Iranian Journal of Pharmaceutical Research (2004) 4: 193-199
Received: December 2003
Accepted: May 2004

Original Article

Lysosomal Oxidative Stress Cytotoxicity Induced By Para-phenylenediamine Redox Cycling In Hepatocytes

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Abstract

It has already been reported that muscle necrosis induced by various phenylenediamine derivatives are correlated with their autoxidation rate. Now in a more detailed investigation of the cytotoxic mechanism using a model system of isolated hepatocytes and ring-methylated structural isomer durenediamine (DD) we have shown that under aerobic conditions, phenylenediamine induced cytotoxicity and ROS formation were markedly increased by inactivating DT-diaphorase but were prevented by a subtoxic concentration of the mitochondrial respiratory inhibitor cyanide. This suggests that the H_2O_2 generation could be attributed to a futile two electron redox cycle involving oxidation of phenylenediamine to the corresponding diimine by the mitochondrial electron transfer chain and re-reduction by the DT- diaphorase. The subcellular organelle oxidative stress effects leading to cytotoxicity has not yet been identified. Hepatocyte mitochondrial membrane potential was only slightly decreased by phenylenediamine before cytotoxicity ensued. However phenylenediamine induced lysosomal damage and hepatocyte protease activation. Endocytosis inhibitors, lysosomotropic agents or lysosomal protease inhibitors also prevented phenylenediamine induced cytotoxicity.

Furthermore desferoxamine (a ferric chelator), antioxidants or ROS scavengers (catalase, mannitol, tempol or dimethylsulfoxide) prevented phenylenediamine cytotoxicity. It is concluded that H_2O_2 reacts with lysosomal Fe^{2+} to form "ROS" which causes lysosomal lipid peroxidation, membrane disruption, protease release and cell death.

Keywords: Durenediamine; Phenyenediamines; Oxidative stress; Lysosome; Hepatocytes.

Introduction

Phenylenediamines are present in hair dye constituents (1), photographic developing agents in photography (2), vulcanizing agents in rubber industry (3) and as industrial antioxidants (4). Rats given subcutaneous injections of N, N, N', N'-tetramethylphenylenediamine (TMPD) developed degeneration and dissolution of skeletal muscle fibers, necrotic lesions on the tongue, cytoplasmic vacuolation in cardiac muscle and lesions in the gastrocnemius (5,6,7). TMPD was also cytotoxic towards cultured rat myofibers (8). The ring-methylated structural isomer durenediamine (2,3,5,6-tetramethylphenylenediamine (DD)) also caused severe skeletal muscle necrosis in rats (9,10). The target site specificity of the various phenylenediamines may involve mitochondrial oxidation of these compounds (7, 9, 11). However, the effectiveness of various ring-methylated phenylenediamines at inducing muscle necrosis is related with their autoxidation rate (9).

Previously it was shown that incubation of isolated rat hepatocytes with DD results in a nearly stoichiometric oxidation of GSH to GSSG, which rapidly effluxes the cell (12). The generation of H_2O_2 that oxidized GSH resulted from a two electron redox cycle involving oxidation of DD to the corresponding diimine and its subsequent reduction (12).

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In the following, however, we provide evidence that the cytotoxicity of durenediamine and other phenylenediamines may involve oxygen activation and ROS formation caused by futile intracellular redox cycling that includes oxidation by the

mitochondrial respiratory chain and subsequent rereduction by DT-diaphorase. Eventually, the hepatocyte is unable to maintain redox homeostasis and all of the DD becomes oxidized. The cytotoxic process that causes plasma membrane disruption is probably mediated by lysosomal membrane damage caused by the ROS formation and release of deadly proteases.

Experimental

Chemicals

Durenediamine (2,3,5,6-tetramethylphenylenediamine), collagenase (from Clostridium histolyticum), bovine serum albumin (BSA), Hepes, trypan blue, d mannitol, dimethylsulfoxide, catalase, superoxide dismutase, chloroquine diphosphate, methylamine HCl, 3 methyl adenine, monensin sodium, leupeptin, pepstatin, tempol, ethyleneglycol bis (p aminoethyl ether) N,NN',N' tetra acetic acid (EGTA), sodium pentobarbital and heparin were obtained from Sigma (St. Louis, MO, USA). Acridine orange and dichlorofluorescin diacetate was purchased from Molecular Probes (Eugene, Ore, USA). Desferoxamine was a gift from Ciba Geigy Canada Ltd. (Toronto, ON, Canada). All chemicals were of the highest commercial grade available.

Animals

Male Sprague-Dawley rats (280-300 g), fed with a standard chow diet and given water *ad libitum*, were used in all experiments.

Isolation and incubation of hepatocytes

Hepatocytes were obtained by collagenase perfusion of the liver as described by Pourahmad and O'Brien, 2000 (13). Approximately 85-90% of the hepatocytes excluded trypan blue. Cells were suspended at a density of 10^6 cells/ml in round bottomed flasks rotating in a water bath maintained at 37°C in Krebs-Henseleit buffer (pH 7.4), supplemented with 12.5 mM Hepes under an atmosphere of 10% O_2, 85% N_2, 5% CO_2. Each flask contained 10 ml of hepatocyte suspension. Hepatocytes were preincubated for 30 min prior to addition of chemicals. Stock solutions of all chemicals (×100 concentrated for the water solutions or ×1000 concentrated for the methanolic solutions) were prepared fresh prior to use. To avoid either non toxic or very toxic conditions in this study we used ED_{50} concentration for DD on the isolated hepatocytes (140 μM). The ED_{50} of a chemical in hepatocyte cytotoxicity assessment technique (with the total 3 h incubation period), is defined as the concentration which decreases the hepatocyte viability down to 50% following the 2 h of incubation (14). In order to determine this value for the investigated compound dose-response curves were plotted and then ED_{50} was determined based on a regression plot of three different concentrations (data and curves not shown). For the chemicals which dissolved in water, we added 100 μl sample of its concentrated stock solution (×100 concentrated) to one rotating flask containing 10 ml hepatocyte suspension. For the chemicals which were soluble in methanol we prepared methanolic stock solutions (×1000 concentrated), and to obtain the required concentration in the hepatocytes, we added 10 μl samples of the stock solution to the 10 ml cell suspension. Ten microlitres of methanol did not affect the hepatocyte viability after 3 h incubation (data not shown).

Cell viability

The viability of isolated hepatocytes was assessed from the intactness of the plasma membrane as determined by the trypan blue (0.2% w/v) exclusion test (13). Aliquots of the hepatocyte incubate were taken at different time points during the 3 hour incubation period. At least 80-90% of the control cells were still viable after 3 hours.

Determination of reactive oxygen species "ROS"

To determine the rate of hepatocyte "ROS"

generation, dichlorofluorescin diacetate was added to the hepatocyte incubate as it penetrates hepatocytes and becomes hydrolysed to non-fluorescent dichlorofluorescein The latter then reacts with "ROS" to form the highly fluorescent dichlorofluorescein which effluxes the cell. Hepatocytes (1×10^6 cells/ml) were suspended in 10 ml modified Hank's balanced salt solution (HBS), adjusted to pH 7.4 with 10 mM Hepes (HBSH) and were incubated with DD at 37°C for 3 hours. After centrifugation (50 × g. 1 min), the cells were resuspended in HBS adjusted to pH 7.4 with 50 mM Tris-HCl and loaded with dichlorofluorescein by incubating with 1.6µl dichlorofluorescein diacetate for 2 min at 37°C. The fluorescence intensity of the "ROS" product was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500nm and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (15).

Lysosomal membrane stability assay

Hepatocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange (16). Aliquots of the cell suspension (0.5 ml) that were previously stained with acridine orange 5 µM, were separated from the incubation medium by 1 minute centrifugation at 1000 rpm. The cell pellet was then resuspended in 2 ml of fresh incubation medium. This washing process was carried out for two times to remove the fluorescent dye from the media. Acridine orange redistribution in the cell suspension was then measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 495 nm excitation and 530 nm emission wavelengths.

Determination of proteolysis

To determine the rate of hepatocyte "ROS" generation, dichlorofluorescin diacetate was added to the hepatocyte incubate as it penetrates hepatocytes and becomes hydrolysed to non-fluorescent dichlorofluorescein The latter then reacts with "ROS" to form the highly fluorescent dichlorofluorescein which effluxes the cell. Hepatocytes (1×10^6 cells/ml) were suspended in 10 ml modified Hank's balanced salt solution (HBS), adjusted to pH 7.4 with 10 mM Hepes (HBSH) and were incubated with DD at 37°C for 3 hours. After centrifugation (50 × g. 1 min), the cells were resuspended in HBS adjusted to pH 7.4 with 50 mM Tris-HCl and loaded with dichlorofluorescein by incubating with 1.6 l dichlorofluorescein diacetate for 2 min at 37°C. The fluorescence intensity of the "ROS" product was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500nm and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (15).

Statistical analysis

The statistical significance of differences between the control and treatment groups in these studies was determined using a one-way analysis of variance (ANOVA) and the Bartlett's test for homogeneity of variances. Results represent the mean±standard error of the mean (SEM) of triplicate samples. The minimal level of significance chosen was $P < 0.001$.

Table 1. Preventing DD induced hepatocyte necrosis and "ROS" formation by antioxidants, "ROS" scavengers, endocytosis inhibitors, and inhibitor of mitochondrial cytochrome oxidase

Addition	%Cytotoxicity 3h	"ROS" 3h
None	20±2	85±7
*Durenediamine (*70 µM*)*	39±3	272±7 [a]
*Durenediamine (*140 µM*)*	73±8 [a]	458±6
+Catalase (200 U/ml)	37±4 [b]	103±6 [b]
+Tempol (300 µM)	41±4 [b]	114±8 [b]
+Dimethyl sulfoxide (150 µM)	36±4 [b]	101±6 [b]
+Mannitol (50 mM)	37±4 [b]	103±7 [b]
+Desferoxamine (200 µM)	36±4 [b]	100±8 [b]
+α-Tocopherol succinate (100µM)	38±4 [b]	106±5 [b]
+Sodium azide (4 mM)	83±6 [b]	611±7 [b]
+Cyanamide (100 µM)	86±8 [b]	615±8 [b]
+Potassium cyanide (300 µM)	36±4 [b]	109±5 [b]
+Dicumarol (30 µM)	96±4 [b]	605±6 [b]
+Monensin (10 µM)	35±4 [b]	119±5 [b]
+Methylamine (30 mM)	34±3 [b]	116±7 [b]
+Chloroquine (100 µM)	45±4 [b]	117±8 [b]
+3-Methyladenine (5 mM)	37±4 [b]	111±6 [b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs -Henseleit buffer pH 7.4 at 37C for 3.0 hrs

Following the addition of durene diamine. Cytotoxicity was determined as the percentage of cells that take up trypan blue (13)

DCF formation was expressed as fluorescent intensity units (15).

Values are expressed as means of three separate experiments (S.D.)

a: Significant difference in comparison with control hepatocytes ($P < 0.001$)

b: Significant difference in comparison with DD treated hepatocytes ($P < 0.001$)

Table 2. Preventing DD induced hepatocyte lysosomal membrane damage by inhibitors of oxidative stress , mitochondrial repiratory chain and endocy tosis

Addition	(Acridine orange red istribution)		
	15 min	30 min	60 min
None	3±1	4±1	4±1
Durenediamine (140 μM*)*	69±4 [a]	118±7 [a]	195±8 [a]
+Catalase (200 u/ml)	3±1 [b]	3±1 [b]	5±1 [b]
+SOD (100 u/ml)	6±1 [b]	7±1 [b]	11±2 [b]
+Dimethylsulfoxide (150 μM)	3±1 [b]	4±1 [b]	7±1 [b]
+Mannitol (50 mM)	6±1 [b]	5±1 [b]	8±2 [b]
+Desferoxamine (200 μM)	3±1 [b]	3±1 [b]	3±1 [b]
+Potassium cyanide (300 μM)	3±1 [b]	3±1 [b]	6±1 [b]
+Dicumarol (250 μM)	99±6 [b]	144±6 [b]	255±7 [b]
+Monensin (10 μM)	7±1 [b]	8±1 [b]	10±1 [b]
+Methylamine (30mM)	6±1 [b]	5±1 [b]	6±1 [b]
+Chloroquine (100 μM)	5±1 [b]	8±1 [b]	9±1 [b]
+3-Methyladenine (5mM)	5±1 [b]	8±1 [b]	10±1 [b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs -Henseleit buffer pH 7.4 at 37 °C.
Lysosomal membrane damage was determined as inte nsity unit of diffuse cytosolic green fluorescence induced by acridine orange following the release from lysosomes (16).
Values are expressed as means of three separate experiments (S.D.)
a: Significant difference in comparison with control hepatocytes ($P < 0.001$)
b: Significant difference in comparison with DD treated hepatocytes ($P < 0.001$)

Table 3. Preventing DD induced hepatocyte proteolysis with inhibitors of oxidative stress or endocytosis or lysosomal protease inhibitors

Addition	Hepatocyte tyrosine release (μM)		
	30 min	60 min	120 min
None	13±1	15±1	21±2
Durenediamine (140 μM*)*	39±4 [a]	48±6 [a]	65±7 [a]
+Catalase (200 u/ml)	15±2 [b]	17±2 [b]	27±3 [b]
+SOD (100 u/ml)	14±2 [b]	17±2 [b]	23±2 [b]
+Dimethylsulfoxide (150mM)	15±2 [b]	19±2 [b]	28±3 [b]
+Mannitol (50 mM)	14±2 [b]	16±2 [b]	20±2 [b]
+Desferal (200 μM)	12±2 [b]	14±2 [b]	20±2 [b]
+Potassium cyanide (300 μM)	13±2 [b]	14±2 [b]	18±2 [b]
+Dicumarol (250 μM)	59±6 [b]	78±6 [b]	105±8 [b]
+Monensin (10 μM)	7±1 [b]	14±2 [b]	15±1 [b]
+Methylamine (30mM)	10±1 [b]	15±3 [b]	16±2 [b]
+Chloroquine (100 μM)	11±1 [b]	18±5 [b]	19±7 [b]
+3-methyladenine (30mM)	12±2 [b]	12±2 [b]	10±2 [b]
+Leupeptin (100 μM)	12±2 [b]	12±2 [b]	17±2 [b]
+Pepstatin (100 μM)	12±2 [b]	13±2 [b]	18±2 [b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs -Henseleit buffer pH 7.4 at 37 °C.
Lysosomal induced proteolysis was determined by measuring the cellular release of tyrosine into the media (17).
Values are expressed as means of three separate experiments (S.D.)
a: Significant difference in comparison with control hepatocytes ($P < 0.001$)
b: Significant difference in comparison with DD treated hepatocytes ($P < 0.001$)

Results and Discussion

When hepatocytes were incubated with 140 μM DD the formation of "ROS" was increased very rapidly (peak in about 60 minutes) in a concentration dependent fashion (Fig. 1, Table 1). The antioxidants, α-tocopheryl succinate, catalase, superoxide dismutase (SOD) and "ROS" scavengers (18), tempol, mannitol and dimethylsulfoxide (DMSO) protected the hepatocytes against DD induced cytotoxicity as well as "ROS" generation. On the other hand catalase inhibitors, sodium azide (19) and cyanamide (20) significantly increased DD induced cytotoxicity, and "ROS" formation (Table 1). Neither of these agents showed any toxic effect on hepatocytes at concentrations used (data not shown).

The DT-diaphorase inhibitor, dicumarol (12) markedly increased DD induced cell lysis and "ROS" generation (Table 1). However the CYP2E1 inhibitor phenylimidazole (18, 19) and P450 reductase inhibitor, diphenyliodonium chloride (DPI) (18, 19) did not show any significant effect on DD induced cell lysis and "ROS" formation (data not shown). On the other hand subtoxic concentration of mitochondrial cytochrome oxidase inhibitor, potassium cyanide (300 μM) protected hepatocytes against DD induced cytotoxicity and "ROS" formation (Table 1). Endocytosis inhibitors including lysosomotropic agents, chloroquine (21), methylamine (22), monensin a Na^+ ionophore that inhibits hepatocyte endosomal acidification (23) and 3-Methyladenine, an inhibitor of hepatocyte autophagy (24) also protected the hepatocytes against DD induced cell lysis and "ROS" formation (Table 1). Neither of these agents showed any toxic effect on hepatocytes at concentrations used (data not shown).

When hepatocyte lysosomes were preloaded with acridine orange, a release of acridine orange into the cytosolic fraction ensued within 60 minutes after treating the loaded hepatocytes with 140 μM DD (Table 2). The DD induced acridine orange release was prevented by dimethylsulfoxide, mannitol, catalase, superoxide dismutase (SOD) or the ferric chelator desferoxamine (Table 2). All endocytosis inhibitors and potassium cyanide (KCN) also inhibited DD induced acridine orange release (Table 2). On the other hand DT-diaphorase inhibitor, dicumarol significantly increased DD induced acridine orange release

(Table 2).

Hepatocyte proteolysis as determined by the release of the amino acid tyrosine into the extracellular medium over 120 minutes was markedly increased when hepatocytes were incubated with DD (Table 3). The DD induced tyrosine release was completely prevented by the lysosomal protease inhibitors; leupeptin (25) and pepstatin (26) (Table 3). Dimethylsulfoxide, mannitol, catalase, SOD, deferoxamine, potassium cyanide (KCN), 3-methyladenine, methylamine and chloroquine also inhibited DD induced tyrosine release (Table 3). On the otherhand DT-diaphorase inhibitor, dicumarol significantly increased DD induced tyrosine release (Table 3).

ROS formation contributes to DD induced cell lysis was markedly increased following the treatment of hepatocytes with DD and the antioxidants and "ROS" scavengers prevented both DD induced "ROS" formation and cytotoxicity (Table 1).

It was already suggested that an oxidative stress without "ROS" generation is responsible for DD induced cytotoxicity (12). However in our study, we determined huge increase in "ROS" formation following the treatment of hepatocytes with DD (Fig. 1). As shown here pretreatment of hepatocytes with subtoxic concentration of KCN (an inhibitor of mitochondrial cytochrome oxidase) prevented DD induced cytotoxicity, "ROS" formation, lysosomal membrane damage and proteolysis (Tables 1, 2, and 3), which suggests that mitochondrial respiratory chain cytochrome oxidase activates DD.

DD induced hepatocyte lysosomal disruption ensued within 60 min following its addition (Table 2). A similar release of acridine orange occurred when acridine orange loaded hepatocytes were treated with hydrogen peroxide generated by glucose/glucose oxidase (23, 27, 28) or nitrofurantoin (29). Hepatocyte proteolysis markedly increased following lysosomal disruption by DD which was inhibited by the lysosomal protease inhibitors leupeptin and pepstatin (Table 3). Furthermore, DD induced cytotoxicity was also prevented by leupeptin or pepstatin (data not shown). Besides, DD induced cytotoxicity, "ROS" formation,

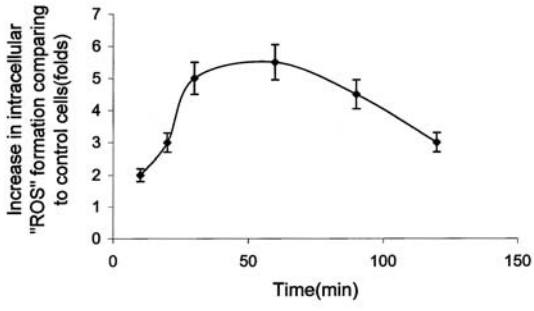

Figure 1. Huge increase (more than 5 folds) in "ROS" formation was observed following the treatment of hepatocytes with DD (peak at 60 minutes).

lysosomal membrane damage and proteolysis were also prevented by the hepatocyte endocytosis inhibitors; methylamine, chloroquine, monensin and 3-methyladenine (Tables 1, 2, and 3).

Methylamine or chloroquine or the ferric chelator desferoxamine also prevented hepatocyte cytotoxicity induced by hydrogen peroxide generated by glucose/glucose oxidase (23, 30), xanthine/xanthine oxidase (31) and nitrofurantoin (29).

In conclusion as shown in Fig. 2 these results suggest that DD induced hepatocyte toxicity involves oxidation by mitochondrial respiratory chain cytochrome oxidase to form the corresponding diimine which undergoes futile redox cycling resulting in the formation of H_2O_2 which is the cause of GSH oxidation. Then, H_2O_2 diffuses inside the lysosomes and interacts with lysosomal Fe^{2+}/Cu^{+} leading to hydroxyl radical formation (Haber-weiss reaction). Hydroxy radicals cause lysosomal membrane damage and deadly protease release.

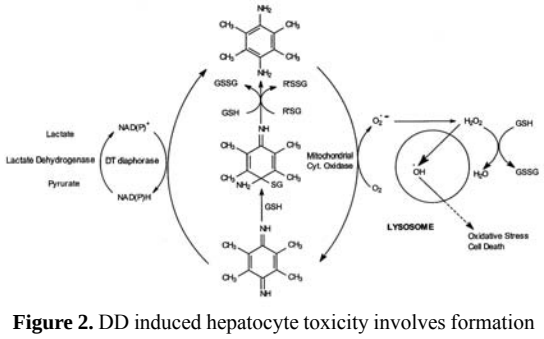

Figure 2. DD induced hepatocyte toxicity involves formation of H_2O_2 which is the cause of GSH oxidation. H_2O_2 generation attributed to a futile two electron redox cycle involving oxidation of phenylenediamine Then H

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