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Effects of Bleomycin on Liver Antioxidant Enzymes and the Electron Transport System From Ad Libitum-Fed and Dietary-Restricted Female and Male Fischer 344 Rats

Varsha G. Desai, Anane Aidoo, Jing Li, Lascelles E. Lyn-Cook, Daniel A. Casciano, and Ritchie J. Feuers

Abstract: *Dietary restriction (DR) is the only known intervention that delays aging and age-related diseases. Mechanisms proposed to explain this DR effect include a decline in free radical production and an increase in free radical detoxification. In the present study the effect of bleomycin (BLM) as a reactive oxygen species-generating antitumor drug has been evaluated on antioxidant enzymes and the electron transport system in different cellular fractions of liver in female and male Fischer 344 rats. Animals were fed ad libitum (AL) or 60% of the AL intake (DR) and were given a single intraperitoneal injection of 2.5, 5, or 10 mg BLM/kg body wt. After four weeks, BLM significantly increased glutathione peroxidase and lactate dehydrogenase activities in liver cytosol of female AL rats and increased activity even more in male rats. Similar changes were also noted for glutathione reductase and glucose 6-phosphate dehydrogenase activities in BLM-treated AL rats. In liver mitochondria, glutathione peroxidase was increased in female and male AL rats but was increased more in female rats. Drug treatment had no significant effect on these enzyme activities in cytosolic or mitochondrial fractions of DR animals. Profound effects of BLM were noted in activities of complexes I, III, and IV of the electron transport system in AL and DR female and male rats; however, complex II demonstrated no significant diet or treatment effect. Induced antioxidant enzyme activities in BLM-treated AL rats may be a response to excessive free radical generation due to BLM metabolism in AL animals that is mitigated by DR. Furthermore, dysfunction of the electron transport system might suggest its role in a secondary generation of free radicals during BLM metabolism contributing to its toxicity.*

Introduction

Aerobic organisms are continuously under attack by reactive oxygen species generated from cellular sources such as neutrophils, macrophages, and oxidative phosphorylation (1). Another important source of reactive oxygen species may result from oxidation of a variety of cellular and chemical cytotoxic agents. By damaging proteins, lipids, DNA, and other macromolecules, these reactive oxygen species may have a significant role in aging and age-related diseases such as atherosclerosis, Parkinson's disease, and Alzheimer's disease (2–4).

The damaging effects of free radicals are controlled to some extent through cellular enzymatic and nonenzymatic antioxidant defenses. Glutathione peroxidase, catalase, and superoxide dismutase, which are considered the primary antioxidant enzymes, are involved in the direct elimination of reactive oxygen species with the help of secondary enzymes such as glutathione reductase and glucose 6-phosphate dehydrogenase. The secondary enzymes help maintain a steady supply of the metabolic intermediates required by the primary antioxidant enzymes (5). The nonenzymatic defenses include reduced glutathione, uric acid, bilirubin, and ubiquinol (4). Despite these systems, accumulation of oxidative damage may result in altered tissue function and ultimately affect health status.

This reactive oxygen species-mediated degradation of biological systems might be prevented by maintaining the balance between free radical production and its detoxification. Dietary restriction (DR) has been shown to reduce free radicals in biological systems by increasing their removal (6–8) and/or by lowering their production (9). This effect of DR may contribute to its ability to extend the maximum achievable life span in rodents and in other organisms (10) and also delay various age-related disorders.

Bleomycin (BLM) is a radiomimetic drug used in the treatment of different types of cancer. BLM cytotoxicity has been demonstrated to be a result of reactive oxygen species generated during its metabolism, where BLM interacts with molecular oxygen and iron, producing superoxide anion and other oxygen metabolites (11). Genotoxic effects of BLM include the release of free base residues and the creation of single- and double-strand breaks in nuclear (12,13) as well

The authors are affiliated with the Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research, Food and Drug Administration, Department of Health and Human Services, Jefferson, AR 72079.

as mitochondrial DNA (14). The genotoxic effects of BLM have primarily been studied with regard to nuclear DNA. Thus little information is available regarding BLM-induced oxidative injury to mitochondrial DNA. Unlike nuclear DNA, mitochondrial DNA lacks introns and protective histones (4). Also, mitochondria are capable of limited DNA repair (4). In addition to these characteristics, its proximity to the respiratory chain makes mitochondrial DNA a more likely target for free radical attack.

Mitochondrial DNA is a double-stranded, closed circular molecule that encodes 11 subunits of the electron transport system complexes. The mitochondrial electron transport system is composed of four complexes: complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome *c* oxidoreductase), and complex IV (cytochrome *c* oxidase). Nuclear and mitochondrial genomes contribute to the electron transport function by interacting to affect the synthesis and assembly of the electron transport system proteins (15). Oxidative damage to these genomes may lead to altered protein synthesis, ultimately resulting in electron transport dysfunction and, thus, generation of more free radicals. The present study was carried out to determine whether DR might inhibit the toxic effects of BLM, which are believed to be mediated by reactive oxygen species.

Material and Methods

Experimental Design

Animals: Forty female and 40 male Fischer 344 rats were obtained from the breeding colony of the National Center for Toxicological Research. The maintenance and handling of the animals were based on the *Guide for the Care and Use of Laboratory Animals* (16).

Treatment regimen: All 80 animals were weaned at 3 weeks of age and fed the standard NIH-31 diet (containing 0.315% vitamin mixture and 0.185% mineral mixture) until they reached 14 weeks of age. The rats were then randomly assigned to a control group that was fed the standard NIH-31 diet ad libitum (AL) or a group that received 60% of the AL intake of the NIH-31 diet supplemented with vitamins and a mineral mixture at 1.67 times that in the standard diet (DR). AL and DR animals received a single dose of 2.5, 5, or 10 mg BLM/kg body wt (Sigma Chemical, St. Louis, MO) or the BLM vehicle, phosphate-buffered saline, pH 7.4, at 18 weeks of age. Each group consisted of five animals. All animals were sacrificed by CO_2 asphyxiation four weeks after mutagen treatment. The livers were aseptically removed and stored at –80°C.

Tissue preparation: The livers were thawed, weighed, minced into small pieces, and rinsed twice with ice-cold homogenization buffer [250 mM sucrose, 5 mM tris(hydroxymethyl)aminomethane·HCl (pH 7.4), 1 mM EDTA] at 4°C according to the method of Ji and co-workers (17). The buffer was discarded, and the tissues were homogenized in five volumes of ice-cold homogenization buffer with a motor-driven Teflon pestle. The homogenates were centrifuged twice at 1,000 *g* for 10 minutes. The pellets were discarded, and supernatants were recentrifuged at 12,000 *g* for 20 minutes. The resultant pellets were resuspended in 0.5 ml of homogenization buffer and labeled as "mitochondrial fractions." The supernatants were subjected to further centrifugation at 105,000 *g* for 60 minutes. These final supernatants correspond to the "cytosolic fractions." All the centrifugation steps were carried out at 4°C, and all the fractions were stored at –70°C for subsequent analysis.

Antioxidant enzyme assays: Enzyme activities were measured spectrophotometrically on a Cobas FARA autoanalyzer (Roche, Nutley, NJ). Glutathione peroxidase and glutathione reductase activities were measured according to Wheeler and colleagues (18). Glutathione peroxidase activity was determined using a reaction mixture containing phosphate buffer (100 mM, 1 mM EDTA, pH 7.0), NADPH (2.25 mM), glutathione reductase (100 U/ml), and reduced glutathione (37.5 mM). Activity was measured as a decrease in absorbance at 340 nm at 30°C as H_2O_2 (15 mM) was added to the reaction mixture containing the tissue fraction. Glutathione reductase assay was performed by adding the tissue fraction to the reaction mixture containing oxidized glutathione (3.7 mM) and NADPH (2 mM) in potassium phosphate buffer (147 mM with 0.74 mM EDTA, pH 7.2). Activity was measured as the rate of oxidation of NADPH at 340 nm at 30°C. Glucose 6-phosphate dehydrogenase analysis was carried out according to Bergmeyer (19). The reaction mixture contained glucose 6-phosphate (6 mM) and NADP (1.6 mM) in triethanolamine·HCl buffer (172 mM, with 13.8 mM $MgCl_2$, pH 7.6). The activity was determined as an increase in absorbance at 340 nm at 30°C by addition of cytosolic fraction to the mixture. Cytosolic lactate dehydrogenase activity was measured spectrophotometrically (20) as oxidation of NADH (1.6 mM) by pyruvic acid (2.39 mM) in 0.2 M potassium phosphate buffer (pH 7.4) at 30°C. Activity was expressed in terms of the decrease in absorbance at 340 nm.

Mitochondrial electron transport system enzyme assays: Activities of all four electron transport system complexes were determined spectrophotometrically on a Cobas FARA autoanalyzer by modifications of the method of Ragan and co-workers (21). Complex I (NADH-ubiquinone oxidoreductase) activity was measured by monitoring the oxidation of NADH by ubiquinone-1 at 30°C. The reaction mixture contained potassium phosphate buffer (10 mM, pH 8.0), NADH (5 mM), lecithin (15 mg/ml), and the mitochondrial fraction. The reaction was started by addition of ubiquinone-1 (10 mM) to the reaction mixture, and the decrease in absorbance at 340 nm was monitored. Complex II (succinate-ubiquinone oxidoreductase) activity was determined

as the rate of reduction of ubiquinone-2 at 30°C followed by the secondary reduction of 2,6-dichlorophenolindophenol by the ubiquinol formed. The reaction mixture contained potassium phosphate buffer (1 mM, pH 7.4), sodium succinate (1 M, pH 7.4), and EDTA (10 mM, pH 7.3). The decrease in absorbance was measured at 600 nm after addition of a mixture of ubiquinone-2 (2.5 mM), 2,6-dichlorophenolindophenol (4.65 mM), and the mitochondrial fraction to the mixture described above. Complex III (ubiquinol-cytochrome *c* oxidoreductase) activity was assayed by following the rate of reduction of cytochrome *c* by ubiquinol-2 at 30°C. The reaction was started by addition of ubiquinol-2 (1 mM) to the reaction mixture containing potassium phosphate buffer (50 mM), EDTA (100 mM), cytochrome *c* (1 mM), potassium cyanide (50 mM), and the mitochondrial fraction diluted 1:10 with sucrose (250 mM) and tris(hydroxymethyl)aminomethane·HCl (10 mM) buffer (pH 7.8). The reduced cytochrome *c* was measured as an increase in absorbance at 550 nm. Complex IV (cytochrome *c* oxidase; EC 1.9.3.1) activity was measured as the rate of oxidation of reduced cytochrome *c* (10 μM) by the enzyme at 550 nm. Total protein contents of the cytosolic and mitochondrial fractions were measured by using a kit supplied by Cobas FARA, with bovine serum albumin as a standard. The enzyme activities were expressed as nanomoles per minute per milligram of protein.

Statistical Analysis

A three-way analysis of variance was used to test the main effect of gender, diet, and dose as well as their interactions. When interactions were significant, main effects were tested separately using two-way analysis of variance with post hoc multiple-comparison (Bonferroni) tests between the control and treated groups. Differences were considered significant at $p < 0.05$. Values are means ± SEM.

Results

The results were subjected to three-way analysis of variance for statistical significance at the 95% confidence level, and the data are represented graphically in Figures 1–6. A significant diet, dose, and gender interaction was noted in cytosolic glutathione peroxidase activity. All male AL and DR rats treated or untreated with BLM had significantly higher ($p < 0.05$) glutathione peroxidase activity than female rats. Glutathione peroxidase showed tendencies toward increased activity in BLM-treated AL animals in both genders compared with controls. However, significant induction was noted only in cytosol of male rats treated with 5 mg BLM/kg. Glutathione peroxidase activity was higher in the cytosolic fraction from AL female rats than in that from DR female rats. Conversely, glutathione peroxidase activity was higher in the mitochondrial fraction from DR female rats than in that from AL female rats. However, these changes were statistically nonsignificant (Figures 1 and 2). Glutathione peroxidase activity was higher in cytosolic and mitochondrial fractions of DR male rats than AL male rats. A statistically significant ($p < 0.001$) diet × gender interaction was noted in mitochondrial glutathione peroxidase.

Similar to glutathione peroxidase, a gender-related significant difference in glutathione reductase activity was observed in cytosol, with male rats showing higher activity than female rats in both diet groups, either treated or untreated. Glutathione reductase (Figures 1 and 2) was higher in all DR animals in both cellular fractions than in their AL counterparts, with a significant ($p < 0.05$) increase noted at 2.5 (15%) and 10 (30%) mg BLM/kg in cytosol and in mitochondria, respectively, in females. Glutathione reductase activity rose significantly in mitochondria of male DR control rats and male rats treated with 2.5 mg BLM/kg.

In female AL rats, cytosolic glucose 6-phosphate dehydrogenase activity was not affected by BLM treatment (Figure 1). However, a significant ($p < 0.05$) decrease of >45% in glucose 6-phosphate dehydrogenase activity was observed in DR female rats compared with their AL counterparts. Enzyme activity was also significantly decreased in male DR rats treated with 2.5 and 10 mg BLM/kg compared with their AL counterparts and with untreated DR controls. However, BLM significantly induced glucose 6-phosphate dehydrogenase activity at the highest dose in AL male rats compared with untreated controls.

Lactate dehydrogenase activity was altered (Figure 3) in a manner similar to cytosolic glutathione peroxidase in BLM-treated AL rats of both genders. Diet, dose, and gender interaction was also noted in lactate dehydrogenase activity ($p < 0.05$) in cytosol. Enzyme activity was induced by BLM in AL male rats by >35% of controls, with the highest twofold increase at 5 mg/kg, but not in female rats. No BLM treatment effect was noted in female or male DR rats. All DR animals had lower lactate dehydrogenase activity than their AL counterparts. The decrease was statistically significant at all BLM doses tested in male rats, whereas in female rats the decrease was significant only in untreated DR rats and in DR rats treated with 5 mg BLM/kg.

BLM produced significant ($p < 0.05$) effects on the activities of the electron transport system complexes I, III, and IV, but not complex II, in DR and AL rats (Figure 4). The diet × gender interaction was statistically significant in complex I. Complex I activity was significantly higher in female AL rats than in male AL rats. Complex I showed a tendency toward a dose-dependent increase in its activity in female and male AL rats due to BLM compared with controls. However, this increase was significant at the highest dose only in female rats. DR animals also showed changes similar to AL animals in female rats, and their activities were lower than those of their AL counterparts. In contrast, in male rats, complex I activity was significantly higher in DR rats than in their AL counterparts. In the case of complex II activity, female and male rats had a nonsignificant diet × treatment effect in AL as well as in DR animals. All controls and treated male rats had significantly ($p < 0.001$) higher complex II activity than female rats.

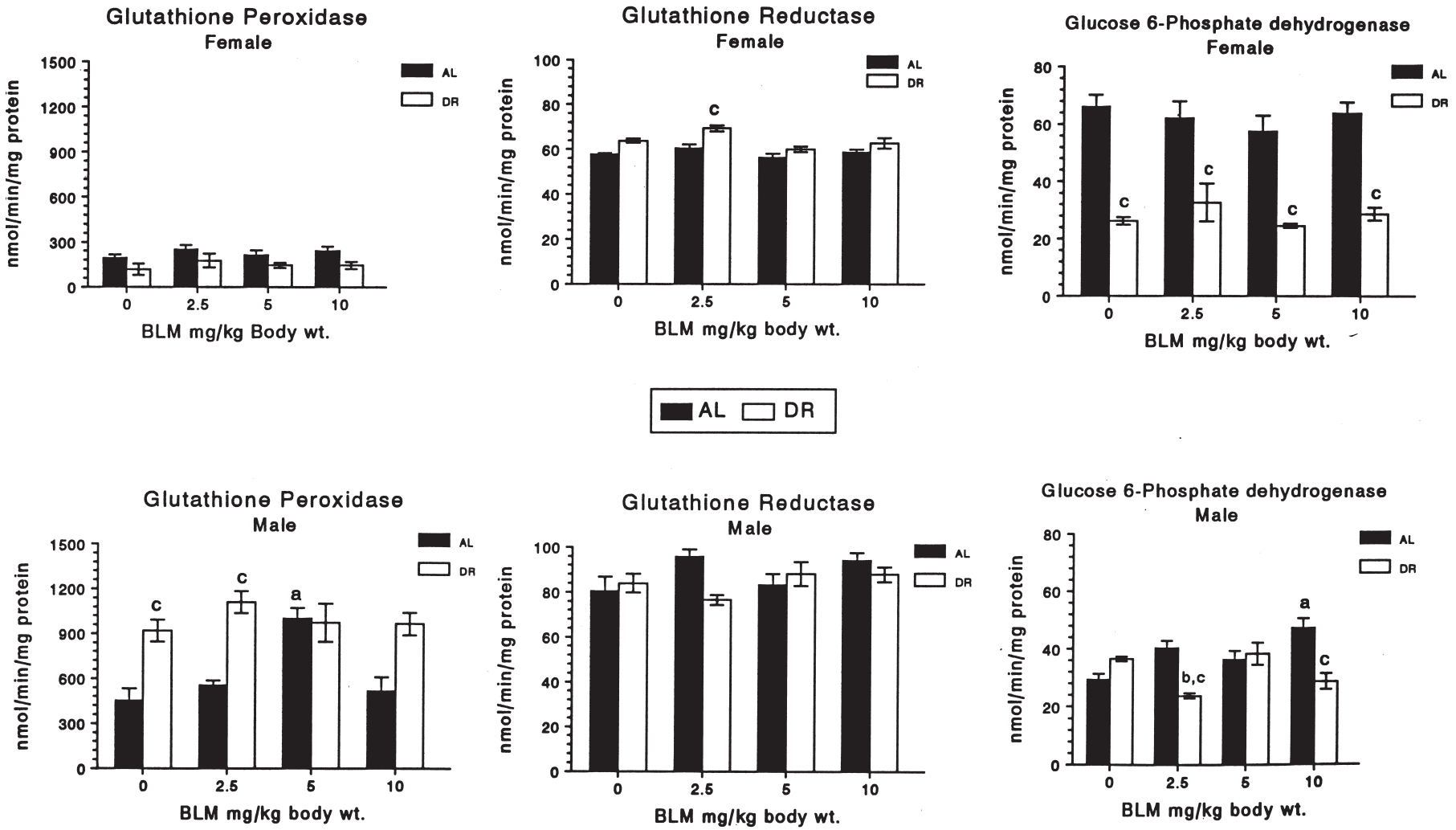

Figure 1. Glutathione peroxidase, glutathione reductase, and glucose 6-phosphate dehydrogenase activities in liver cytosol of ad libitum-fed (AL) and dietary-restricted (DR) female and male rats treated with different doses of bleomycin (BLM). a, $p < 0.05$ compared with AL at 0 mg BLM/kg; b, $p < 0.05$ compared with DR at 0 mg BLM/kg; c, $p < 0.05$ compared with respective AL counterparts.

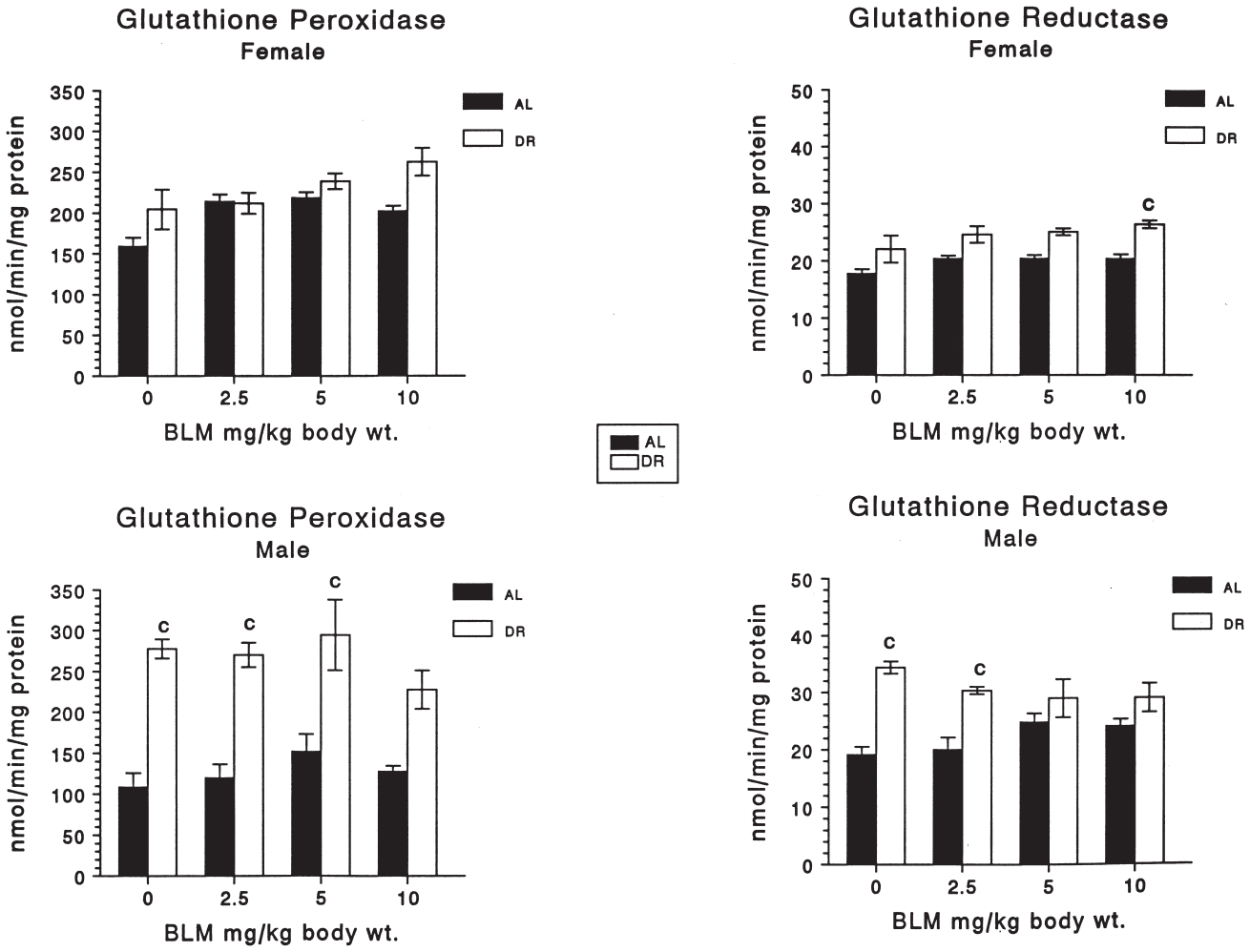

Figure 2. Glutathione peroxidase and glutathione reductase activities in liver mitochondria of AL and DR female and male rats treated with different doses of BLM. c, $p < 0.05$ compared with respective AL counterparts.

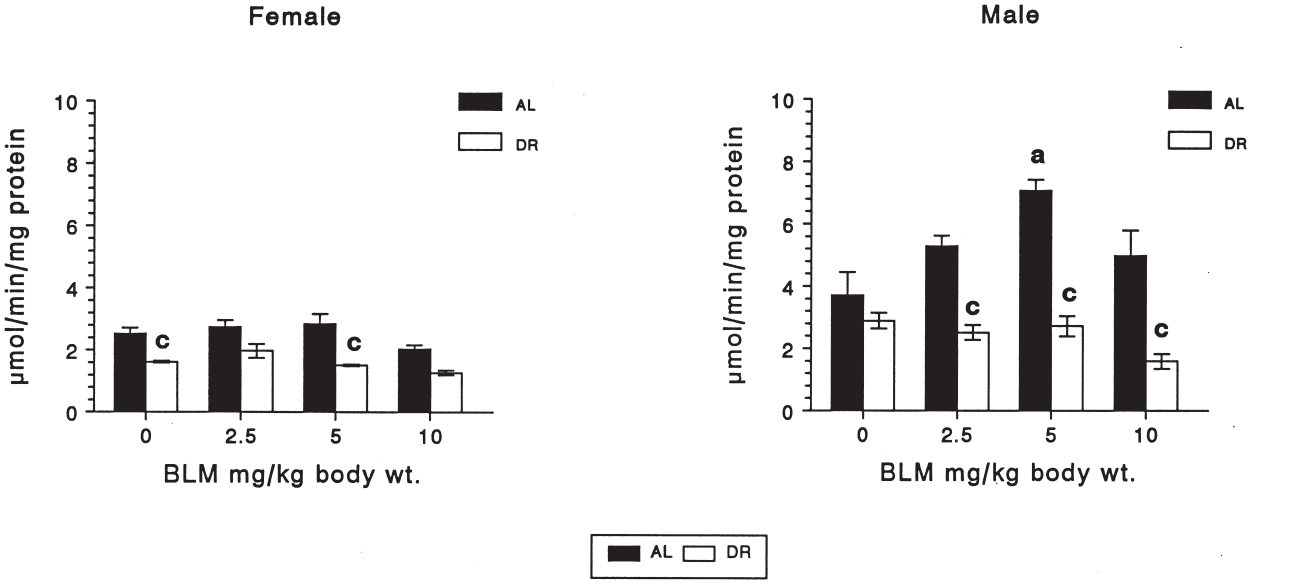

Figure 3. Lactate dehydrogenase activity in liver cytosol of AL and DR female and male rats treated with different doses of BLM. a, $p < 0.05$ compared with AL at 0 mg BLM/kg; c, $p < 0.05$ compared with respective AL counterparts.

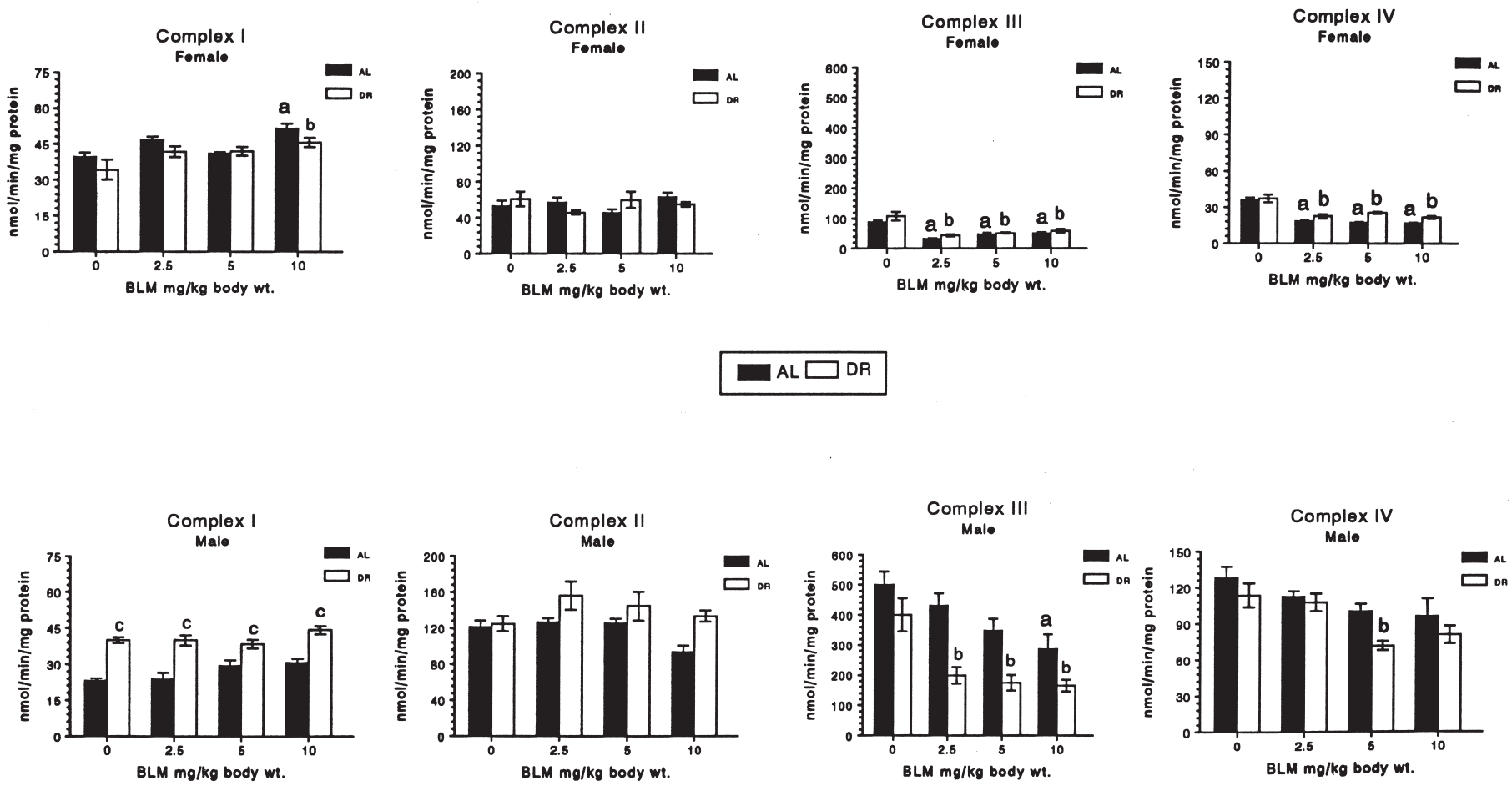

Figure 4. Electron transport system enzyme activities in liver mitochondria of AL and DR female and male rats treated with different doses of BLM. a, $p < 0.05$ compared with AL at 0 mg BLM/kg; b, $p < 0.05$ compared with DR at 0 mg BLM/kg; c, $p < 0.05$ compared with respective AL counterparts.

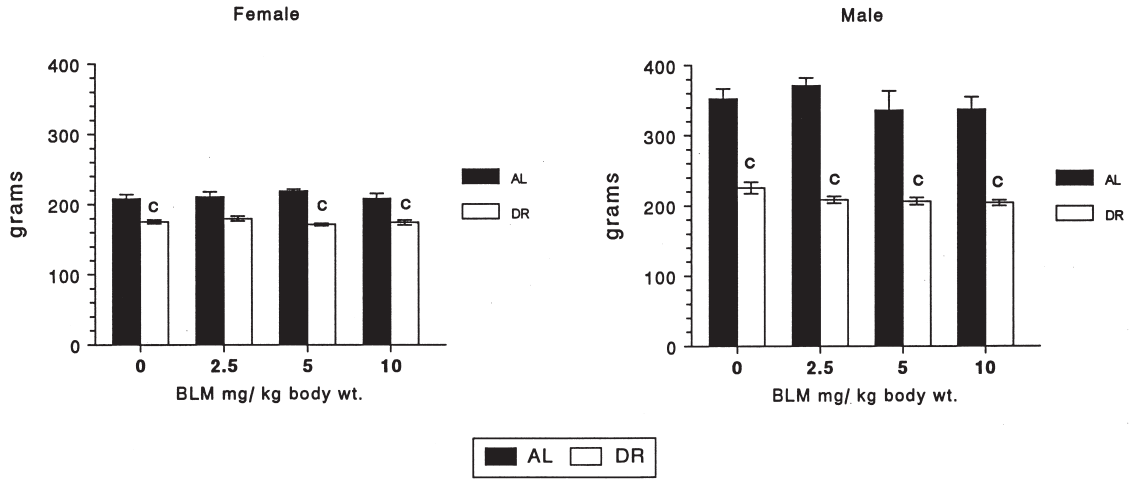

Figure 5. Body weights of AL and DR female and male rats treated with different doses of BLM. c, $p < 0.05$ compared with respective AL counterparts.

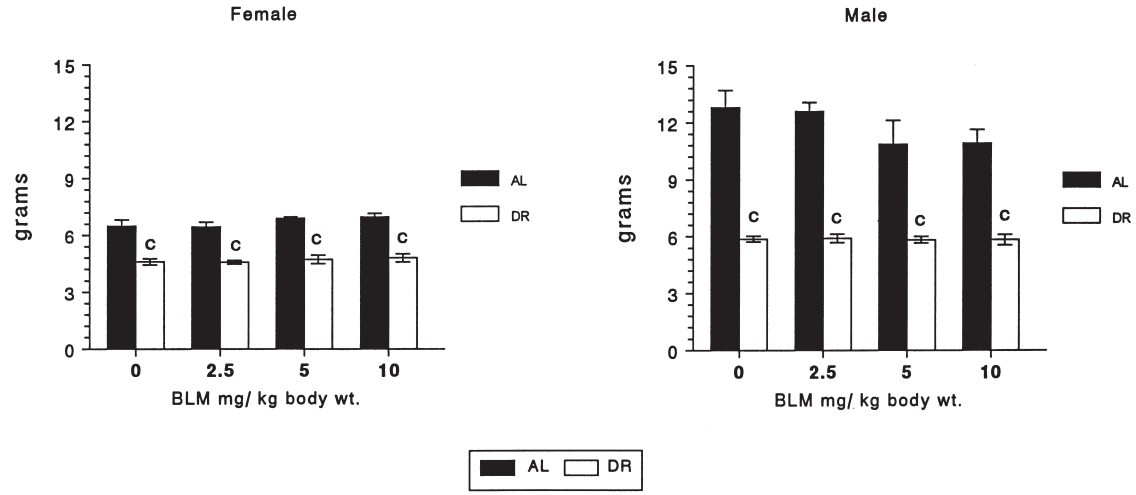

Figure 6. Liver weights of AL and DR female and male rats treated with different doses of BLM. c, $p < 0.05$ compared with respective AL counterparts.

In contrast to complex I activity, a severe decline was observed in the activities of complexes III and IV in BLM-treated AL and DR rats compared with untreated controls of both genders. This effect was more prominent in female rats, where the decrease was >45% ($p < 0.05$) for all doses of BLM, in contrast to a <40% decrease in male rats (Figure 4). In female rats, the percent decrease in complex III and IV activities was less in DR than AL rats, in which activities were reduced to approximately 50% of the controls. In male rats, complex III activity appeared to be more severely affected than complex IV activity. All female DR animals had higher complex III and IV activities than their AL counterparts, whereas male DR rats had lower enzyme activities than their respective AL counterparts. All BLM-treated DR male rats had significantly lower complex III activity than untreated DR controls. Complex III and IV activities were significantly higher in male than in female AL and DR animals. Diet × gender and dose × gender interactions were significant for both of these complexes.

Female and male rats showed a significant diet effect in their body weights as well as liver weights compared with their respective AL counterparts (Figures 5 and 6). Body and liver weights were significantly decreased ($p < 0.05$) in all DR animals compared with their AL counterparts. A significant gender × diet interaction ($p < 0.05$) was also noted in body weights and in liver weights, with male rats showing larger body as well as liver weights than female rats.

Discussion

BLM is a chemotherapeutic drug used in the treatment of various types of cancers. Its mode of action appears to be through the generation of reactive oxygen species in the presence of iron and oxygen, yielding oxidative damage to DNA (13). Various approaches have been attempted to reduce reactive oxygen species-mediated cytotoxicity in normal tissues by supplementation of antioxidants (22–24).

However, this strategy has met with little success. DR seems to limit free radical production, improve detoxification (6,25), and improve repair of oxidative damage (7). Additionally, DR seems to improve xenobiotic- and drug-metabolizing systems (26). These actions of DR may contribute not only to the extension of life span, but also to the reduction in appearance and degree of numerous pathologies (27,28).

Our data demonstrate higher cytosolic glutathione peroxidase activity in BLM-treated female and male rats. However, in a previous study we observed a nonsignificant decrease in glutathione peroxidase activity in cytosol of BLM-treated male AL rats (24). In this experiment, rats were kept on a 12:12-hour light-dark cycle, and BLM was injected 2 hours after lights-off, whereas in the previous study a light-dark cycle was not maintained. The light-dark cycle might have changed the physiological condition of the rats, such that BLM metabolism was altered, thus culminating in changes in antioxidant enzyme activities. Higher cytosolic glutathione peroxidase activity in this study could be the result of higher steady-state levels of reactive oxygen species due to BLM metabolism within the cytosol of AL rodent liver, leading to enzyme induction. We previously noted induction of antioxidant activities associated with increased reactive oxygen species during aging in AL animals (25).

BLM affected cytosolic lactate dehydrogenase in a manner similar to cytosolic glutathione peroxidase in AL rats. Induction of lactate dehydrogenase activity was >40% in BLM-treated male AL rats (with a maximum of 91% at 5 mg BLM/kg) as opposed to only 20% in BLM-treated female AL rats. This provides evidence that BLM was more toxic in male than in female rats and suggests a gender-specific effect of BLM exposure. Supporting this conclusion is the result of a concurrent mutagenicity experiment with BLM at the *Hprt* locus (data not shown) that indicated an increase in mutant frequency in male compared with female AL rats. The toxicity was modulated by diet restriction, inasmuch as lactate dehydrogenase activity was maintained at the control level in female and male DR rats. Higher cytosolic glutathione peroxidase activities in male DR rats could be responsible for limiting reactive oxygen species level, consequently reducing BLM toxicity. A significant decrease was also observed in body and liver weights of DR animals compared with AL animals, irrespective of BLM treatment. The process of cellular attrition might have contributed to the reduction in body and liver weights of the DR animals. It has been indicated that the rate of apoptotic cell death is increased in tissues of DR animals relative to their AL counterparts (29). However, irrespective of dietary intake, the male rats appeared larger than the female rats.

Cytosolic glucose 6-phosphate dehydrogenase activity was unaffected by BLM in AL or DR female rats, whereas activity was induced in male AL rats. However, significantly lower enzyme activity in all female DR rats than in their AL counterparts was not unexpected, since glucose 6-phosphate dehydrogenase has been reported to be higher in AL than in DR animals when activity is measured at saturated substrate concentrations (30). This allosteric enzyme does not fully activate at low endogenous substrate levels in AL tissues, whereas in DR animals full activation occurs. This seems to result in excess synthesis of this enzyme as a compensatory mechanism in AL, whereas DR animals seem to maintain low levels of total enzyme with higher activity at low substrate concentration.

Like glucose 6-phosphate dehydrogenase, cytosolic glutathione reductase activity was not affected by BLM in AL female rats but was increased in BLM-treated AL male rats. However, in mitochondria, glutathione reductase showed a tendency toward an increase in activity in female and male AL animals. Glutathione reductase levels were higher in tissues of DR animals than in their AL counterparts. Glutathione reductase is responsible for reduction of the oxidized form of glutathione (GSSG) to its reduced form (GSH), which is required by glutathione peroxidase. This glutathione peroxidase-glutathione reductase-catalyzed GSH-GSSG cycle plays a key role in detoxification of H_2O_2 and various organic hydroperoxides, which are generated primarily in mitochondria (17). Thus higher glutathione peroxidase and glutathione reductase activities within mitochondria of DR animals may provide an advantage in detoxification of reactive oxygen species.

Some BLM toxicity may be manifested through mitochondrial effects, inasmuch as BLM treatment had significant effects on electron transport activity in AL and DR animals. BLM induced an increase in complex I activity in AL and DR female and male rats. The mechanism by which BLM causes an increase in complex I activity is unclear. If BLM reduced ATP production due to a severely altered electron transport chain, which is a prime site of ATP generation, the glycolytic pathway might be induced as a secondary source of ATP. This could ultimately lead to more NADH production, causing substrate-induced higher complex I activity.

In contrast to complex I, BLM caused a significant decline in the activities of complexes III and IV in AL and DR animals compared with untreated controls of both genders. However, activities were higher in DR female rats and lower in DR male rats than in their AL counterparts. The decrease in activities of complexes III and IV due to BLM exposure in AL rats indicates that complexes were more severely altered in female than in male rats. The greater induction of glutathione peroxidase within mitochondria of female than of male rats might be in response to higher levels of reactive oxygen species, which in turn would provide protection against these mitochondrial effects of BLM. Complex II, which is totally nuclear in origin, was not affected by BLM in AL or DR animals. Thus it might be argued that BLM produces free radical damage at the level of mitochondrial DNA. Enzyme analyses were carried out four weeks after treatment; thus it seems unlikely that damage at the protein or membrane level would yield these responses.

As mentioned earlier, animals in this study also showed dose-dependent mutant frequency in the *Hprt* locus. Direct effects on the mutant frequency at this gene locus might be the result of oxidative damage. The changes in activity associated with complexes I, III, and IV suggest additional BLM toxicity involving reactive oxygen species-induced mitochondrial DNA damage. Because subunits of complexes I, III, and IV are also encoded by nuclear DNA, the idea that the responses to BLM seen for complexes I, III, and IV may involve reactive oxygen species-induced nuclear DNA damage cannot be ruled out. Nevertheless, the fact that no change occurred in complex II activity at any dose of BLM strongly suggests some mitochondrial DNA involvement.

The deleterious effects of BLM on complex III and IV activity may produce a situation where additional reactive oxygen species are generated from a dysfunctional electron transport system. We previously reported similar responses of these respiratory chain complexes during aging, where altered kinetic properties (9) yield an impedance against electron flow with a concomitant increase in free radical production during oxidative phosphorylation. If this is the case, BLM produces genetic damage with multiple and even long-term consequences. These arguments are further supported by various *in vitro* studies that have demonstrated BLM-induced oxidative damage to mitochondrial DNA, causing single-strand breaks at multiple sites on double-stranded DNA (14) in addition to damage at bases and abasic sites (31). Unlike nuclear DNA, mitochondrial DNA has very limited DNA repair (32). Hence, damage persists longer and stimulates secondary production of reactive oxygen species due to altered electron transport function (33).

In conclusion, moderate restriction of energy intake (40% less than the ad libitum intake) in BLM-treated rats significantly modulated the activities of free radical-scavenging enzymes as well as the activities of the electron transport system complexes. This suggests the role of free radical generation in the metabolism of BLM.

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