H_2O_2 [11].

There is a clear rationale for combining ascorbate with appropriate cytotoxic agents. Cells contain an antioxidant network to prevent or repair the damage caused by reactive oxygen species. Catalase and peroxidases remove H_2O_2, reducing it to water. Glutathione peroxidase (GPx) requires several co-factors and secondary enzymes. If removal of H_2O_2 generated by pharmacological ascorbate is inhibited, then pancreatic cancer cells should be more effectively killed because of direct toxicity resulting from H_2O_2-mediated damage. There are a number of compounds that can inhibit the removal of H_2O_2 by the antioxidant network. 1,3 *bis* (2-Chloroethyl)-1-nitrosurea (BCNU) is a clinically used chemotherapeutic agent that causes DNA alkylation but also inhibits glutathione disulfide reductase (GR) *via* carbamoylation. If GR is inhibited, cells have reduced ability to remove H_2O_2 *via* the glutathione peroxidase system. 2-Deoxy-D-glucose (2DG) is a relatively non-toxic analog of glucose that competes with glucose for uptake *via* the glucose transporters; it is phosphorylated by hexokinase at the entry point to glycolysis. Competition between 2DG and glucose is thought to cause inhibition of glucose metabolism, thereby creating a chemically induced state of glucose deprivation resulting in inhibition of hydroperoxide detoxification [12]. Combinations of these chemical inhibitors of glucose and

Correspondence: Joseph J. Cullen, M.D., 1528 JCP, Department of Radiation Oncology and Surgery, University of Iowa Hospitals and Clinics, Iowa City, IA 52242, USA. Office: +(319) 353-8297. Fax: +(319) 356-8378. E-mail: joseph-cullen@uiowa.edu

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hydroperoxide metabolism would be predicted to enhance ascorbate toxicity in tumor cells.

In this study, we used aminotriazole-mediated inhibition of endogenous catalase to determine ascorbate-induced changes in intracellular H_2O_2. We provide evidence that supports the proposal that the toxicity of pharmacological ascorbate is mediated by H_2O_2 formed from ascorbate oxidation. We demonstrate that chemical inhibitors of hydroperoxide detoxification further increase the level of intracellular H_2O_2 produced by pharmacological ascorbate thereby enhancing its toxicity.

Methods

Cell lines and chemicals

The human pancreatic cancer cell line MIA PaCa-2 was cultured in DMEM high glucose supplemented with 10% FBS and 2.5% horse serum. Human pancreatic cancer AsPC-1 cells were maintained in RPMI 1640 medium supplemented with 20 % FBS, sodium pyruvate (1 mM), and 1 % penicillin-streptomycin. Cells were maintained in a humidified atmosphere of 95 % air/5 % CO_2 at 37°C.

L-ascorbic acid was purchased from Macron Chemicals (Center Valley, PA). Stock solutions of ascorbate (1.0 M) were made as previously described [13]. L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (A2P), 2-deoxy -glucose (2DG), 1,3-*bis* (2-chloroethyl)-1-nitrosourea (BCNU), and 3-amino-1,2,4-triazole (3-AT) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). siRNA targeted against glutathione disulfide reductase was designed and manufactured by Ambion (Austin, TX). siRNA ID #: s6249, sense (GUAUCACGCAGUUAC-CAAATT), antisense (UUUGGUAACUGCGUGAUA-CAT); Silencer Select Negative control (Product #: 4390843) was from Applied Biosystem (Foster City, CA). siNeg (siRNAs with sequences that do not target any gene product) was used to determine the transfection efficiency and to control for the effects of the siRNA delivery method. MIA PaCa-2 cells were transfected with siRNA (20 pmole/2 × 105 cells by Lipofectamine 2000, Invitrogen) for 24 hours. The cells were incubated in full media for specified times prior to experiments.

Oxygen consumption rate (OCR) determination

The rate of oxygen consumption by ascorbate ($AscH^-$) oxidation was monitored using a BioStat™ multi-mode electrochemical system (ESA Biosciences, Chelmsford, MA) in conjunction with a Clark electrode (YSI oxygen probe 5331, Yellow Springs, OH). Three milliliters of DMEM growth media was transferred to the reaction chamber, stirring for 2 min. After inserting the electrode, the baseline was recorded for 2 min. Ascorbate (2, 5, 10 and 20 mM) was added to the reaction chamber using a gas tight Hamilton syringe. The oxygen consumption was monitored for 3 to 5 min. Since the measurements are displayed in nanoamps (nA), the baseline measured in nA is equal to initial concentration of oxygen 258 μM, at room temperature [14]. OCR was determined from the initial slope after introduction of $AscH^-$.

Determination of intracellular hydrogen peroxide

Intracellular H_2O_2 concentrations were determined by analysis of the rate of aminotriazole-mediated inactivation of endogenous catalase activity [15]. Catalase is irreversibly inactivated by aminotriazole (3-AT, 3-amino-1,2,4-triazole (Sigma)) in the presence of H_2O_2; following the rate of inactivation of intracellular catalase allows determination of the intracellular concentration of H_2O_2. Cells grown in 150-mM culture dishes were treated with ascorbate (20 mM) in the presence of 3-AT (20 mM) for 0, 5, 10, 20, 30, 60, and 120 min at 37°C. Cells were washed with ice-cold PBS and harvested and lysed by freeze/thaw methodology. To determine the amount of fully active cellular catalase, cell lysate (2.00 mL) was introduced into the reaction chamber of the oxygen monitor (YSI model 5300, YSI Inc., Yellow Springs, OH). Then 333 μM of H_2O_2 was injected into the reaction chamber and the rate of production of oxygen was continuously recorded for 5 min or until the curve reached a plateau. The rate of appearance of dioxygen reflects the amount of active catalase in the cell lysate. Cell protein was determined by Bio-Rad DC protein assay. The intracellular steady-state concentration of H_2O_2 was calculated from the equation $[H_2O_2]_{ss} = k_{inactivation}/k_1$ where $k_{inactivation}$ is the experimental pseudo first-order rate constant of catalase inactivation, and the value k_1 is $1.7 \times 10^7\ M^{-1}\ s^{-1}$, the rate constant for the formation of catalase compound I.

Intracellular GSH and GSSG determination

Cells grown in 100-mM dishes were treated with ascorbate (0, 2, 5, 10, and 20 mM) for 0, 2, 6, and 24 h. After treatment, cells were washed with PBS and harvested by trypsinization. Cells were placed in 5 % perchloric acid/100 μM diethylenetriaminepentaacetic acid (DETAPAC; Sigma-Aldrich Chemical Co, St Louis, MO); this precipitates the protein and preserves glutathione (GSH) and glutathione disulfide (GSSG). The sample was centrifuged to pellet the protein (4000 g, 5 min). Supernatants were stored at − 80°C or immediately analyzed. To determine the status of the GSSG,$2H^+$/2GSH couple in treated cells we used HPLC with electrochemical detection following the protocol outlined by Park et al. [16]. For the analysis, 20 μL of each sample was injected and allowed to run for 1 hour. The method is based on an electrochemical detection (ECD) system using a boron-doped diamond (BDD) electrode (Model 5040, ESA Biosciences, Chelmsford, MA). The BDD electrode is an excellent detector for thiol and disulfide compounds as these analytes require a high electrode potential. Derivatization of the sample is not required allowing higher throughput. With the minimal sample processing required there is less opportunity for the sample to oxidize, which would lead to overestimation of GSSG [17]. The results

from the HPLC/BDD in conjunction with a cell count using a Z2 Coulter Counter® and the known intracellular volume (MIA PaCa-2: 2.0 pL [14]) allow for the determination of the intracellular concentrations of GSH and GSSG. These concentrations in conjunction with the Nernst equation at room temperature conditions and pH 7.4 were used to determine the intracellular half-cell reduction of the GSSG,$2H^+$/2GSH couple (E_{hc}) [18].

Glutathione disulfide reductase activity assay

Cells treated with BCNU or siRNA against GR were harvested by trypsinization. Cell pellets were lysed in phosphate buffer. Glutathione reductase catalyzes NADPH-dependent reduction of GSSG to 2GSH. The oxidation of NADPH to $NADP^+$ was monitored at 340 nM as an indicator of GR activity [19].

Western blotting

Cells were collected by scraping; protein concentrations were determined using a Bio-Rad DC Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA). Protein (30 μg) was electrophoresed in a Bio-Rad 4-20 % Ready gel at 100 V. The proteins were electrotransferred onto a PVDF membrane (Millipore, Billerica, CA) and blocked with 5 % nonfat dry milk in Tween-PBS (TPBS) for 60 min. The membranes were incubated with a rabbit polyclonal anti-glutathione reductase antibody (1:1000, Abcam, Cambridge, MA) at 4˚C overnight. Membranes were washed 5 times with 0.1 % TPBS and incubated with secondary antibodies conjugated with horseradish peroxidase (1:50 000, CHEMICON International, Temecula, CA). GAPDH (1:5000, Millipore Corporation, Billerica, MA) was used as a loading control. After wash with TPBS, membranes were stained with Super Signal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) and exposed to Classic Blue Autoradiography Film (Molecular Technologies, St Louis, MO).

Clonogenic assay

Cells (2×10^5) were seeded in 60 mM culture dishes and treated 48 h later. After treatment, cells were trypsinized and seeded into 6-well plates at 400 cell/well in 4 mL growth media. Colonies formed between 10 and 14 days at 37°C were fixed with 70 % ethanol and stained with Coomasie blue. Colonies with more than 50 cells were counted.

Cell viability

As an indicator of cell metabolic viability, the MTT assay was used. Cells were seeded at 1×10^4 cells per well in a 96-well plate in full media. After 48 h, cells were treated with ascorbate for 1 h. For the cell viability experiments, all cell lines were treated in DMEM media for consistency. Then the cells were washed with PBS and incubated with fresh media for an additional 24 h. MTT (3-[4,5-dimethylthiazol-2-yl](-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO) 1 mg/mL was added to the wells and incubated at 37ºC for 3 h. At the end of incubation, media were removed and 100 μL of DMSO was added to each well for cell lysis. The plate was read at 590 nM on a Tecan SpectraFluor Plus plate reader (Tecan, Research Triangle Park, NC).

Statistical analysis

Statistical analyses were performed by means of Systat (Systat Inc., Evanston Ill). A single factor ANOVA, followed by post-hoc Tukey test, was used to determine statistical differences between means. All means were calculated from at least three different experiments and error bars represent standard error of mean (SEM). All western blots were repeated at least twice and activity assays were performed in triplicate.

Results

Pharmacological ascorbate in the media increases OCR

As shown in Table I, the OCR of 2 mM ascorbate in DMEM growth media was 61 ± 12 nM s^{-1} (no cells). Assuming all oxygen consumed upon oxidation of pharmacological ascorbate is converted to H_2O_2, then H_2O_2 was produced at the rate of 61 ± 12 nM s^{-1}. For 10 x 10^6 MIA PaCa-2 cells grown in 10 mL media treated with 2 mM ascorbate, each cell was exposed to H_2O_2 at the rate of 61 x 10^{-18} mol $cell^{-1}$ s^{-1}, *i.e.* 61 amol $cell^{-1}$ s^{-1}; for 20 mM ascorbate, the rate was 334 amol $cell^{-1}$ s^{-1}. As context, the OCR of MiaPa-2 cells is 57 amol $cell^{-1}$ s^{-1} [14]. If 1 % of this metabolically consumed oxygen is converted to H_2O_2, then the rate of metabolic production of H_2O_2 would be 0.6 amol $cell^{-1}$ s^{-1}. Thus, the flux of H_2O_2 per cell due to pharmacological ascorbate is on the order of 100 times that from metabolically produced H_2O_2 in this experimental configuration. Thus, pharmacological ascorbate exposes cells to a very high flux of H_2O_2.

Pharmacological ascorbate increases intracellular H_2O_2

Pharmacological ascorbate has been shown to form H_2O_2 in extracellular media through the ascorbate radical

Table I. OCR of pharmacological ascorbate in DMEM growth media.*

Ascorbate (mM)	OCR (nM s^{-1})
0	$< 1^{\dagger}$
2	61 ± 12
5	114 ± 16
10	224 ± 1
20	334 ± 45

*This is the rate of oxygen uptake by the media upon addition of ascorbate. No cells were present. n = 3.
†Sometimes minor electrode drift contributed to an apparent low OCR. This was always much less than with ascorbate.

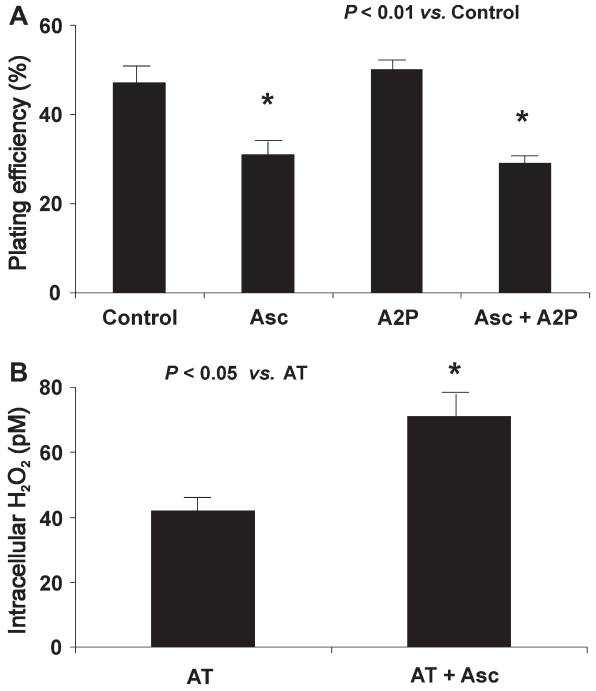

Figure 1. Ascorbate increases intracellular concentration of H_2O_2. A. MIA PaCa-2 cells were treated with L-ascorbate 2-phosphate ([A2P] = 100 μM) for 24 h and ascorbate (2 mM) for 1 h and clonogenic survival was determined. A combination of A2P (100 μM) and ascorbate (2 mM) did show a significant decrease in survival compared to controls. (*$P < 0.01$ *vs.* control, means ± SEM, $n = 3$). However, this treatment did not show significant decreases in clonogenic survival when compared to ascorbate-treated cells, suggesting that increased intracellular ascorbate levels were not responsible for ascorbate-induced cytotoxicity under our experimental conditions. B. MIA PaCa-2 cells were treated with 20 mM ascorbate in the presence of 20 mM aminotriazole (AT) and the concentration of intracellular H_2O_2 was determined using aminotriazole-mediated inactivation of endogenous catalase. Cells treated with ascorbate showed a significant 66 % increase in intracellular H_2O_2 when compared to cells that were treated with AT only, *$P < 0.05$ *vs.* AT, means ± SEM, $n = 4$.

intermediate [4]. We hypothesized that ascorbate-induced cytotoxicity is due to the formation of extracellular H_2O_2, which then diffuses into the cell and causes cytotoxicity as opposed to intracellular ascorbate oxidation. L-Ascorbate 2-phosphate (A2P) is a form of ascorbate that is protected from oxidation by the presence of a phosphate moiety. Although A2P is not a substrate of sodium-dependent vitamin C transporters (SVCTs), the phosphate group is most likely hydrolyzed by cell membrane esterases with the resulting ascorbate being transported into cells [20–22]. The addition of A2P to cell culture media will increase intracellular ascorbate to millimolar levels [23]. Figure 1A shows that addition of A2P to treatment with pharmacological ascorbate does not enhance cytotoxicity. MIA PaCa-2 cells treated with A2P (100 μM) for 24 h showed no changes in clonogenic survival when compared to controls, while cells treated with ascorbate (2 mM) had significant decreases in clonogenic survival. More importantly, clonogenic survival was similar in cells treated with the combination of A2P and ascorbate when compared to ascorbate alone. This suggests that increases in intracellular ascorbate are not responsible for ascorbate-induced cytotoxicity observed when ascorbate is added to the extracellular medium.

Previous studies have demonstrated that pharmacological ascorbate increases extracellular H_2O_2 generation [5]. To determine changes in intracellular H_2O_2 following exposure to pharmacological ascorbate we measured aminotriazole-mediated inactivation of endogenous catalase. We hypothesized that ascorbate would cause an increase in intracellular H_2O_2, thereby enhancing cytotoxicity. As shown in Figure 1B, MIA PaCa-2 cells treated with ascorbate (20 mM) for 1 h demonstrated a significant increase in intracellular H_2O_2 as determined by the rate of inactivation of intracellular catalase. Control cells had steady-state concentrations of H_2O_2 of 43 ± 5 pM, which increased to 71 ± 15 pM with ascorbate (20 mM) ($n = 3$, $P < 0.05$).

Ascorbate leads to oxidation of the GSSG/2GSH couple

Increases in intracellular H_2O_2 may lead to changes in the redox state of the intracellular redox buffer. Peroxide-induced oxidative stress can cause a decrease in the amount of intracellular GSH and an increase in GSSG [24,25]. We hypothesized that treatment with ascorbate would increase intracellular H_2O_2 leading to a decrease in intracellular GSH and changes in the important GSSG/2GSH redox couple. Indeed, our results show a time- and dose-dependent decrease in intracellular GSH (Figure 2) when cells are exposed to ascorbate (2 mM) for 0, 2, 6, and 24 h. As the time of exposure to ascorbate increased, the half-cell reduction potential (E_{hc}) of the GSSG/2GSH couple became more oxidized (Figure 2B). In addition, when MIA PaCa-2 cells were exposed to varying concentrations of extracellular ascorbate (2, 5, 10, and 20 mM), the intracellular concentration of GSH decreased (Figure 2C) with a concomitant increase in E_{hc}, *i.e.* GSSG/2GSH couple became more oxidized (Figure 2D). These results further demonstrate that exposure of cells to high levels of extracellular ascorbate induces intracellular oxidative stress by increasing intracellular H_2O_2.

Inhibitors of peroxide metabolism enhance ascorbate-induced cytotoxicity and intracellular H_2O_2 levels

Ascorbate can be cytotoxic to pancreatic cancer cells, and this cytotoxicity is most likely due to an H_2O_2-mediated mechanism [5]. We hypothesized that using inhibitors of H_2O_2 metabolism would enhance ascorbate-induced cytotoxicity by increasing the levels of H_2O_2 in the cell. 1,3-*bis*-Chloroethyl-l-nitrosourea (BCNU) is a chemotherapy drug that decomposes in aqueous buffer at physiological pH to form an alkylating moiety and a carbamoylation moiety. The alkylating moiety reacts in the cell to alkylate purines or pyrimidines, resulting in DNA and RNA cross-linking.

The carbamoylation moiety acts on nucleophilic alkyl side chain groups of amino acids inactivating proteins, including glutathione disulfide reductase [26,27]. After exposure to BCNU, cells increase the synthesis of new glutathione (GSH) [28] and also increase the percentage of glutathione disulfide (GSSG) [29], most likely due to

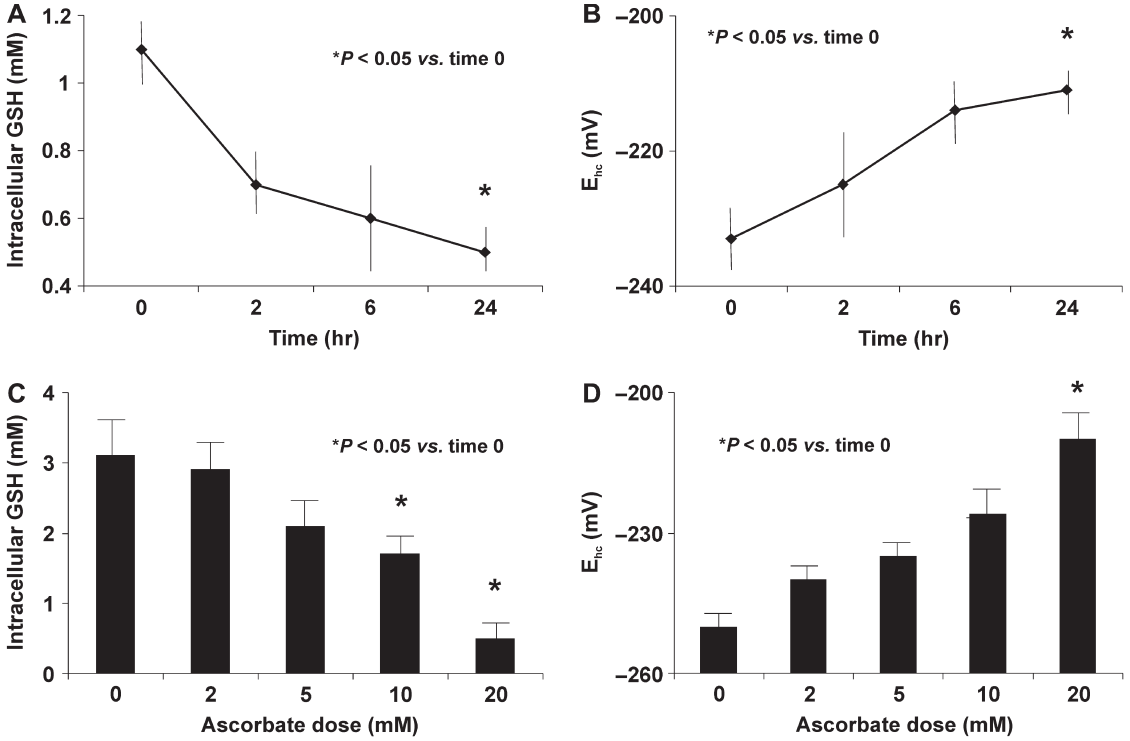

Figure 2. Ascorbate treatment induces oxidative stress. A. MIA PaCa-2 cells were treated with ascorbate (2 mM) for 0, 2, 6, and 24 h and intracellular GSH levels were measured. Ascorbate (2 mM) treatment decreases intracellular GSH. $^*P < 0.05$ *vs*. the 0 time point, means ± SEM, $n = 3$. B. Ascorbate (2 mM) increases (more positive) the intracellular half-cell reduction potential (E_{hc}) of the intracellular GSSG/2GSH couple (intracellular redox buffer) of MIA PaCa-2 cells, a clear indication of increased intracellular oxidations. $^*P < 0.05$ *vs*. the 0 time point, means ± SEM, $n = 3$. C. Intracellular GSH decreases with increasing concentrations of extracellular ascorbate (2 – 20 mM for 1 hour). $^*P < 0.05$ *vs*. the 0 mM dose, means ± SEM, $n = 3$. D. The half-cell reduction potential of MIA PaCa-2 cells increases in a dose-dependent manner demonstrating ascorbate-induced oxidation. $^*P < 0.05$ *vs*. the 0 mM dose, means ± SEM, $n = 3$.

the inactivation of GR, which reduces GSSG to GSH. If GR is inhibited, the ability of cells to remove hydrogen peroxide will be compromised. Pretreatment of MIA PaCa-2 cells with BCNU decreases GR activity in a dose-dependent manner (Figure 3A). Additionally, BCNU (25 μM) decreases GR activity in a time-dependent manner (Figure 3B). Cells treated with BCNU (25 μM) alone had no effect on clonogenic survival (Figure 3C). However, the combination of BCNU and ascorbate (2 mM) dramatically decreased clonogenic survival ($n = 3$, $^*P < 0.001$ *vs*. control, $^{**}P < 0.001$ *vs*. ascorbate). The combination of BCNU and ascorbate had similar effects in another pancreatic cancer cell line, AsPC-1 (Figure 3D). Plating efficiency was 44 ± 2 in control cells and decreased to 8 ± 1 with ascorbate (2 mM) and 13 ± 1 with BCNU (25 μM). However, the combination of ascorbate and BCNU further decreased plating efficiency to 2 ± 1 ($n = 3$, $^*P < 0.001$ *vs*. control, $^{**}P < 0.05$ *vs*. ascorbate). The MTT assay also demonstrated significant decreases in cell viability when MIA PaCa-2 and AsPC-1 cells were treated with the combination of ascorbate and BCNU. Ascorbate decreased viability to 15 ± 0.1 % of control while the combination of ascorbate and BCNU further decreased viability to 10 ± 0.1 % ($n = 4$, $^*P < 0.01$ *vs*. control, $^{**}P < 0.05$ *vs*. ascorbate) (Figure 3E).

As mentioned, BCNU has other effects in addition to inhibition of GR. To determine if changes in the activity of GR alone can modulate clonogenic survival, we used siRNA targeted to GR (Figure 4). MIA PaCa-2 cells transfected with siGR demonstrated decreases in GR protein compared to the siNegative control (Figure 4A). In addition, GR activity assays demonstrated > 50 % decrease in GR activity in cells treated with siGR (Figure 4B). After demonstrating that the siGR reduced the amount GR protein and decreased overall activity, a clonogenic survival assay was performed. Cells transfected with siGR had similar clonogenic survival when compared to controls. Ascorbate (2 mM) decreased clonogenic survival from 23 ± 0.3 % to 7 ± 0.1 % ($n = 3$, $P < 0.001$). In contrast, the combination of ascorbate and siGR enhanced the cytotoxicity of ascorbate leading to a further decrease in clonogenic survival down to 2.5 ± 0.5 % ($P < 0.001$ compared to ascorbate alone, Figure 4C).

2-Deoxy-D-glucose (2DG) is thought to cause inhibition of glucose metabolism and the pentose phosphate pathway, thereby creating a chemically induced state of glucose deprivation resulting in inhibition of hydroperoxide detoxification due to decreased NADPH [12]. When MIA PaCa-2 cells were treated with 2DG (25 mM) for 1 h clonogenic survival was similar to controls (controls 48 ± 4 %; 2DG 40 ± 0.1 %), Figure 5A. Treatment of cells with ascorbate (2 mM) decreased clonogenic survival to 22 ± 2 %. However, the combination of ascorbate and 2DG enhanced these effects, resulting in clonogenic survival of only 0.5 ± 0.1 % ($n = 3$, $P < 0.001$). Similar results were seen in AsPC-1 cells as the combination of

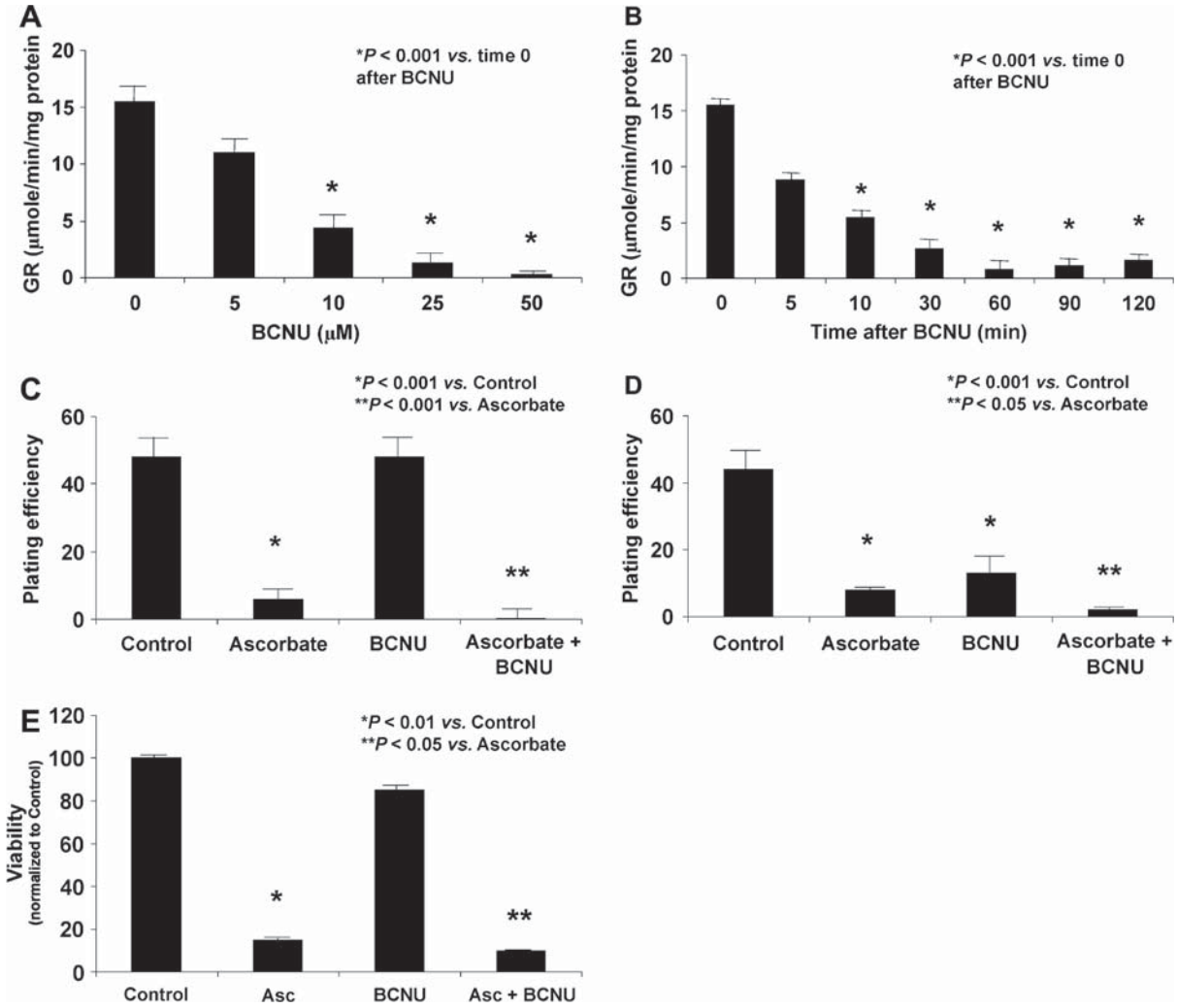

Figure 3. BCNU enhances ascorbate-induced cytotoxicity through the inhibition of glutathione disulfide reductase. A. BCNU decreases the activity of glutathione disulfide reductase in a dose-dependent manner. $^{*}P < 0.05$ *vs*. the BCNU 0 μM dose, means ± SEM, $n = 3$. B. MIA PaCa-2 cells treated with BCNU (25 μM) showed decreasing activity of GR over time. $^{*}P < 0.05$ *vs*. the BCNU 0 μM dose, means ± SEM, $n = 3$. C. MIA PaCa-2 cells were treated with BCNU (25 μM, 2 h) and ascorbate (2 mM, 1 h) and clonogenic survival was determined. Ascorbate caused a decrease in clonogenic survival, $^{*}P < 0.001$ *vs*. control, mean ± SEM, $n = 3$. D. AsPC-1 cells were treated with BCNU (25 μM) and ascorbate (2 mM) and clonogenic survival was determined. Ascorbate caused a decrease in clonogenic survival, $^{*}P < 0.001$ *vs*. control, mean ± SEM, $n = 3$. E. MIA PaCa-2 cells were treated with BCNU (25 μM, 2 h) and ascorbate (2 mM, 1 h) and cell viability with a MTT assay was determined. Ascorbate caused a decrease in cell viability, $^{*}P < 0.01$ *vs*. control, mean ± SEM, $n = 4$. Treatment with BCNU alone showed no significant effect on cytotoxicity, but a combination of BCNU and ascorbate enhanced ascorbate-induced cytotoxicity, $^{**}P < 0.05$ *vs*. ascorbate, means ± SEM, $n = 4$. Similar trends were seen with AsPC-1 cells treated with ascorbate ± BCNU (data not shown).

ascorbate and 2DG enhanced ascorbate-induced cytotoxicity (Figure 5B). Significant decreases in cell viability were also demonstrated when MIA PaCa-2 and AsPC-1 cells were treated with the combination of ascorbate and 2DG. Ascorbate decreased viability to 15 ± 0.1% of control while the combination of ascorbate and 2DG further decreased viability to 10 ± 0.1 % ($n = 4$, $^{*}P < 0.05$ *vs*. control, $^{**}P < 0.05$ *vs*. ascorbate) (Figure 5C). Taken together with the mechanism of 2DG-induced inhibition of hydroperoxide inhibition, these results are consistent with the hypothesis that H_2O_2 is a key species responsible for the toxic effects of pharmacological ascorbate.

To further elucidate the mechanism of ascorbate-induced cytotoxicity, cells were treated with a scavenger of H_2O_2, pyruvate; indeed, pyruvate rescues MIA PaCa-2 cells from ascorbate-induced cytotoxicity, Figure 5D. MIA PaCa-2 cells treated with ascorbate (2 mM) showed a significant decrease in clonogenic survival from 49 ± 3 % to 20 ± 0.5 % ($n = 3$, $P < 0.001$). However, pyruvate reversed ascorbate-induced cytotoxicity, while treatment with pyruvate alone (5 mM) had little effect on clonogenic survival. Again, these observations are consistent with a peroxide-mediated mechanism for the toxicity of pharmacological ascorbate.

Since exposure of cells to pharmacological ascorbate leads to an increase in intracellular H_2O_2 and inhibitors of peroxide-removal enhance ascorbate-induced cytotoxicity, we hypothesized that inclusion of inhibitors of H_2O_2 metabolism while cells were exposed to pharmacological ascorbate would further increase intracellular H_2O_2. We determined the changes in intracellular H_2O_2 that accompany treatments with inhibitors of H_2O_2 metabolism

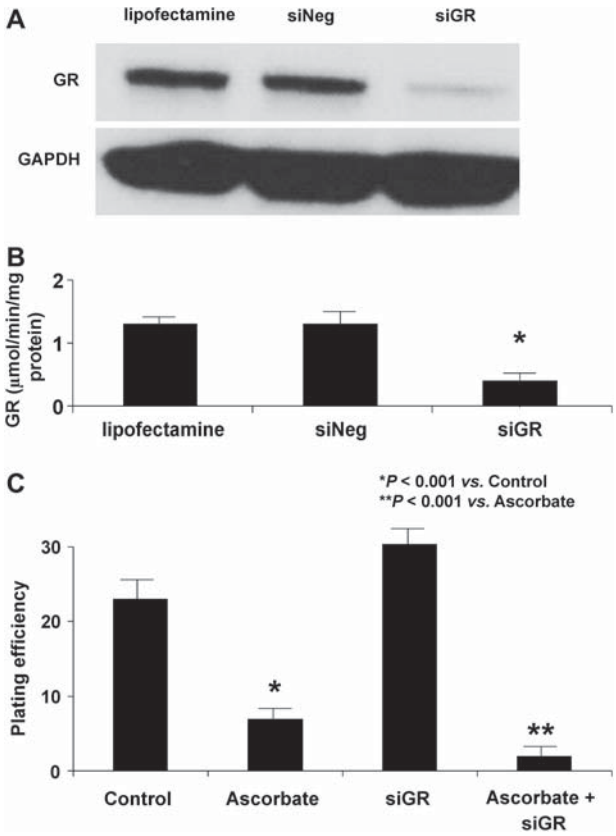

Figure 4. siRNA to GR enhances ascorbate-induced cytotoxicity. A. Cells were transfected with a siRNA against GR. The Western blot shows that GR protein was significantly decreased in siGR-transfected cells in comparison to controls. B. GR activity was decreased in cells treated with siGR compared to controls or cells treated with siNEG. C. MIA PaCa-2 cells were transfected with a siRNA against GR and treated with ascorbate (2 mM for 1 h) and clonogenic survival was determined. Treatment with siGR had little effect on clonogenic survival while cells treated with ascorbate showed a significant decrease in clonogenic survival, $^{*}P < 0.001$ *vs*. control, means ± SEM, $n = 3$. Additionally, treatment with a combination of siGR and ascorbate enhanced ascorbate-induced cytotoxicity ($^{**}P < 0.001$ *vs*. ascorbate, means ± SEM, $n = 3$).

through the use of aminotriazole-mediated inactivation of endogenous catalase, Figure 5E. BCNU (25 µM) combined with ascorbate (20 mM) increased intracellular H_2O_2 by 35 pM compared to cells treated with BCNU only ($P < 0.001$). Treatment with siRNA against GR in addition to ascorbate significantly increased intracellular H_2O_2 by 47 pM over cells treated with siRNA against GR only ($P < 0.05$). MIA PaCa-2 cells treated with 2DG (25 mM) and ascorbate (20 mM) for 1 h also showed a significant increase in intracellular H_2O_2 by 85 pM when compared with cells that were treated with 2DG alone ($P < 0.05$). These data demonstrate that inhibitors of H_2O_2 metabolism enhance the increase in the intracellular steady-state concentration of H_2O_2 caused by pharmacological ascorbate.

Pharmacological ascorbate increases intracellular H_2O_2. Pyruvate (5 mM) negated the increase in intracellular H_2O_2 seen upon exposure of cells to extracellular ascorbate (20 mM), Figure 5F. Consistent with this we found that pyruvate also reversed ascorbate-induced cytotoxicity, Figure 5D. These observations indicate that under our experimental conditions, extracellular H_2O_2 produced by the oxidation of pharmacological ascorbate is the primary species responsible for the observed cytotoxicity. This H_2O_2 diffuses into cells where it is removed by the intracellular antioxidant system. Agents that enhance peroxide removal, *e.g*. pyruvate, are protective; agents that inhibit intracellular peroxide removal sensitize cells to exposure to pharmacological ascorbate.

Discussion

Recent studies have demonstrated that pharmacological ascorbate induces cytotoxicity in pancreatic cancer cells and maybe a potential treatment for pancreatic cancer [5,30]. Our previous studies suggested that ascorbate-induced cytotoxicity is due to an H_2O_2-mediated mechanism, where H_2O_2 is formed in the extracellular fluid and diffuses into the cell, resulting in cytotoxicity [5]. Utilizing a reliable kinetic assay for the intracellular concentration of H_2O_2, *i.e*. the aminotriazole-mediated inactivation of endogenous catalase, we were able to measure ascorbate-induced changes in intracellular H_2O_2. Taken together with our previous studies [4,5], our current findings suggest that ascorbate oxidizes extracellularly producing H_2O_2, which diffuses into the cell to cause cytotoxicity. Using A2P, a form of ascorbate that increases intracellular ascorbate but does not oxidize in cell culture media, we demonstrated that increases of intracellular ascorbate did not attenuate the effect of extracellular ascorbate-induced cytotoxicity. We further demonstrated that the H_2O_2 produced by ascorbate extracellularly diffuses into the cell to induce cytotoxicity. Most importantly, our results showed that treatment of ascorbate (extracellular) increases the levels of intracellular H_2O_2. Consistent with our hypothesis, addition of pyruvate, a compound that scavenges H_2O_2, reversed ascorbate-induced increases in intracellular H_2O_2 as well as rescues cells from ascorbate-induced cytotoxicity. Additionally we demonstrated that increased levels of peroxide lead to oxidation of the intracellular redox buffer; intracellular GSH decreases in a dose- and time-dependent manner. Thus, ascorbate-induced generation of H_2O_2 leads to toxicity through an oxidative stress mechanism.

Due to ascorbate's role in the increase in intracellular H_2O_2, augmentation of the intracellular H_2O_2 concentration could potentially enhance cytotoxicity to pancreatic cancer cells. One method that can be used to increase the intracellular concentration of H_2O_2 is to inhibit peroxide removal. The GPx system is an important system in the detoxification of peroxide, and GR is an essential enzyme in this system [31]. We demonstrated that inhibiting GR with BCNU or siGR reduces the cell's ability to remove H_2O_2. Likewise, the GR system is dependent on glucose, and using 2DG, a competitive inhibitor of glucose, prevents the removal of H_2O_2. Our results showed that the combination of inhibitors of peroxide detoxification with ascorbate increased intracellular H_2O_2 concentration and cytotoxicity. Interesting for future research will be the targeting of the thioredoxin/peroxiredoxin node of the

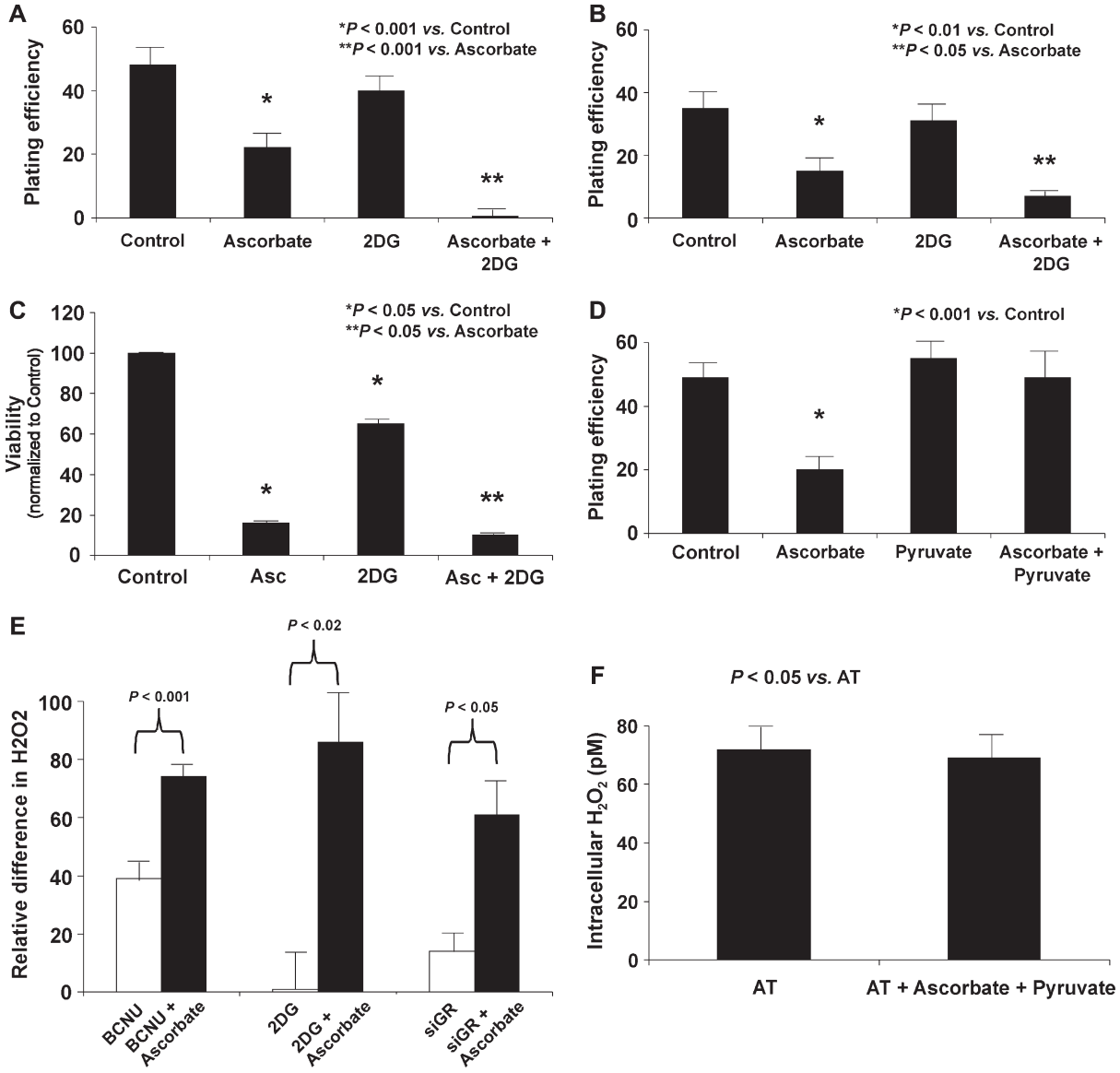

Figure 5. Inhibitors of H2O2 removal enhance ascorbate-induced cytotoxicity and increase intracellular concentrations of H2O2. A. Mia PaCa-2 cells were treated with 2DG (25 mM) and ascorbate (2 mM) for 1 h and clonogenic survival was determined. Ascorbate treatment caused a decrease in clonogenic survival ($^*P < 0.001$ *vs.* control, means ± SEM, $n = 12$). Treatment with 2DG (25 mM) did not affect clonogenic survival but the combination of 2DG (25 mM) and ascorbate showed a significant enhancement in ascorbate-induced cytotoxicity, $^{**}P < 0.001$ *vs*. ascorbate, means ± SEM, $n = 6$. B. AsPC-1 cells were treated with 2DG (25 mM) and ascorbate (2 mM) for 1 h and clonogenic survival was determined. Ascorbate treatment caused a decrease in clonogenic survival ($^*P < 0.01$ *vs*. control, means ± SEM, $n = 3$). Treatment with 2DG (25 mM) did not affect clonogenic survival but the combination of 2DG (25 mM) and ascorbate showed a significant enhancement in ascorbate-induced cytotoxicity, $^{**}P < 0.05$ *vs*. ascorbate, means ± SEM, $n = 3$. C. MTT assay demonstrated significant decreases in cell viability in MIA PaCa-2 and AsPC-1 cells treated with the combination of ascorbate and 2DG. Ascorbate significantly decreased viability while the combination of ascorbate and 2DG further decreased viability *(*$n = 4$, $^*P < 0.05$ *vs*. control, $^{**}P < 0.05$ *vs*. ascorbate). Similar trends were seen with AsPC-1 cells treated with ascorbate ± 2DG (data not shown). D. Cells were treated with pyruvate (5 mM) and ascorbate (2 mM) for 1 h and clonogenic survival determined. Cells treated with ascorbate showed a significant decrease in clonogenic survival ($^*P < 0.001$ *vs*. control, means ± SEM, $n = 3$). Pyruvate (5 mM), a scavenger of H_2O_2 was able to reverse the toxicity of ascorbate. Pyruvate alone (5 mM) did not affect clonogenic survival. E. MIA PaCa-2 cells were treated with BCNU (25 μM), 2DG (25 mM), or siGR and ascorbate (20 mM) for 1 h, and the concentration of intracellular H_2O_2 was measured. All cells treated with a combination of their respective inhibitor of hydroperoxide metabolism and ascorbate showed significant increases in intracellular H_2O_2 concentrations when compared to the inhibitors of hydroperoxide removal alone (Means ± SEM, $n = 3$). F. MIA PaCa-2 cells were treated with pyruvate (5 mM) and ascorbate (20 mM) for 1 h, and the concentration of intracellular H_2O_2 was measured. Cells treated with a combination of pyruvate and ascorbate showed no change in intracellular H_2O_2 concentration when compared to control cells (Means ± SEM, $n = 3$).

peroxide removal system to determine if the cytotoxicity induced by this H_2O_2 can be increased further.

Besides inhibition of GR *via* carbamoylation, BCNU also causes DNA or RNA crosslinking *via* alkylation. Thus, the dual effect of BCNU on enhanced cell killing may be due to the alkylating effect, as well as (or instead of) the inhibition of GR. In support of the carbamoylation effect of BCNU and subsequent inhibition of GR, Nathan and Cohn demonstrated that BCNU could enhance the antitumor effect of H_2O_2 [32]. Other methods to enhance

cancer cell toxicity by increasing intracellular peroxide include increased expression of manganese superoxide dismutase (MnSOD) [33]. Elevating the MnSOD activity in cells can increase the intracellular flux of superoxide ($O_2^{\bullet -}$) and subsequently H_2O_2 [33]. Inhibition of the GR activity in cells by BCNU resulted in inhibition of peroxide removal through the GPx pathway [34]. Zhong et al. [34] demonstrated a significant correlation between the sensitivity of glioma cells to BCNU and catalase levels suggesting that inhibition of the glutathione system shifts the burden to catalase for protecting against peroxide toxicity.

In summary, treatment of pancreatic cancer cells with pharmacological ascorbate results in extracellular generation of H_2O_2; this H_2O_2 diffuses into the cell resulting in an increase in intracellular H_2O_2 and cytotoxicity. Exciting is that inhibitors of H_2O_2 removal can enhance the cytotoxicity of pharmacological ascorbate. Thus, treatments consisting of a combination of ascorbate and inhibitors of the removal of H_2O_2 may potentially be an effective therapy for pancreatic adenocarcinoma. Pharmacological ascorbate in combination with appropriate adjuvants appears to be an immediately available and more clinically applicable treatment for pancreatic adenocarcinoma at this time compared to gene-directed therapies in their current state of development.

Declaration of interest

The authors report no declarations of interest. The author alone are responsible for the content and writing of the paper.

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