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Original Article

Involvement of Cytochrome P-450 in *n*-Butyl Nitrite-Induced Hepatocyte Cytotoxicity

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Abstract

Addition of *n*-butyl nitrite to isolated rat hepatocytes caused an immediate glutathione depletion followed by an inhibition of mitochondrial respiration, inhibition of glycolysis and ATP depletion. At cytotoxic butyl nitrite concentrations, lipid peroxidation occurred before the plasma membrane was disrupted. Cytochrome P-450 inhibitors inhibited peroxynitrite formation and prevented butyl nitrite-induced mitochondrial respiration inhibition, ATP depletion, lipid peroxidation and plasma membrane disruption. However, glutathione depletion, *S*-nitroso-glutathione (GSNO) formation, or the inhibition of glycolysis was not affected by cytochrome P-450 inhibitors. Glutathione-depleted hepatocytes were resistant to butyl nitrite which suggests that cytotoxicity and peroxynitrite formation results from GSNO formation. Peroxynitrite formation was also inhibited by reactive oxygen scavengers. These findings suggest that cytochrome P-450 isoforms (particularly CYP2E1) act as a source of superoxide anion radicals in the formation of cytotoxic peroxynitrite from nitric oxide.

Keywords: *n*-Butyl nitrite; Cytochrome P-450; Nitrosoglutathione; Peroxynitrite; Cytotoxicity; ATP depletion.
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1. Introduction

The vasodilator isoamyl nitrite has been used for years to relieve angina pectoris [1]. The structural isomer *n*-butyl nitrite (BN) is used as a drug of abuse to produce a euphoric-like state [2]. Both of these distinctive features have been attributed to the vasodilatory action of nitric oxide (NO) formed during the metabolism of isoamyl nitrite [1], or when isoamyl nitrite is reduced by ascorbate or dithiothreitol [3]. Reports have also appeared suggesting that the abuse of nitrite inhalants is a cofactor in AIDS [4, 5], or Kaposi's sarcoma in AIDS patients [6]. BN has also been shown to be cytotoxic to lymphocytes [7]. Furthermore, T-lymphocyte blastogenesis and antibody responsiveness is decreased 90% when mice are exposed to

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BN at levels similar to those used by chronic users [8, 9]. Mortality was found to occur in rats exposed to ≥ 600 ppm BN vapours for 14 days and hepatocellular cytoplasmic vacuolization was noted [10].

Previously, we showed that BN readily induces cytotoxicity in isolated hepatocytes. The molecular cytotoxic mechanisms involved the immediate formation of *S*-nitrosoglutathione (GSNO) as well as a concomitant decrease in protein thiols, followed by a marked ATP depletion and finally lipid peroxidation [11]. Furthermore, GSH-depleted hepatocytes were resistant to BN suggesting that GSNO formed in normal hepatocytes treated with BN contributes to ATP depletion, lipid peroxidation and cytotoxicity. We also showed that the cytotoxicity of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) towards isolated hepatocytes could be attributed to GSNO formation and was prevented by cytochrome P-450 inhibitors [12]. In the present study, we have also found that cytochrome P-450 inhibitors prevented BN cytotoxic effects including inhibition of mitochondrial respiration, ATP depletion, lipid peroxidation and plasma membrane disruption.

2. Materials and methods

2.1. Chemicals

Collagenase (from *Clostridium histolyticum*), HEPES and bovine serum albumin were obtained from Boehringer-Mannheim (Montreal, P.Q., Canada). Trypan blue, metyrapone, imidazole and isoniazid were obtained from the Sigma Chemical Co. (St. Louis, MS, USA). Desferoxamine was a gift from Ciba Geigy (Mississuga, ON, Canada). Isopropanol and toluene were purchased from BDH Chemicals (Toronto, ON, Canada) and acetone was obtained from Anachemia Canada Inc. (Toronto, ON, Canada). SKF 525A was obtained from Smith Kline Beecham Pharmaceuticals (King of Prussa, PA, USA) and HPLC grade solvents were purchased from Caledon (Georgetown, ON, Canada). *n*-Butyl nitrite was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (carboxy-PTIO) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). Other chemicals were of the highest grade available.

2.2. Isolation and incubation of hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (250-300g), maintained on a standard chow diet, by collagenase perfusion of the liver, as previously described [13]. Cell viability was assessed by determining the percentage of the hepatocytes which excluded trypan blue. Routinely, 85 to 90% of hepatocytes excluded trypan blue immediately after isolation. GSH-depleted hepatocytes were prepared by preincubating with *n*-bromoheptane as previously described [14].

Hepatocytes (10^6 cells/ml) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES. All incubations were performed in rotating, round bottomed flasks at 37 °C under a continuous flow of 95% O_2 and 5% CO_2 or 1% O_2, 94% N_2, and 5% CO_2, where indicated. Reactions were started by the addition of BN. Aliquots of the incubation mixture were taken at various time points for biochemical analysis and cell viability determination.

2.3. Biochemical assays

Lipid peroxidation was measured by treating 1 ml aliquots of hepatocytes with 1 ml 20% trichloroacetic acid, 1 ml 0.8% thiobarbituric acid, and the mixture was heated for 20 min. Samples were centrifuged for 5 min at 2500 g and the supernatant was monitored at 535 nm [15]. nATP levels in hepatocytes were determined by alkaline extraction and quantified by HPLC using a C18

μBondapak reverse phase column (Waters Associates, Milford, MA, USA) [16]. Glycolysis was determined by measuring lactate formation from fructose. Lactate was measured by the formation of NADH from NAD^+ by lactate dehydrogenase as previously described [17]. Total GSH and GSSG content of hepatocytes were measured by an HPLC procedure using a μBondapak NH_2 column, a Waters 6000 A solvent delivery system, a WISP 710A automatic injector and Data Module (Water Associates, Milford, MA, USA) [18].

2.4. Peroxynitrite/NO_2 assay

The rate of peroxynitrite formation from BN was determined using the chemiluminescence method described by Radi *et al.* [19]. BN was added to 1 ml of hepatocytes (10^6 cells/ml) in the presence of 1 mM luminol and the relative light unit (RLU) was recorded every 6 seconds, using a Luminometer LB 9501-Berthold Lumat.

2.5. Hepatocyte respiration measurement

Hepatocyte respiration was measured at determined time points after the addition of BN to the incubation of hepatocytes, using a Clark-type oxygen electrode (Model 5300; Yellow-Spring Instrument Co., Inc.) in a 2 ml chamber maintained at 37 °C. Prior to oxygen consumption measurement, hepatocytes (10^6 cells/ml) were kept at 37 °C in Krebs-Henseleit buffer plus 12.5 mM HEPES, pH 7.4 under 95% O_2 and 5% CO_2.

2.6. Statistics

Values are means ± SD of three separate experiments unless otherwise stated. Statistically significant differences between control and experimental groups were obtained using one way ANOVA.

3. Results

BN cytotoxicity was dose-dependent and became marked above 100 μM of BN at 95% O_2. Plasma membrane bleb formation was seen as early as 30 min after 200 μM BN was added, with cytotoxicity commencing around 60 min and 60% cytotoxicity occurring at about 120 min. However, as shown in Table 1, BN cytotoxicity was prevented if the hepatocytes were preincubated for 15 min with the cytochrome P-4502E1 inhibitors acetone, and 2-hexanone. Similarly, the P-450 inhibitors phenylimidazole, SKF 525A, metyrapone, isoniazid, piperonyl butoxide, and imidazole also prevented or markedly delayed cytotoxicity. On the other hand, carboxy-PTIO, which converts NO to NO_2 radical [20], increased BN cytotoxicity. At low oxygen concentrations, BN was five times less toxic as the ED_{50} for 2 hr determined at 1% O_2 (1 mM) was five times higher than that determined at 95% O_2 (0.2 mM).

Lipid peroxidation, as measured by malondialdehyde formation, occurred only at BN concentrations which were toxic to the

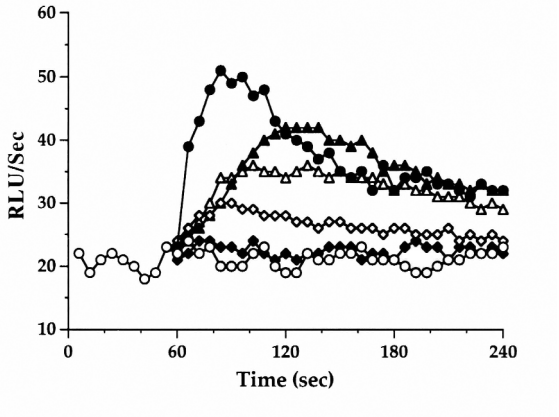

Figure 1. Peroxynitrite formation from butyl nitrite. Butyl nitrite was added to 1 ml of hepatocytes (10^6 cells/ml) incubated with 1 mM luminol at 1 min and the relative light unit (RLU) was measured every 6 second. GSH-depleted hepatocytes were prepared by preincubation with bromoheptane; P-450 inhibition was achieved by preincubating hepatocytes with phenyl imidazole (0.3 mM) for 15 min. (_) No addition, (_) butyl nitrite 200 μM, (◊) GSH depleted hepatocytes + butyl nitrite 200 μM, (_) P-450 inhibited hepatocytes + butyl nitrite 200 μM, (Δ) butyl nitrite 200 μM + morin 100 μM, (_) butyl nitrite 200 μM + hepatocytes incubated at 1% O_2. Background chemiluminescence in GSH depleted or P-450 inhibited hepatocytes were not significantly different from control hepatocytes.

cell. As shown in Table 2, lipid peroxidation was also prevented by the P-450 inhibitors SKF 525A, phenylimidazole, piperonyl butoxide, and metyrapone, if added before BN but not if added 30 min after BN. Lipid peroxidation was increased by carboxy-PTIO and did not occur at a low 1% O_2 concentration.

Table 1. Effects of cytochrome P-450 inhibitors on BN cytotoxicity.

Addition	Cytotoxicity (%)			
	30 min	60 min	120 min	180 min
None	15±2	16±3	18±3	20±3
Butyl nitrite (0.2 mM)	22±3	49±3	65±5	80±5[a]
+ Piperonyl butoxide (0.1 mM)	21±3	27±3	38±3	62±4
+ SKF 525A (50 µM)	20±2	31±3	37±3	45±4[b]
+ Metyrapone (1 mM)	22±3	34±3	42±4	47±5[b]
+ Acetone (2 mM)	17±2	17±2	19±3	25±3[b]
+ 2-Hexanone (2 mM)	18±3	19±2	23±3	25±3[b]
+ Isoniazid (5 mM)	22±2	29±3	33±3	60±4
+ Imidazole (5 mM)	23±2	25±3	29±4	63±5
+ Phenylimidazole (0.3 mM)	21±3	28±3	34±4	38±4[b]
+ Carboxy-PTIO[c] (0.2 mM)	36±3	92±5	100[b]	100[b]
+ 1% O_2	16±2	18±3	20±3	25±3

Note: Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer (pH 7.4) at 37 °C under an atmosphere of 95% O_2 and 5% CO_2 with P-450 inhibitors for 20 min before addition of butyl nitrite. Cytotoxicity was determined as the percentage of cells taken up trypan blue. Values are shown as means ± SD of at least three separate experiments. [a]Significantly different from untreated cells (p<0.001). [b]Significantly different from butyl nitrite treated cells (p<0.005). [c]Carboxy-PTIO, a nitric oxide oxidizing agent, was not toxic at the concentration used.

Furthermore, prior GSH depletion or low oxygen concentrations prevented peroxynitrite formation from BN. Antioxidants and P-450 inhibitors eg. phenylimidazole decreased the rate of peroxynitrite formation (Figure 1).

Table 2. Effect of cytochrome P-450 inhibitors on BN-induced hepatocyte lipid peroxidation.

Addition	Lipid peroxidation (nmoles MDA*/10^6cells)		
	30 min	60 min	120 min
None	0.12±0.04	0.42±0.11	0.50±0.12
Butyl nitrite (0.2 mM)	0.45±0.10	2.30±0.34	3.24±0.47[a]
+ SKF 525A (50 µM)	0.21±0.05	0.72±0.10	0.82±0.22[b]
+ Phenylimidazole (0.3 mM)	0.41±0.15	0.47±0.12	0.56±0.21[b]
+ Piperonyl butoxide (0.1 mM)	0.40±0.10	0.43±0.13	0.49±0.17[b]
+ Metyrapone (1 mM)	0.32±0.12	0.80±0.26	1.10±0.31[b]
+ Tempol (0.3 mM)	0.15±0.06	0.46±0.08	0.63±0.12
+ Carboxy-PTIO (0.2 mM)	0.72±0.13	3.27±0.36[b]	4.80±0.50[b]
+ 1% O_2	0.10±0.02	0.15±0.08	0.21±0.07[b]

Note: Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer (pH 7.4) at 37 °C under an atmosphere of 95% O_2 and 5% CO_2 with P-450 inhibitors for 20 min before addition of butyl nitrite. Values are shown as means (± SD) of at least three separate experiments. *MDA: Malondialdehyde. [a]Significantly different from untreated cells (p<0.01). [b]Significantly different from butyl nitrite treated cells (p<0.01).

The addition of BN to hepatocytes caused peroxynitrite formation as determined by luminol chemiluminescence.

Hepatocyte GSH levels were rapidly depleted upon addition of BN even if the hepatocytes were pretreated with P-450 inhibitors (data not shown).

ATP depletion preceded cytotoxicity and

unlike lipid peroxidation, occurred with subtoxic concentrations of BN [11]. As shown in Table 3, ATP depletion was not prevented by antioxidants or desferoxamine but was prevented if the hepatocytes were preincubated with the P-450 inhibitors SKF 525A, phenylimidazole, piperonyl butoxide, metyrapone or 2-hexanone. Hepatocyte ATP was not depleted at 1% O_2 with 0.2 mM BN, even though initial ATP levels were lower than levels found at 95% O_2.

Table 3. Effect of cytochrome P-450 inhibitors or reactive oxygen scavengers on butyl nitrite-induced ATP depletion.

Addition	ATP (nmoles / 10^6 cells)		
	30 min	60 min	120 min
95% O_2			
None	28±3	28±4	23±3
Butyl nitrite (0.2 mM)	11±2	8±2	5±2[a]
+ SKF 525A (50 μM)	22±2	20±3	17±3[b]
+ Phenylimidazole (0.3 mM)	27±2	22±3	18±3[b]
+ Piperonyl butoxide (0.1 mM)	25±3	19±2	15±2[b]
+ Metyrapone (1 mM)	22±2	20±3	16±2[b]
+ 2-Hexanone (2 mM)	27±3	26±3	21±3[b]
+ 4-Hydroxyanisole (50 μM)	11±2	8±2	6±2
+ Desferoxamine (0.1 mM)	14±2	12±2	9±2
+ Tempol (300 μM)	15±2	11±2	10±2
+ Purpurogallin (100μM)	12±2	9±2	7±2
1% O_2			
None	19±2	17±2	17±2
Butyl nitrite (0.2 mM)	16±2	15±2	15±2

Note: Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer (pH 7.4) at 37 °C under an atmosphere of 95% O_2 and 5% CO_2 with P-450 inhibitors or antioxidants for 15 min before addition of butyl nitrite. Values are shown as means (± SD) of three separate experiments. [a]Significantly different from untreated cells (p<0.01). [b]Significantly different from butyl nitrite treated cells (p<0.03).

Table 4. Effect of butyl nitrite on hepatocyte respiration.

Addition	Oxygen uptake (nmoles O_2/10^6 cells)	
	5min	30 min
Control	12.3±1.8	12.8±1.3
BN 0.3 mM	6.1±1.0[a]	4.0±0.7[a]
+ GSH depletion	11.2±1.4	10.4±1.1
+ P-450 inhibition	10.3±1.6	8.9±1.7
+ Carboxy-PTIO	4.6±0.8[a]	2.4±0.4[a]

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer, pH 7.4 under 95% O_2 and 5% CO_2. Oxygen uptake was measured with a Clark type electrode and a 2 ml chamber. GSH depleted hepatocytes were prepared by preincubating hepatocytes with bromoheptane. P-450 inhibition was achieved by preincubating hepatocytes with phenyl imidazole (300 μM) for 15 min. [a]Significantly different from control. GSH depleted or P-450 inhibited hepatocytes were not significantly different from control. Carboxy-PTIO (100 μM) did not affect respiration of untreated hepatocytes.

As shown in Table 4, BN (300 μM) decreased hepatocyte respiration to about 50% and 31% of control value by 5 min and 30 min, respectively. Prior GSH depletion or P-450 inhibition prevented the inhibition of hepatocyte respiration by BN. On the other hand, the NO-oxidizing agent carboxy-PTIO [20] slightly increased the inhibition of mitochondrial respiration by BN. The rate of glycolysis was also markedly inhibited by BN; however, the inhibition of glycolysis was little affected by most P-450 inhibitors or carboxy-PTIO (Table 5).

4. Discussion

It has been previously shown that BN cytotoxicity towards isolated hepatocytes involves ATP depletion, lipid peroxidation, and plasma membrane disruption [11]. Membrane disruption and lipid peroxidation but not ATP depletion were prevented by antioxidants. However, BN-induced ATP depletion, cytotoxicity and lipid peroxidation could be prevented, if GSH was depleted before the addition of BN. This suggest-

ed that GSH was required for the cytotoxicity of BN. Similar results were also obtained for the NO donor MNNG [12].

Table 5. Effect of cytochrome P-450 inhibitors on butyl nitrite-induced inhibition of glycolysis.

Addition	Lactate (nmoles / 10^6 cells)		
	30 min	60 min	120 min
None	1235±153	1475±88	1534±105
Fructose 5 mM	1577±139	2533±433	3531±463[a]
+ Butyl nitrite 200 µM	1179±153	1234±147	1493±230[b]
+ Phenylimidazole 0.3 mM	1491±125	1534±98	1680±166[b]
+ Metyrapone 1 mM	1450±102	1548±155	1674±137[b]
+ SKF 525A 50 µM	1251±77	1315±95	1330±114[b]
+ Piperonyl butoxide 0.1 mM	1213±127	1261±86	1358±105[b]
+ 2-Hexanone 2 mM	1175±120	1200±132	1404±157[b]
+ Carboxy-PTIO 100 µM	1156±87	1150±112	1288±125[b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer (pH 7.4) at 37 °C under an atmosphere of 95% O_2 and 5% CO_2 with P-450 inhibitors for 15 min before the addition of butyl nitrite. Fructose was added after butyl nitrite. Values are shown as means ± SD of at least three separate experiments. [a]Significantly different from untreated cells (p<0.001). [b]Significantly different from frucctose treated cells (p<0.001).

S-Nitrosoglutathione formation from organic nitrites has been shown [21] to be catalyzed by human GSH transferases particularly M1a-1a and A 1-1 but not P1-1 (eq. 1).

$$RCH_2ONO + GSH \_ GSNO + RCH_2OH \quad \text{(eq. 1)}$$

Furthermore, whilst stable in solution, GSNO slowly decomposes in the presence of GSH or ascorbate to form NO, apparently catalyzed by trace metals [22]. GSNO may thus act as an intracellular NO store which contributes to the cytotoxicity of BN.

$$2\ GSNO \_ 2\ NO\cdot + GSSG \quad \text{(eq. 2)}$$

The present study shows that cytochrome P-450 also contributes to the BN cytotoxic mechanisms as various P-450 inhibitors or substrates prevented cytotoxicity. Furthermore, P-450 inhibitors partially prevented the BN-induced inhibition of mitochondrial respiration, ATP depletion, and lipid peroxidation. Other investigators have shown that sodium nitrite reacts with Fe^{2+}-P-450 to form an unstable NO-Fe^{2+}-P-450 complex which is converted to a NO-Fe^{2+}-P-420 complex [23, 24]. Our results suggest that a similar unstable complex is also formed when BN or GSNO reacts with reduced cytochrome P-450 which suggests that reduced P-450 can reduce BN or GSNO to form nitric oxide (eq. 3, 4).

$$RCH_2ONO + \text{P-450-}Fe^{2+} \_ \text{P-450-}Fe^{3+} + NO\cdot + RCH_2O^- \quad \text{(eq. 3)}$$

$$GSNO + \text{P-450-}Fe^{2+} \_ NO\cdot + GS^- + \text{P-450-}Fe^{3+} \quad \text{(eq. 4)}$$

BN also markedly increased peroxynitrite/$NO_2^{\bullet}$ formation in normal hepatocytes which was prevented if GSH depleted hepatocytes were used. This suggests that peroxynitrite formation from BN catalyzed by hepatocytes is dependent on GSNO formation and could be responsible for the cytotoxicity. Peroxynitrite has been shown to cause lipid peroxidation [25] and could therefore be responsible for BN-induced lipid peroxidation and cytotoxicity at high O_2 concentrations. Lipid peroxidation could also be initiated by nitroxyl radicals or nitrogen dioxide formed from the peroxynitrite anion [26] as the NO oxidizing agent carboxy-PTIO, which converts NO to $NO_2^{\bullet}$ [20], further increased cytotoxicity and lipid

peroxidation induced by BN. The high oxygen requirement for BN-induced lipid peroxidation and ATP depletion further suggests that peroxynitrite could be the toxic species responsible for the inhibition of mitochondrial respiration and lipid peroxidation. Thus GSH was instantly depleted to the same extent upon addition of BN at 1% or 95% O_2 (data not shown), whereas peroxynitrite formation, ATP depletion or lipid peroxidation did not occur at 1% but at 95% O_2. Furthermore, the NO-oxidant carboxy-PTIO also increased the inhibition of mitochondrial respiration and ATP depletion induced by BN at 95% O_2, further suggesting that an oxidation product of NO was responsible for the inhibition of mitochondrial respiration.

Cytochrome P-450 inhibitors, reactive oxygen scavengers, or desferoxamine partially inhibited peroxynitrite/$NO_2^{\bullet}$ formation from BN. It is, therefore, more likely that cytochrome P-450 plays a role in BN cytotoxicity by catalyzing the formation of peroxynitrite (eq. 5, 6) or by acting as a source of superoxide radicals which are involved in the formation of cytotoxic peroxynitrite as shown in equation 7.

$NO^{\cdot}$ + P-450-Fe^{2+} _ P-450-$Fe^{2+}NO$ (eq. 5)

P450-$Fe^{2+}NO$ + O_2 _ P-450-Fe^{3+} + $ONOO^{-}$(eq. 6)

$NO^{\cdot}$ + $O_2^{\cdot -}$ _ $ONOO^{-}$ (eq. 7)

It is possible that cytochrome P-450 activates peroxynitrite by catalyzing its conversion to NO_2 which also readily causes lipid peroxidation [26]. Such a reaction (eq. 9) has also been suggested to explain the activation of peroxynitrite by peroxidases in which peroxidase compound II is formed [27].

ONOOH + Fe^{3+} _ NO_2 + $Fe^{4+}O$ (complex II) (eq. 8)

BN markedly depletes hepatocytes ATP prior to lipid peroxidation even at noncytotoxic doses [11]. As hepatocyte respiration was partially inhibited when ATP depletion occurred, it is possible that ATP depletion arose because: (a) NO complexed the iron-sulfur proteins of the respiratory chain [28]; (b) peroxynitrite inhibited mitochondrial respiration by inactivating cytochrome *C* oxidase or succinate dehydrogenase [29, 30] or by inactivating aconitase of the citric acid cycle [31, 32]; and/or (c) GSNO (probably via NO^{+}) inhibits glycolysis by inactivating glyceraldehyde-3-phosphate dehydrogenase by *S*-nitrosylating the active site which causes nonenzymatic auto-ADP-ribosylation by NAD^{+} [33-35]. Cytochrome P-450 inhibitors prevented mitochondrial respiration inhibition and ATP depletion induced by BN, without affecting the inhibition of glycolysis by BN, which suggests that ATP depletion primarily results from mitochondrial toxicity caused by peroxynitrite, whereas, glycolysis inhibition can be attributed to NO. Previously, it has been shown that prior hepatocyte GSH depletion prevented the inhibition of glycolysis by BN [11].

In conclusion, a metabolite of BN, most likely peroxynitrite (or its breakdown products) formed from GSNO, is responsible for the inhibition of mitochondrial respiration, ATP depletion, lipid peroxidation and cytotoxicity induced by BN. Furthermore, cytochrome P-450 also seems to be involved in the reductive activation of BN or GSNO to form reactive metabolites eg. peroxynitrite which causes lipid peroxidation that mediates BN cytotoxicity. Cytochrome P-450 inhibitors or substrates prevented the inhibition of hepatocyte respiration but did not prevent the inactivation of glycolysis by BN. It is interesting to speculate whether NO or GSNO inhibits glycolysis whereas peroxynitrite inhibits mitochondrial respiration.

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