Intracellular glutathione levels are involved in carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone-induced apoptosis in As4.1 juxtaglomerular cells

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Abstract. Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) is an uncoupler of mitochondrial oxidative phosphorylation in eukaryotic cells. In the present study, we investigated the involvement of reactive oxygen species (ROS) and glutathione (GSH) in FCCP-induced As4.1 juxtaglomerular cell death. Intracellular ROS levels were decreased by FCCP at the early time points (10-150 min) and increased at 48 h. FCCP inhibited the activity of Mn-superoxide dismutase (Mn-SOD) via down-regulating its protein expression. Ebselen (an antioxidant) significantly attenuated ROS levels in FCCP-treated cells, but did not prevent FCCP-induced cell death. Moreover, intracellular GSH content was rapidly diminished within 10 min of FCCP treatment, which was accompanied by a reduction of the mitochondrial membrane potential [MMP ($\Delta\psi$m)]. L-buthionine sulfoximine (BSO, a GSH synthesis inhibitor) significantly augmented As4.1 cell death by FCCP. However, N-acetylcysteine (NAC, a GSH precursor and antioxidant) attenuated GSH depletion, MMP ($\Delta\psi$m) loss and cell death in FCCP-treated As4.1 cells. In addition, NAC increased Mn-SOD activity and decreased ROS levels in FCCP-treated As4.1 cells. In conclusion, these results suggest that compared to ROS levels, intracellular GSH levels are more closely linked to FCCP-induced apoptosis in As4.1 juxtaglomerular cells.

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Abbreviations: FCCP, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; ROS, reactive oxygen species; PARP, poly(ADP-ribose) polymerase; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; PS, phosphatidylserine; FITC, fluorescein isothiocyanate; GSH, glutathione; CMFDA, 5-chloromethyl-fluorescein diacetate; NAC, N-acetylcysteine; BSO, L-buthionine sulfoximine; SOD, superoxide dismutase; Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one; DCF, 2,7-dichlorofluorescein; MMP ($\Delta\psi$m), mitochondrial membrane potential; DNP, 2'4-dinitrophenol

Key words: FCCP, ROS, As4.1, GSH, mitochondrial membrane potential

Introduction

Juxtaglomerular cell tumors, first described in the late 1960s (1), are benign tumors of the kidney. Reninomas are understood to arise from juxtaglomerular cells. Clinically, the patients suffer from headaches, polyuria, nocturia and dizziness among other symptoms. As4.1 cells, a model for juxtaglomerular cells, were isolated from a kidney neoplasm of a transgenic mouse containing a renin SV40 T-antigen transgene (2). Although JGA cell tumors are considered benign, with only one report of metastasis, they are potentially lethal if left untreated (3).

Reactive oxygen species (ROS) include the hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) and the hydroxyl radical ($^{\bullet}OH$). These molecules can regulate many important cellular functions such as transcription factor activation, gene expression, cell differentiation and proliferation (4). Principal antioxidant enzymes include superoxide dismutase (SOD), which is expressed as extracellular (EC-SOD), cytoplasmic (Cu/Zn-SOD) and mitochondrial isoforms (Mn-SOD). These isoforms metabolize O_2^- to H_2O_2. Further metabolism of H_2O_2 by catalase and glutathione (GSH) peroxidase yields O_2 and H_2O (5). GSH is a main non-protein and thiol antioxidant in the cell and is crucial for regulation of cell proliferation and cell signaling (6) and is known to protect cells from toxic insults through detoxification of toxic metabolites of drugs and scavenging ROS (7). In addition, alterations in its concentration have been demonstrated as a common feature of many pathological situations including cancer and neuro-degenerative diseases (8). Moreover, high GSH levels have been associated with an apoptotic resistant phenotype in various cells (9).

Mitochondria are cellular organelles that perform pivotal functions essential for ATP production, ROS generation and metabolism. Mitochondria are also involved in cell death (or apoptosis) and cell survival pathways (10). Therefore, specific drugs that damage the mitochondria may provide a foundation

to treat a variety of diseases, especially cancer, wherein these functions are deregulated. For instance, a mitochondria uncoupler can rapidly dissipate the gradient of protons across the inner mitochondrial membrane, trigger ROS production, reduce MMP ($\Delta\psi m$) and consequently induce cell death (11,12). Recently, we demonstrated that 2,4-dinitrophenol (DNP) as a mitochondrial uncoupler induces apoptosis in As4.1 cells via the loss of MMP ($\Delta\psi m$) and alterations of GSH levels (13). In the present study, we evaluated the involvement of ROS and GSH in the carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP)-induced death of As4.1 juxtaglomerular cells and examined the effects of 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen), L-buthionine sulfoximine (BSO) and N-acetylcysteine (NAC) on FCCP-treated As4.1 cells in relation to cell death, ROS and GSH.

Materials and methods

Cell culture. As4.1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in a humidified incubator containing 5% CO_2 at 37˚C. As4.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco-BRL, Grand Island, NY). Cells were routinely grown in 100-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark).

Reagents. DMEM, FBS, dimethylsulfoxide (DMSO), L-BSO, propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), FCCP and ebselen were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO). FCCP was dissolved in ethanol at 200 mM as a stock solution. BSO and ebselen were dissolved in water at 100 mM as stock solutions. The non-toxic dietary glutathione precursor, NAC was obtained from Sigma and was dissolved in designated solution buffer (500 mM sodium bicarbonate) at 500 mM as a stock solution. The control cells also contained the appropriated amounts of vehicles, which had no effect on cell death. All of the stock solutions were kept at -20˚C.

Detection of intracellular ROS and GSH level. Intracellular ROS and GSH were detected using the probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Ex/Em, 495 nm/529 nm) and 5-chlorometylfluorescein diacetate (CMFDA; Ex/Em, 522 nm/595 nm) (Invitrogen Molecular Probes, Eugene, OR) as previously described (14). Negative CMF staining (GSH depleted) cells were expressed as the percent of negative CMF cells. The CMF (excluding GSH-depleted cells) and ROS levels were expressed as mean fluorescence intensity (MFI), which was calculated with the CellQuest software (Becton-Dickinson).

Measurement of cellular SOD activity. The level of cellular SOD enzyme activity was measured using the SOD assay kit, WST (Fluka Co., Milwaukee, WI) as indicated by the manufacturer. Activities between Mn-SOD and Cu/Zn-SOD were distinguished by differential sensitivity to 1 mM potassium cyanide blocking the Cu/Zn-SOD activity (15). The value for the experimental group was converted to the percentage of the control group.

Measurement of cellular catalase activity. The level of cellular catalase enzyme activity was measured using the catalase assay kit from Sigma-Aldrich Chemical Company as indicated by the manufacturer. The value for the experimental group was converted to the percentage of control group.

Western blot analysis. Protein expression was evaluated using Western blot analysis, as previously described (16). In brief, $1x10^6$ cells were incubated with 20 μM of FCCP for 12-48 h with or without NAC. The cells were then washed in PBS and suspended in five volumes of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP-40, 0.5 mM DTT, 1% protease inhibitor cocktail). Lysates were collected and stored at -20˚C until further use. Supernatant protein concentrations were determined using the Bradford method. Supernatant samples containing 30 μg total protein were resolved by SDS-PAGE gel depending on the target protein sizes, and were then transferred onto an Immobilon-P PVDF membranes (Millipore, MA) by electro-blotting. The membranes were probed with antibodies against ß-actin, PARP, Cu/Zn-SOD, and Mn-SOD (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Blots were developed using an ECL kit (Amersham, Arlington Heights, IL).

Measurement of MMP ($\Delta\psi m$). MMP ($\Delta\psi m$) levels were measured using rhodamine 123 fluorescent dye (Sigma-Aldrich Chemical Company; Ex/Em, 485nm/535nm) as previously described (17). Rhodamine 123 staining intensity was determined by flow cytometry (Becton-Dickinson). An absence of rhodamine 123 from the cells indicated the loss of MMP ($\Delta\psi m$) in cells. The MMP ($\Delta\psi m$) levels in viable cells (excluding MMP ($\Delta\psi m$) loss cells) were expressed as the MFI, which was calculated by the CellQuest software (Becton-Dickinson).

Annexin V-fluorescein isothiocyanate (FITC) staining for apoptotic detection. Apoptosis was determined by staining cells with annexin V-FITC (Invitrogen Corporation, Branford, CT; Ex/Em, 488 nm/519 nm). In brief, $1x10^6$ cells were incubated with the designated doses of FCCP with or without NAC, BSO and ebselen for 48 h. The cells were washed twice with cold PBS, and then resuspended in 500 μl of binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of $1x10^6$ cells/ml. Annexin V-FITC (5 μl) was then added to these cells, which were analyzed with a FACStar flow cytometer (Becton-Dickinson).

Statistical analysis. The results represent the mean of at least three independent experiments (mean ± SD). The data were analyzed using Instat software (GraphPad Prism4, San Diego, CA). The Student's t-test or one-way analysis of variance (ANOVA) with a post-hoc analysis using the Tukey's

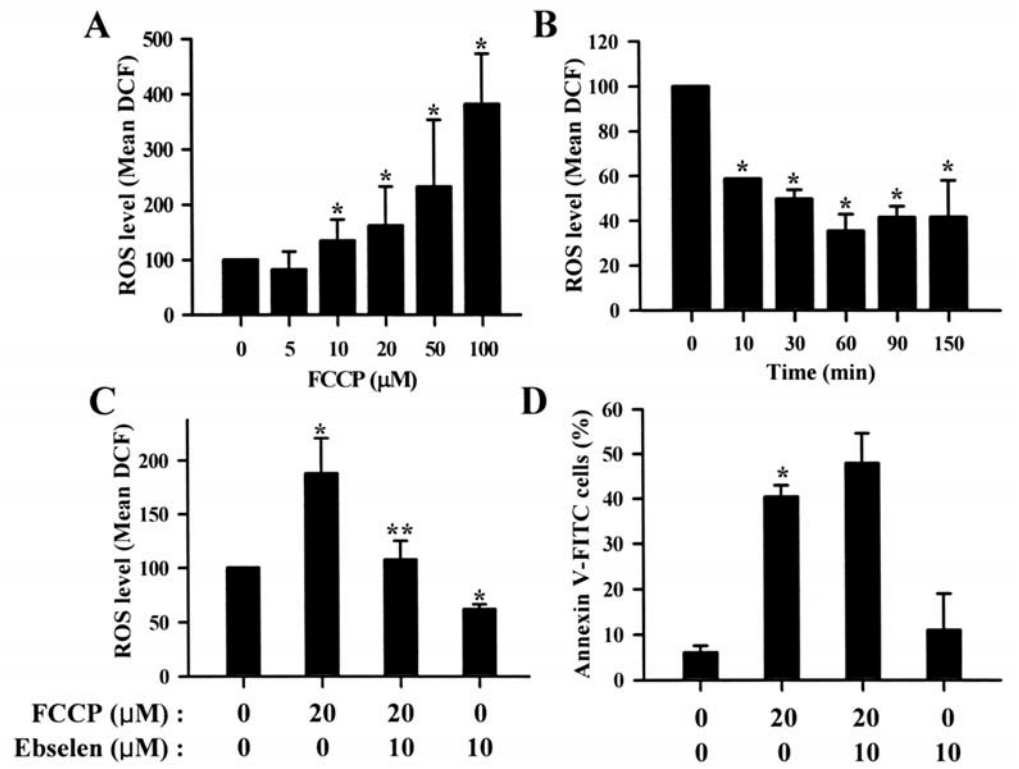

Figure 1. Effects of FCCP on ROS levels in As4.1 cells. (A and B) The graphs show intracellular ROS levels in 5-100 μM FCCP-treated cells at 48 h (A) and in 20 μM FCCP-treated cells for 10-150 min (B). (C) The graph shows intracellular ROS levels in FCCP-treated cells at 48 h following pre-treatment with 10 μM ebselen for 30 min. (D) The graph shows the percent of annexin V-FITC cells in As4.1 cell treated with FCCP and/or ebselen. *P<0.05 compared with the control. **P<0.05 compared with cells treated only with FCCP.

multiple comparison test for parametric data. Statistical significance was defined as $p<0.05$.

Results

Effects of FCCP on ROS level in As4.1 cells. We have previously observed that FCCP inhibited the growth of As4.1 cells with an IC_{50} of approximately 10 μM FCCP and induced apoptosis at 48 h (18). We first screened As4.1 cells with a wide range FCCP concentrations and chose the optimal doses (5-100 μM) and incubation times (10-150 min or 48 h) for further experiments. As shown in Fig. 1A, increases in ROS level were detected in a dose-dependent manner at 48 h. The level of ROS in As4.1 cells treated with 100 μM FCCP was approximately 4x higher than that of the control cells (Fig. 1A). Treatment with 20 μM FCCP, considered to be a suitable dose to differentiate the level of apoptosis, significantly decreased ROS levels at the early times points of 10-150 min (Fig. 1B).

To test whether ROS act as signaling molecules to induce cell death in FCCP-treated As4.1 cells, cells were treated with ebselen for 30 min prior to FCCP exposure. Pre-treatment with 10 μM ebselen efficiently reduced ROS levels in 20 μM FCCP treated- and untreated-cells at early time points (data not shown) and at 48 h (Fig. 1C). However, ebselen did not protect As4.1 cells against FCCP insults, as shown by the annexin V-positive staining of As4.1 cells (Fig. 1D). Collectively, FCCP reduced ROS levels for the early time phases, and finally increased the level at the late time phase of 48 h. In addition, FCCP induced apoptosis in As4.1 cells, which was not prevented by ebselen.

Effects of FCCP on SOD and catalase in As4.1 cells. Because FCCP increased ROS levels at 48 h, we attempted to evaluate whether FCCP affects these antioxidant enzymes in As4.1 cells. Treatment with 20 μM FCCP significantly inhibited the Mn-SOD activity in a time-dependent manner, while the activity of Cu/Zn-SOD was not changed for 48 h (Fig. 2A). In addition, treatment with FCCP enhanced the activity of catalase in As4.1 cells for 12 h. After 12 h, catalase activity was diminished (Fig. 2A). Treatment with FCCP also down-regulated Mn-SOD protein expression and did not change Cu/Zn-SOD protein expression during the tested time (Fig. 2B).

Effects of FCCP on the intracellular GSH levels in As4.1 cells. Next, we analyzed the changes of GSH levels in As4.1 cells using the CMF dye. The M1 regions in Fig. 3A demonstrate the depletion of GSH content in As4.1 cells. FCCP significantly elevated the numbers of cells in the M1 region at 48 h in a dose-dependent manner, indicating that FCCP induced a dose-dependent depletion of the intracellular GSH contents in As4.1 cells. Treatment with 20 μM FCCP for short time periods, decreased GSH levels (mean CMF fluorescence) within 10 min, and the decrease in GSH levels remained for the tested time of 150 min (Fig. 3B).

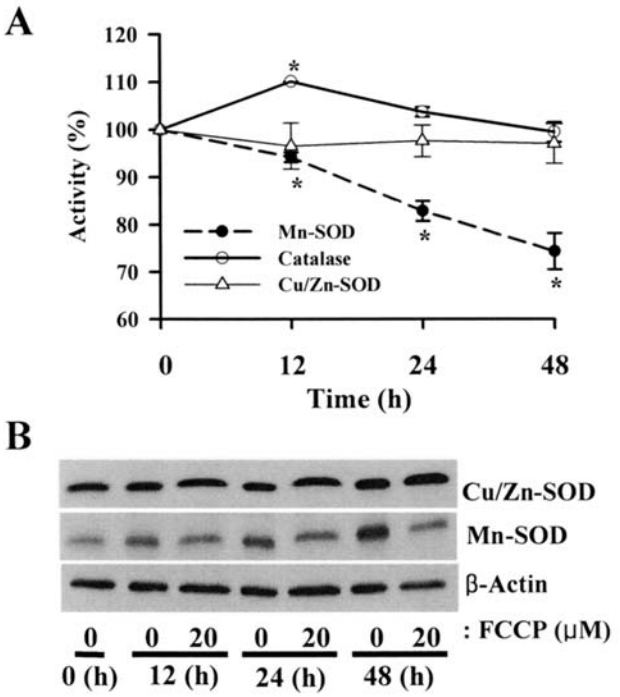

Figure 2. Effects of FCCP on SOD and catalase in As4.1 cells. (A) The graph shows the activities of Cu/Zn-SOD, Mn-SOD and catalase. Results shown are the means of three independent experiments. *P<0.05 compared with the control group. (B) Protein extracts were separated by SDS-PAGE gel, transferred onto the PVDF membrane and immunoblotted with the indicated antibodies, Cu/Zn-SOD, Mn-SOD and ß-actin.

Effects of BSO on GSH level, ROS level, and apoptosis in FCCP-treated As4.1 cells. To verify the possibility that a decrease in GSH levls is relevant to FCCP-induced As4.1 cell death, the reduction of GSH levels was induced by pre-treatment with 20 μM BSO (a GSH synthesis inhibitor) (19). BSO diminished the intracellular GSH level in FCCP-treated As4.1 cells and control cells (Fig. 3C). Coincidentally, BSO intensified FCCP-induced apoptosis in As4.1 cells whereas this agent alone did not induce apoptosis in As4.1 control cells (Fig. 3D). Concerning the intracellular ROS levels, BSO strongly exaggerated intracellular ROS level in FCCP-treated As4.1 cells (Fig. 3E). BSO alone increased intracellular ROS levels as well (Fig. 3E).

Effects of NAC on GSH levels, ROS levels and apoptosis in FCCP-treated As4.1 cells. We investigated whether ROS and GSH levels in FCCP-treated As4.1 cells were altered by a well known antioxidant, NAC. Pre-treatment of As4.1 cells with 2 mM NAC for 30 min prevented FCCP-mediated GSH reduction at early time points (30-120 min) (Fig. 4A). Intracellular GSH contents were slightly increased in NAC-treated control cells (Fig. 4A). However, the increase in GSH did not appear to be statistically significant. Treatment with 2 mM NAC significantly prevented the GSH depletion level in FCCP-treated As4.1 cells at 48 h (Fig. 4B) and decreased the ROS (Fig. 4C). NAC alone, did not affect GSH and ROS levels in control cells (data not shown). NAC restored Mn-SOD activity and its expression was reduced by FCCP at 48 h (Fig. 4D and E).

Next, we examined whether NAC inhibited FCCP-induced apoptosis. As shown in Fig. 5A, NAC rescued some cells

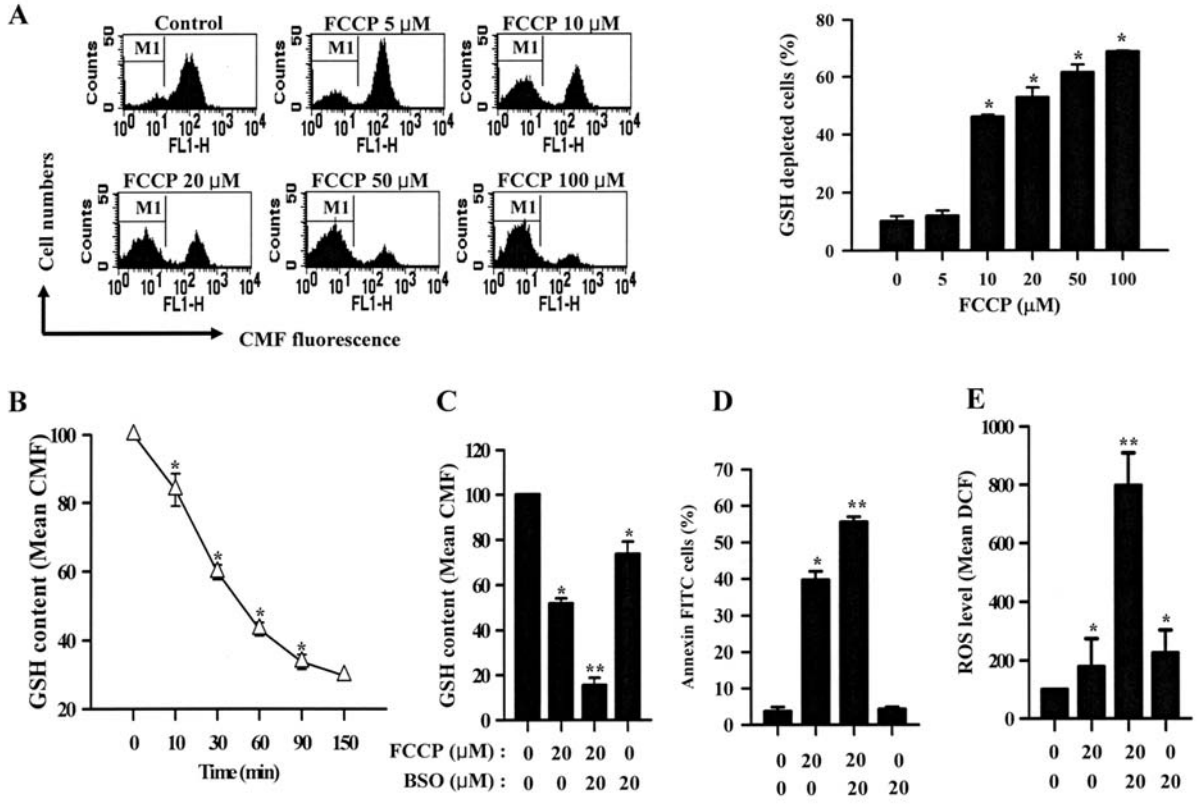

Figure 3. Effects of FCCP and/or BSO on intracellular GSH, ROS and apoptosis in As4.1 cells. (A) The M1 region indicates GSH-depleted (CMF negative) cells. The graph shows the percent of M1 region cells in FCCP-treated As4.1 cells at 48 h. (B) The graph shows the levels of mean CMF fluorescence in As4.1 cells. (C-E) Graphs show intracellular GSH contents (C), annexin V FITC-stained cells (D) and intracellular ROS level (E). *P<0.05 compared with the control group. **P<0.05 compared with the FCCP only-treated cells.

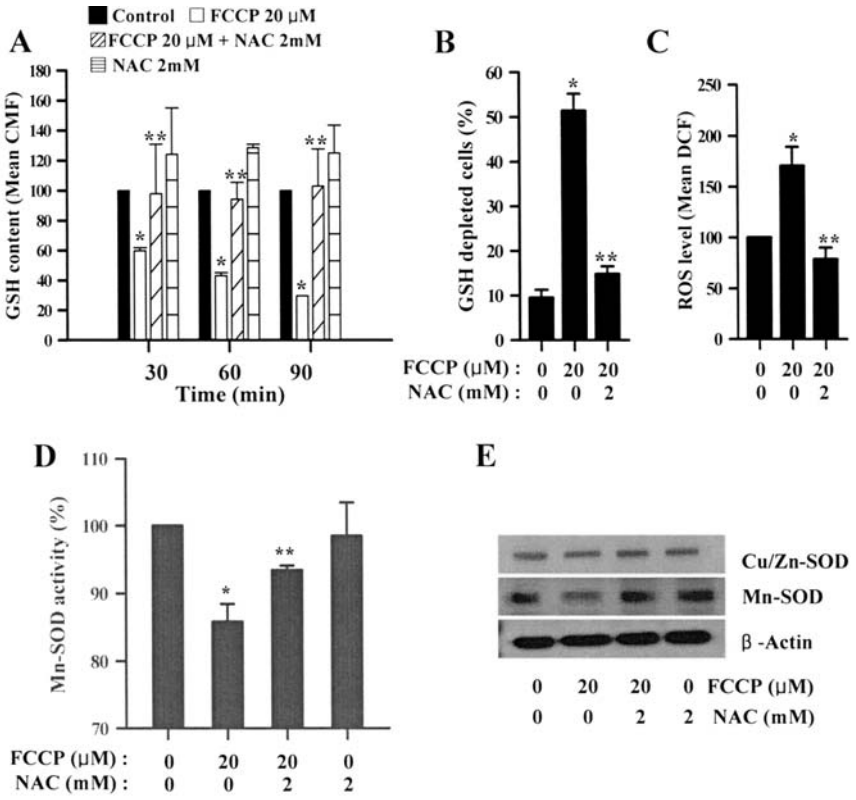

Figure 4. Effects of NAC on GSH, ROS and SOD activity in FCCP-treated As4.1 cells. Exponentially growing cells were pretreated with NAC for 30 min and exposed to 20 μM FCCP for the indicated times or 48 h. (A-D) The graphs show intracellular GSH levels (A), percent of GSH depleted cells (B), intracellular ROS levels (C) and Mn-SOD activity (D). (E) Immnunoblot analysis of Cu/Zn-SOD and Mn-SOD protein. *P<0.05 compared with the control group; **P<0.05 compared with the FCCP only-treated cells.

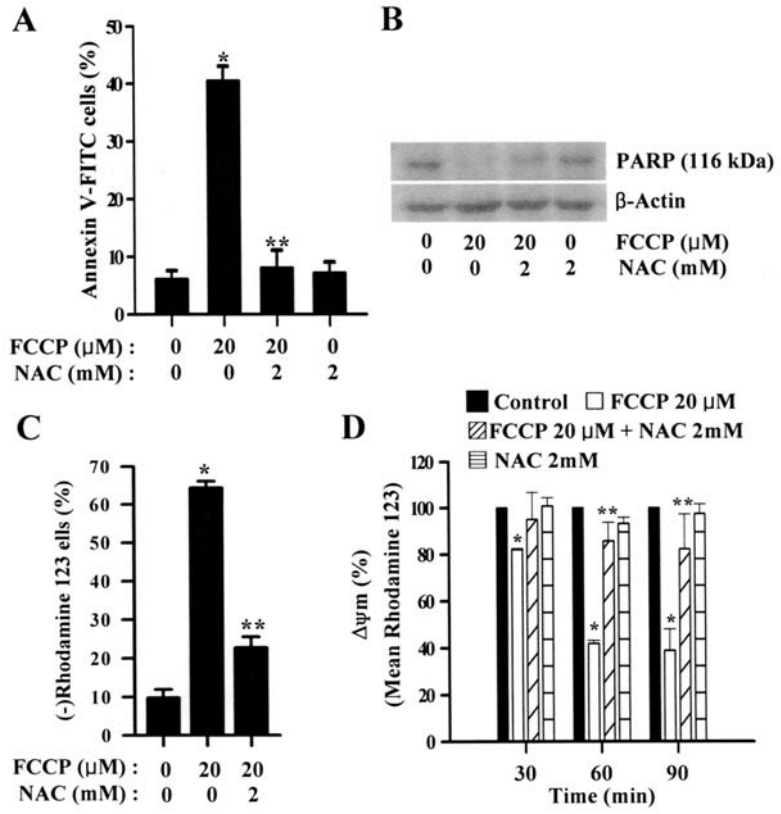

Figure 5. Effects of NAC on apoptosis and MMP ($\Delta\psi$m) in FCCP-treated As4.1 cells. Following treatment with 2 mM NAC for 30 min, cells were treated with 20 μM FCCP for the designated times or 48 h. (A) Percent of annexin V-positive cells. (B) Immnunoblot detection of PARP. (C) Percent of rhodamine 123 negative staining [MMP ($\Delta\psi$m) loss] cells. (D) Levels of mean rhodamine 123 in cells except MMP ($\Delta\psi$m) loss cells. *P<0.05 compared with the control group; **P<0.05 compared with the FCCP only-treated cells.

from FCCP insults, as assessed by the percentage of annexin V positive cells. In addition, NAC partially prevented the cleavage of PARP (a hallmark of apoptosis) in FCCP-treated cells (Fig. 5B). NAC also efficiently prevented the loss of MMP ($\Delta\psi m$) in FCCP-treated As4.1 cells at 48 h (Fig. 5C). At the early time points (30-120 min), FCCP significantly reduced the MMP ($\Delta\psi m$) level and NAC attenuated the decreased level (Fig. 5D).

Discussion

In the present study, we focused on the roles of ROS and GSH in FCCP-induced As4.1 cell death. The scheme for FCCP-induced As4.1 cell death according to our data is shown in Fig. 6. ROS and GSH levels were significantly decreased at the early time points. However, FCCP increased intracellular ROS level at 48 h. This result indicates that ROS levels are dependent on incubation times of FCCP and raises the possibility that prolonged incubation with FCCP induces mitochondrial membrane damage. In fact, we observed that FCCP induced the loss of MMP ($\Delta\psi m$) in As4.1 cell. When determining whether a ROS scavenger prevented FCCP-induced cell death, ebselen caused the reduction of ROS levels in FCCP-treated As4.1 cells, but it did not decrease apoptosis levels in these cells. We have also recently reported that ROS scavengers did not prevent apoptosis in FCCP-treated Calu-6 lung cancer cells or DNP-treated As4.1 cells (13,20). Furthermore, BSO strongly increased ROS levels in FCCP-treated As4.1 cells, but it mildly enhanced apoptosis in FCCP-treated cells. BSO alone increased ROS levels to a similar level of FCCP-treated cells without cell death. In contrast, another antioxidant of NAC induced parallel decreases in ROS level and apoptosis in FCCP-treated As4.1 cells. Taken together, these results suggest that intracellular ROS levels are not closely, but to some extent related to FCCP-induced cell death in As4.1 cells.

The high level of ROS in FCCP-treated cells at 48 h was likely to be from the decreased activity of SOD, especially Mn-SOD activity as well as mitochondrial membrane damage. The activity of Mn-SOD was reduced in FCCP-treated As4.1 cells at ~12-48 h. This decrease seemed to down-regulate the dismutation of the O_2^- generated from mitochondria into H_2O_2, resulting in accumulation of ROS in the cells. Interestingly, an increased catalase activity was observed in FCCP-treated As4.1 cells at 12 h and the augmented catalase activity decreased between 12-48 h. Kuruvilla *et al* showed that FCCP down-regulated the gene expression associated with antioxidant enzymes in a human rhabdomyosarcoma cell line (21). Conclusively, these results indicate that intracellular ROS level in FCCP-treated cells is regulated by various redox-related proteins including Mn-SOD and catalase as well as mitochondrial damage.

Intracellular GSH levels have a decisive role in anticancer drug-induced apoptosis, as indicated by the fact that apoptotic effects are inversely comparative to GSH levels (22,23). The reduction of GSH levels in FCCP-treated Calu-6 lung cells has a decisive effect on cell death (20). Likewise, decreased GSH levels and GSH depletion were observed in FCCP-treated As4.1 cells. This finding suggests that FCCP plays a role as a GSH depletor itself in As4.1 cells. Although we cannot exactly explain how FCCP decreased GSH, it is likely that FCCP directly interacts with GSH since FCCP decreased GSH content within 10 min. In addition, inhibition of GSH synthesis by BSO significantly aggravated the reduction of GSH content and cell death in FCCP-treated As4.1 cells. NAC obviously increased GSH levels at the early time points and inhibited depletion of GSH in FCCP-treated As4.1 cells. NAC also prevented apoptosis and PARP cleavage in FCCP-treated As4.1 cells. These results are consistent with other reports that intracellular GSH is rapidly decreased before the commencement of cell death in response to various insults (20,24,25). Notably, BSO alone decreased the intracellular GSH level without cell death in As4.1 cells. These results suggest that a simple decrease in GSH levels is necessary, but not sufficient to trigger apoptosis.

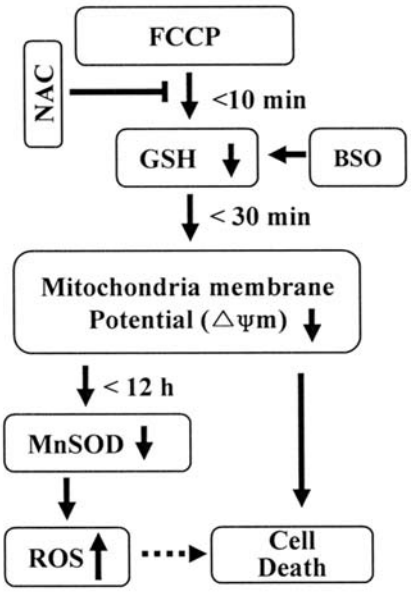

Figure 6. The scheme for FCCP-induced As4.1 cell death.

GSH depletion has been shown to directly modulate both formation of the permeability transition pore and cell death (26-28). According to our results, a decrease in GSH level and MMP ($\Delta\psi m$) were observed in FCCP-treated As4.1 cells at early time points. NAC increased both GSH level and MMP ($\Delta\psi m$) levels in FCCP-treated cells. Interestingly, the decreased GSH level by FCCP at the early time points was higher than the MMP ($\Delta\psi m$) reduction levels. Probably, FCCP primarily decreases intracellular GSH and then induces mitochondrial dysfunction to progress to subsequent steps, such as loss of MMP ($\Delta\psi m$) and generation of ROS.

Taken together, our results suggest that compared to ROS levels, intracellular GSH levels are more closely linked to FCCP-induced apoptosis in As4.1 juxtaglomerular cells.

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