Manganese Superoxide Dismutase Attenuates Cisplatin-Induced Renal Injury: Importance of Superoxide

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Abstract. Cisplatin is a potent chemotherapeutic agent that is used to treat many human malignancies. Unfortunately, in addition to side effects such as ototoxicity, anaphylaxis, and bone marrow suppression, a significant percentage of patients receiving cisplatin develop severe nephrotoxicity. Mitochondrial dysfunction that is mediated via the generation of reactive oxygen species has been implicated in the pathogenesis of cisplatin-induced renal injury. To address the mechanism, it was hypothesized that overexpression of antioxidant enzymes, such as mitochondria-localized manganese superoxide dismutase (MnSOD) or mitochondria-targeted catalase (mito-Cat), would be cytoprotective in cisplatin-induced cell injury. To this end, human MnSOD or a mito-Cat vector were stably transfected into human embryonic kidney 293 cells. Cells that overexpressed MnSOD exhibited significantly less cell rounding and detachment compared with both mito-Cat and vector controls after exposure to 20 μM cisplatin. Cell injury as assessed by DNA fragmentation and annexin V binding assays was significantly decreased in the cells that overexpressed MnSOD compared with vector alone and mito-Cat. In addition, elevated levels of MnSOD were strongly associated with increased clonogenic potential after cisplatin challenge. Thus, overexpression of MnSOD, and not catalase, protects against cisplatin-induced renal epithelial cell injury. These results demonstrate the importance of reactive oxygen species in the mechanism that underlies cisplatin-induced renal injury and specifically implicate the superoxide radical, and not hydrogen peroxide, as the mediator.

Cisplatin is a chemotherapeutic agent that is used in the treatment of a variety of solid-organ cancers, including those of the head, neck, testis, ovary, and breast (1). Unfortunately, in addition to causing bone marrow suppression, ototoxicity, and anaphylaxis, 28 to 36% of patients receiving an initial dose (50 to 100 mg/m²) of cisplatin develop acute renal failure (1,2) due to its preferential accumulation within the proximal tubule cells in the outer medulla of the kidney (2,3). The cellular events in cisplatin-mediated nephrotoxicity, including decreased protein synthesis, membrane permeabilization, mitochondrial dysfunction, and DNA injury, are a consequence of free radical generation and the inability to scavenge such molecules (4–6). These contentions are supported by a variety of studies, including those that demonstrate a protective role for free radical scavengers, such as vitamin E, catalase, and glutathione, in cisplatin-mediated cytotoxicity (7–12). Of particular interest is work by McGinness et al. (11) that demonstrated a reduction in cisplatin-mediated nephrotoxicity in rats that were treated with the superoxide dismutase (SOD) mimetic, orgotein.

Studies from our laboratory and others have demonstrated a protective role for the mitochondrial-localized manganese superoxide dismutase (MnSOD) in several models of free radical–mediated cell injury (13–15). In light of the clear role that reactive oxygen species (ROS) and the mitochondria play in the apoptotic cascade (16) and, more importantly, in cisplatin-induced renal cell injury (4,17,18), we hypothesized that overexpression of MnSOD provides protection against cisplatin-mediated cytotoxicity in cultured renal epithelial cells. Therefore, in an attempt to define the specific role of ROS in cisplatin-mediated toxicity, we challenged renal epithelial cells that stably expressed MnSOD with cisplatin. In addition, we generated cells that overexpressed a mitochondria-targeted catalase (mito-Cat) to evaluate the free radical specificity of cisplatin-induced renal cell injury. Our results demonstrate that overexpression of MnSOD, and not catalase, provides protection against cisplatin in renal epithelial cells, a result that strongly implicates a role for the mitochondria-derived superoxide radical in cisplatin-mediated nephrotoxicity.

Materials and Methods

Reagents

Cisplatin and calf serum were obtained from Sigma Chemical (St. Louis, MO). The cytotoxicity kit for lactate dehydrogenase (LDH) was purchased from Roche (Mannheim, Germany). The apoptosis detection kit was obtained from R & D Systems (Minneapolis MN). A rabbit polyclonal anti-MnSOD antibody was generated in our laboratory by using an internal peptide coupled to hemocyanin. The anti-catalase antibody was purchased from Calbiochem (La Jolla, CA). pPCR-Script Amp (SK)+ was purchased from Stratagene (La Jolla, CA), and the mammalian expression vector pcDNA3.1/Zeocin + and Zeocin were obtained from Invitrogen (Carlsbad, CA).
Cell Culture
Human embryonic kidney 293 (HEK 293) cells (ATCC, Rockville, MD) were cultured in Dulbecco’s modified Eagle’s medium that was supplemented with 4 mM glutamine, 25 mM HEPES, and 10% calf serum at 37°C in 90% room air/10% CO₂.

Plasmid Construction for MnSOD and Mito-Cat Overexpression
An 853-bp MnSOD cDNA was generated by reverse transcriptase–polymerase chain reaction (PCR) from mRNA that had been isolated from human renal proximal tubule cells (Clonetics, Walkersville, MD) by using oligonucleotides that contained HindIII (5’) and EcoRI (3’) restriction sites. The resulting product was ligated into pPCR-Script Amp SK(+) and then directionally cloned into the HindIII/EcoRI sites of pcDNA3.1/Zeo (pcDNA3.1/SOD; Figure 1A). For construction of the mitochondrial leader sequence fused to a human catalase cDNA was removed by complete digestion with Bgl II and NotI of pSVZeomspCAT (kindly provided by A. Melendez and A. M. Rodriguez, Center for Immunology and Microbial Disease, Albany Medical College, Albany, NY) (19). This fragment was then placed downstream of the cytomegalovirus promoter/ enhancer of the mammalian expression vector pcDNA3.1/Zeo+ that had been completely digested with NotI and partially digested with Bgl II (pcDNA3.1/mito-Cat; Figure 2A). The integrity of all constructs was verified by DNA sequencing.

Stable Transfection of MnSOD and Mito-Cat Plasmids
One µg of each expression plasmid was stably transfected into HEK 293 cells by using Lipofectamine Plus (Life Technologies/BRL, Rockville, MD) according to the manufacturer's instructions. Cells were cultured for 48 h and transfected to Dulbecco’s modified Eagle’s medium that contained HEPES (25 mM), glutamine (4 mM), and zeocin (150 µg/ml). Media that contained 150 µg/ml zeocin was replaced every 3 d for approximately 4 wk. Multiple zeocin-resistant colonies were isolated by clonal selection and subcultured without antibiotic before screening for MnSOD or catalase overexpression by immunoblot analysis.

Northern Blot Analysis
Total cellular RNA was isolated from transfected cells by using a modification of the Chomczynski and Sacchi method (20). RNA was quantified and size fractionated on a 1% agarose-formaldehyde gel, blotted onto a nylon membrane, and hybridized with a 32P-labeled human MnSOD cDNA probe. The membrane was washed and subjected to autoradiography.

Immunoblot Analysis
For immunoblot analysis, transfected cells were lysed with buffer that contained Triton X-100 (1%) (Sigma). Protein concentration was determined by the bicinchoninic assay (Pierce, Rockford, IL), and 20 µg of total protein was electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were blocked and incubated for 1 h with anti-MnSOD (polyclonal, 1:600) or anti-catalase (1:500, Calbiochem) and then by washing and incubation with peroxidase-conjugated goat anti-rabbit IgG antibody (1:10,000). Labeled protein bands were visualized by chemiluminescence (Pierce).

Subcellular Fractionation
To confirm mitochondrial targeting of overexpressed catalase, cytosolic and mitochondrial fractionation was performed as described previously (21). Briefly, cells were washed with ice-cold phosphate-buffered saline (PBS), harvested with a cell scraper, and centrifuged at 200 × g for 5 min at 4°C. The pellet was resuspended in 300 µl of ice-cold buffer A (20 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM

Figure 1. Plasmid construction and overexpression of manganese superoxide dismutase (MnSOD). (A) Schematic representation of the MnSOD expression vector pcDNA3.1/SOD and Northern blot analysis of HEK 293 cell transiently transfected with either vector alone or pcDNA3.1/SOD. The endogenous (4 KB and 1 KB) and the transgene- derived (approximately 1.2 KB) transcripts are shown. CMV, cytomegalovirus; polyA, bovine growth hormone polyadenylation signal. (B) Individual clonal cell populations transfected with either vector alone or pcDNA3.1/SOD were analyzed by immunoblot analysis as described in Materials and Methods. The 22-kD MnSOD monomer is shown.

Figure 2. Plasmid construction and overexpression of mitochondria-targeted catalase (mito-Cat). (A) Schematic representation of the mito-Cat expression vector containing an MnSOD mitochondrial leader sequence fused to the human catalase cDNA. (B) Immunoblot analysis of stable cell populations probed with anti-catalase antibody as described in Materials and Methods. The 52-kD band represents the overexpressed catalase protein. (C) Mitochondrial and cytosolic fractionation of vector and mito-Cat cell lines reveals mitochondrial enrichment of catalase as shown by immunoblot analysis.
MgCl₂, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-cotraetraacetic acid, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitors). After incubation on ice for 20 min, cells were disrupted by dounce homogenization (B pestle/40 strokes). The sample was centrifuged at 1000 x g for 10 min at 4°C, and the supernatant was subjected to further centrifugation at 10,000 x g for 15 min. The resulting supernatant (cytosolic fraction) and the mitochondria-enriched pellet (resuspended in buffer B, consisting of Tris [pH 8.0], 0.5% NP-40, and 5 mM CaCl₂) were subjected to immunoblot analysis as described above.

Cell Viability Assays

Cell viability was assayed by phase contrast microscopy, specific LDH release, and trypan blue exclusion. For LDH release, cell lines were split into 24-well tissue culture dishes and incubated at 37°C 24 h before challenge with 20 μM cisplatin or 500 μM hydrogen peroxide (H₂O₂) for the indicated times. LDH release was quantified by measuring the release of LDH from cells. The samples were added to 96-well plates and incubated for 10 min at 4°C. The absorbance at 490 nm was measured in a microplate reader. The LDH release was calculated as a percentage of the total LDH content of the cells.

Agarose Gel Electrophoresis for DNA Fragmentation

DNA fragmentation was performed as described previously (22). Briefly, control and treated cells from 100-mm plates were collected by scraping and were centrifuged at 1000 x g for 5 min at room temperature. Cell pellets were resuspended in 50 μl of Tris-ethylenediaminetetraacetic acid (TE) (10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 8.0), followed by the addition of 900 μl of lysis buffer (TE plus 0.5% Triton X-100) and incubation on ice for 15 min. The samples were centrifuged at 10,000 x g for 10 min, and supernatants were treated with 50 μg/ml ribonuclease A for 1 h at 37°C. Next, 100 μg/ml proteinase K and 0.5% sodium dodecyl sulfate were added and allowed to incubate for 2 h at 50°C. Fragmented DNA was extracted by using equal volumes of phenol/chloroform and ethanol precipitation. The nucleic acid pellets were resuspended in 30 μl of TE and subjected to 2% agarose gel electrophoresis at 100 V for 1 h.

Measurement of Cell Size

Measurements of cell size after cisplatin treatment were assessed by changes in forward-angle light scatter by using a fluorescence-activated cell sorter (FACScan; Becton Dickinson, San Jose, CA) and CellQuest software version 3.3 (Becton Dickinson, San Jose, CA). Forward-angle light scatter is directly proportional to cellular diameter, and a decrease in light scatter is indicative of a reduction in cell size (23). Therefore, normal versus apoptotic cells can be distinguished by this method. Data are presented as the mean differences in cells exhibiting less forward-angle light scatter (smaller size) between the control and treated cells in each of the cell lines.

Annexin V Binding Assay

Annexin V binding assays were performed by using the apoptosis detection kit (R & D Systems) per the manufacturer’s protocol for analysis of adherent cells. Briefly, cell lines were split into 6-well trays 24 h before incubation with 20 μM cisplatin for 5 d. Both floating and adherent cells (removed with a 0.025% trypsin treatment) were collected and pelleted by centrifugation at 500 x g for 5 min. Cell pellets were resuspended in complete media to allow for recovery and cell growth. The concentration of trypsin used does not interfere with the annexin V assay, as shown by negative staining of control cells. Cells were pelleted, resuspended in ice-cold PBS, and centrifuged at 500 x g for 5 min. Control and treated cells were then suspended in binding buffer that contained annexin V-FITC for 15 min. Unbound annexin V-FITC was removed by centrifugation, and the cells were resuspended in excess binding buffer. In each sample, a minimum of 10,000 cells were subjected to fluorescence-activated cell sorting with a Becton Dickinson Biosciences FACScan and CellQuest software version 3.3.

Clonogenic Assay

To evaluate the ability of the vector, mito-Cat, and MnSOD cell lines to survive and proliferate after a cisplatin challenge, equal numbers of cells were plated into 35-mm dishes 24 h before incubation with PBS (control) or 20 μM cisplatin. Twenty-four hours later, cells were washed with PBS, trypsinized, and seeded at a density of 5000 cells per 150-mm dish. They were maintained in culture for 7 d in room air and 10% CO₂ at 37°C. The dishes were then washed with PBS, fixed with cold methanol, and stained with a 0.2% crystal violet solution. Colonies greater than 0.5 mm in size were counted by using a dissecting microscope. The clonogenic capability of each cell line is presented as the percentage of colonies formed in treated versus control cells.

Statistical Analyses

Data are presented as mean ± SEM. The t test was used for comparisons between two groups. For comparisons that involved more than two groups, we used ANOVA and the Newman-Keuls test. Significance is defined as P < 0.05.

Results

Overexpression of MnSOD and Mito-Cat

In an attempt to explore the mechanisms of cisplatin-mediated cytotoxicity in renal epithelial cells, we generated cell lines that overexpressed human MnSOD by using the MnSOD expression vector that is depicted in Figure 1A. To differentiate the exogenously derived transcript from the endogenous mRNA, we designed the pcDNA3.1/SOD construct to include only 853 nucleotides of the human MnSOD cDNA. Northern blot analysis of cells transiently transfected with vector alone or pcDNA3.1/SOD demonstrates overexpression of an approximately 1.2-Kb transcript (Figure 1A). Clonal cell populations of vector alone and pcDNA3.1/SOD were selected, and overexpression of MnSOD protein was confirmed by immunoblot analysis (Figure 1B). Vector clone #2 and pcDNA3.1/SOD#5 were chosen for subsequent experiments.

Previous work has suggested that mitochondrial dysfunction is an early event in cisplatin-induced renal tubular toxicity and that it is potentially mediated by the generation of ROS (4,17). We therefore chose to overexpress another antioxidant enzyme, catalase, with specific targeting to the mitochondrion. The rationale for developing the mito-Cat cell line was provided by the studies of Bai et al. (19), in which cells expressing a mito-Cat displayed increased resistance to oxidant-mediated apoptosis as compared with cytosolic expression of the enzyme. In addition, the mito-Cat cells provided an additional tool to address the ROS specificity and consequently the cellular mechanism of cisplatin-mediated cytotoxicity. Immunoblot analysis after clonal selection demonstrates overexpression of catalase (Figure 2B). Mito-Cat clone #3 was chosen for subsequent experiments. The subcellular localization of catalase in the mito-Cat cell line was confirmed by immunoblot.
analysis, which showed a significant enrichment of catalase in the mitochondrial compartment (Figure 2C).

**Cytoprotective Effects of Mito-Cat in H$_2$O$_2$-Induced Cell Injury**

To assess the functionality of the cells that overexpress mito-Cat, we challenged both vector and mito-Cat cells with 500 µM H$_2$O$_2$. As shown in Figure 3, cells that overexpressed catalase demonstrated significant cytoprotection as assessed by phase contrast microscopy (A) and LDH release (B) when compared with vector. In contrast, overexpression of MnSOD did not confer cytoprotection to H$_2$O$_2$-induced cell injury (data not shown), as reported previously (24).

**Overexpression of MnSOD but Not Catalase Is Cytoprotective in Cisplatin-Induced Injury**

To explore the effects of MnSOD and catalase overexpression in cisplatin-induced cell injury, we exposed vector alone, MnSOD, and mito-Cat cell lines to 20 µM cisplatin for 72 h, an exposure that primarily caused an apoptotic form of cell injury. As shown in Figure 4, significant cytotoxicity, as evidenced by cell rounding, detachment, and decrease in cell size, was observed in the vector and mito-Cat clones, whereas no visible toxicity was evident in the cells that overexpressed MnSOD (Figure 4, D through F). Both floating and adherent cells excluded trypan blue after the 72-h exposure to cisplatin. In addition, no significant increase in LDH release was observed in vector, MnSOD, and mito-Cat cell lines that were exposed to cisplatin.

To further evaluate the cytoprotective potential of MnSOD overexpression, we compared the extent of DNA fragmentation after exposure to 20 µM cisplatin for up to 24 h. We demonstrate significant oligonucleosomal fragmentation of DNA in the vector and mito-Cat overexpression cell lines at 24 h after cisplatin treatment consistent with a predominantly apoptotic form of cell death in our studies (Figure 5). Significantly less DNA laddering was observed in the cell line that overexpressed MnSOD, substantiating the cytoprotective effects of MnSOD expression.

In an effort to quantify cell injury, we employed two methods of flow cytometric analysis, forward-angle light scatter measurements and the annexin V binding assay. As further evidence of the apoptotic nature of cell injury, a significantly higher percentage of cells with decreased forward-angle light scatter were observed in vector cells and cells that expressed mito-Cat as compared with cells that overexpressed MnSOD (vector, 15.94 ± 2.17; mito-Cat, 15.89 ± 7.34; MnSOD; 5.40 ± 3.88; P < 0.05; n = 4).

In early stages of cell injury, phosphatidylserine residues, normally located on the inner leaflet of the membrane bilayer, flip to the outer leaflet. The appearance of these flipped lipid moieties can be quantified by fluorescence-activated cell sorter analysis after incubation with annexin V-FITC in the presence of calcium. In these experiments, cells were exposed to 20 µM cisplatin for 5 d followed by assessment of annexin V binding. Figure 6 is a representative histogram that demonstrates the appearance of a higher number of annexin V–positive cells in both vector and mito-Cat cell lines that are indicative of increased cell injury. Interestingly, cells that overexpressed MnSOD did not demonstrate an increase in annexin V binding.

A quantitative representation that summarizes the results of the annexin V binding is shown in Figure 7. Cell injury as assessed by annexin V binding was significantly decreased in the cells that expressed MnSOD compared with vector alone (vector, 15.84 ± 5.12; MnSOD, 0.84 ± 1.42; P < 0.05) (Figure 7A). Similar cell injury was observed in vector alone and mito-Cat cells, suggesting the inability of catalase to protect renal epithelial cells against cisplatin (vector, 20.79 ± 3.53; mito-Cat, 19.42 ± 5.89; P = NS) (Figure 7B).

To assess the growth potential of the vector, mito-Cat, and MnSOD cell lines after a 24-h, 20 µM cisplatin challenge, a clonogenic assay was performed. No differences in growth potential were observed in each of the control (untreated) cell lines. However, significant differences were observed in the ability of cisplatin-treated cells to form colonies. Cells that overexpressed MnSOD showed significant clonogenic capability after cisplatin compared with vector and mito-Cat cell lines (vector, 0.47 ± 0.25; mito-Cat, 2.86 ± 0.64; MnSOD, 51.84 ± 2.38; n = 4; P < 0.001) (Figure 8), demonstrating the ability

![Figure 3](image-url)
of MnSOD overexpression to abrogate cisplatin-mediated cytotoxicity.

The results presented in this work demonstrate the specificity of ROS generation in response to cisplatin and directly implicate the superoxide radical, and not \( \text{H}_2\text{O}_2 \), as the mediator of cisplatin-induced nephrotoxicity.

**Discussion**

A significant risk of dose-dependent nephrotoxicity exists for patients who receive cisplatin for cancer chemotherapy. Several lines of evidence support a role for ROS in the pathogenesis of cisplatin-induced renal injury (8,9,18). *In vitro* assays have documented the generation of superoxide radicals during interaction of cisplatin with DNA (25), a phenomenon that has been implicated as the mechanism of cisplatin-mediated toxicity in cancer cells (12). In addition, a variety of oxygen radical scavengers have been shown to protect against cisplatin-mediated cytotoxicity *in vitro* and *in vivo*, further substantiating the role of ROS (7–12). The majority of these studies have involved the addition of antioxidants in the form of reconstituted protein (SOD and catalase) to cells in culture (9). In this work, we report the selective and targeted overexpression of two antioxidant enzymes, MnSOD and mito-Cat, in renal epithelial cells. Phenotypic characterization of vector alone, MnSOD, and mito-Cat cell lines demonstrated the ability of overexpressed MnSOD to protect HEK 293 cells against an apoptotic challenge of cisplatin, and catalase, targeted to the mitochondrion (the organelle where cisplatin-mediated injury is prominent), did not afford cytoprotection. These data substantiate the specificity of MnSOD and indicate a significant role for the superoxide radical in cisplatin-induced nephrotoxicity.
The mechanism of cisplatin-mediated cell death, although not yet completely elucidated, is probably pleiotropic. Recently, Lieberthal et al. (9) demonstrated differences in proximal tubule cell phenotype in response to varying cisplatin concentrations. Low doses (<50 \mu M) of cisplatin resulted in apoptosis, but cells challenged with higher concentrations (50 to 800 \mu M) displayed a more necrotic phenotype with cytotoxic swelling and an early loss of plasma membrane integrity. These results are not surprising, because the concentration-dependent mechanism of cisplatin-mediated cell death is similar to that observed with other cytotoxic compounds (6,9). Our data support these studies in that a low dose of cisplatin (20 \mu M) resulted primarily in apoptosis as evidenced by DNA laddering, decreased cell size, the exclusion of trypan blue, lack of LDH release, and annexin V-positive labeling. In addition, the ability of cells to proliferate after exposure to cisplatin was demonstrated by significantly higher colony formation in the cells that overexpressed MnSOD compared with the vector control line or the cells that expressed mito-Cat.

The functionality of the mito-Cat cells was demonstrated by the fact that these cells were resistant to H_2 O_2 but showed no cytoprotection with cisplatin in our studies. Previous studies by Bai et al. (19) have demonstrated the beneficial effects of catalase overexpression in either the mitochondria or cytoplasm after exposure to H_2 O_2 and menadione. In contrast, overexpression of catalase within the cytosol or mitochondrial compartments of Hep G2 cells was shown to exacerbate tumor necrosis factor (TNF)-mediated cell injury (26). We have not documented a significant increase in cisplatin-mediated toxicity in the mito-Cat cell lines as compared with vector alone; therefore, the mechanism of cisplatin-mediated cell death may involve a different pathway than that of TNF. Nonetheless, MnSOD provides protection against both cisplatin (this study) and TNF-mediated cytotoxicity (15).

Similar to these studies, St. Clair et al. (27) have shown that overexpression of MnSOD attenuates the toxic effects of paraquat with no affect on endogenous copper/zinc SOD, catalase, or glutathione peroxidase expression. These results are consistent with our observations with cisplatin in that overexpression of MnSOD also demonstrated the role of superoxide radicals in paraquat cytotoxicity. Furthermore, studies in mouse proximal tubular cells have demonstrated that antioxidants, including SOD and catalase, protect these cells from ROS in apoptosis that is induced by either growth factor deprivation (28) or cisplatin (9). Regarding a cellular mechanism that is associated with MnSOD cytoprotection, Manna et al. (29) have reported that MnSOD blocks TNF-mediated activation of the transcription factors, NF-\kappa B and AP-1, as well as the induction of c-Jun protein kinase and mitogen-activated protein kinase. These investigators also showed that MnSOD overexpression suppresses apoptosis that is induced by okadaic acid, H_2 O_2, and Taxol (Bristol-Myers Squibb) but not by the chemotherapeutic agents, vincristine, vinblastine, and daunomycin. This further demonstrates the unique specificity of MnSOD overexpression in cisplatin-mediated cytotoxicity in renal epithelial cell injury.

In summary, we have demonstrated the ability of MnSOD to protect renal epithelial cells against cisplatin, whereas cells that overexpress mito-Cat do not afford cytoprotection. Our results strongly implicate the superoxide radical in cisplatin-mediated cell death.
nephrotoxicity and provide the impetus for potential targeted gene therapy with MnSOD in high-risk settings of acute renal failure.

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