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Formation of Nitrated and Hydroxylated Aromatic Compounds from Benzene and Peroxynitrite, A Possible Mechanism of Benzene Genotoxicity

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Peroxynitrite, the reaction product of nitric oxide ($NO^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$) produced during immune activation by a variety of inflammatory cells, may contribute to genotoxicity of benzene through its ability to carry out hydroxylation and nitration. After exposure of benzene to synthesised peroxynitrite, phenol, nitrophenols (*p*-nitrophenol, *o*-nitrophenol and *m*-nitrophenol) and nitrobenzene were identified in the reaction mixture by HPLC separation and single UV wavelength and diode array detection. The formation of phenol, nitrophenols and nitrobenzene showed a linear relationship with both benzene and peroxynitrite concentrations. The molar ratio for phenol/(nitrobenzene and nitrophenols) was approximately 9/5 with a total product yield of 14% hydroxylated and nitrated products as based on peroxynitrite. The physiological relevance of the chemical reaction between benzene and peroxynitrite was tested by detecting the reaction products in human neutrophils (2.5×10^7 cells/ml) incubated with 10 mM benzene for 25 min. The concentration of phenol and *p*-nitrophenol were found to be 1.29 ± 0.22 and 1.56 ± 0.61 μM (mean ± SD) in the incubation medium of the neutrophils pretreated with phorbol myristate acetate (500 nM) for 5 min, respectively, whereas no metabolites were detected if the neutrophils were not pretreated. Nitrated aromatic compounds are known to be more carcinogenic than the parent compounds. It is reported that acute and chronic infection increases the risk of cancer at various sites; and that anti-inflammatory agents decrease benzene myelotoxicity. We suggest that the increased production of peroxynitrite during chronic inflammation combined with benzene exposure may increase the carcinogenicity of benzene by a mechanism that includes the formation of metabolites from the chemical reaction between benzene and peroxynitrite. Thus, peroxynitrite mediated hydroxylation and nitration of benzene during immune activation represent a novel *in vivo* mechanism for generation of proximal carcinogens of benzene.

Keywords: Benzene, hydroxylation, nitration, peroxynitrite, neutrophils

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ABBREVIATION

$NO^{\bullet}$, nitric oxide; $O_2^{\bullet -}$, superoxide anion; ROS, reactive oxygen species; RNS, reactive nitrogen species; CYP2E1, cytochrome P-4502E1; PMA, phorbol 12-myristate 13-acetate.

INTRODUCTION

Benzene (C_6H_6) is used in industry as a solvent and significant concentrations of benzene are found in gasoline, car exhaust, tobacco smoke, drinking water, ambient air and certain types of food.[1] Exposure to benzene has been shown to induce bone marrow toxicity and ultimately leukaemia in humans and multiple forms of cancer in rodents.[2–6] The mechanisms underlying benzene carcinogenesis have not been fully elucidated, although it is generally accepted that metabolism is required. Benzene is mainly metabolised by cytochrome P-4502E1 (CYP2E1) in the liver to various phenolic metabolites which accumulate in the bone marrow where toxic metabolites are formed.[7–11] Although phenol is the primary metabolite of benzene, it does not induce cancer in rats as benzene *per se* does.[12–14] In accordance, benzene is classified as a human carcinogen (in group CARC1) while phenol is not.[15,16] Consequently, it can be hypothesised that there are additional pathways involved in the mechanism of toxification.

Peroxynitrite is a powerful reactive species which can be formed in any situation where reactive nitrogen species (RNS) and superoxide anion ($O_2^{\bullet -}$) are generated.[17] Furthermore, it has been reported that benzene exposure can stimulate the generation of $NO^{\bullet}$ and $O_2^{\bullet -}$ both *in vivo* and *in vitro* in the presence of inflammatory mediators.[18–20]

The purpose of this study was to investigate whether peroxynitrite can interact with benzene to form phenolic and nitro-containing products in both non-biological and biological systems.

MATERIALS AND METHODS

Chemicals

Benzene (Riedel-deHaen Co. Seelze, Germany. Purity: 99.7%, CAS No. 71-43-2), Lymphoprep™-1.077 (Nycomed Pharma AS Oslo, Norway), dextran wt: 500,000 (Pharmacia Fine Chemical Lot No: 11648), phorbol 12-myristate 13-acetate (PMA), phenol, *p*-nitrophenol, *o*-nitrophenol, *m*-nitrophenol, nitrobenzene were purchased from Sigma, St. Louis, MO.

Peroxynitrite Synthesis

Peroxynitrite was synthesised with a quench-flow-technique[21] with minor modification. Six ml of 0.6 M $NaNO_2$ (solution 1), 6 ml of 0.6 M H_2O_2 and 0.7 M HCl (solution 2) and 6 ml of 3 M NaOH (solution 3) were poured separately into 3 syringes and cooled down to 2°C. Solutions 1 and 2 were connected via a Y-piece from which 2 cm down another Y-piece was connected to solution 3. The solutions were simultaneously poured out in a beaker on ice within 30 s. The mixture was stirred for 10 min for cooling. Aliquots were stored in −20°C for later use. The concentration was quantified prior to use by measuring the absorbance at 302 nm, using a molar extinction coefficient of 1670 M^{-1} cm^{-1}.

Experiment in Chemical Reaction System

Benzene solutions were prepared in 100 mM KH_2PO_4 (pH 4.6). The different concentrations of peroxynitrite were obtained by spontaneous decomposition with variable storage time. One hundred µl of peroxynitrite solution was added directly on the surface of 5 ml of the benzene solution which was rapidly vortexed. Sixty-six µl of 1 M HCl was added later to keep consistency with the component and pH (3.1) in controls. The controls were designed by decomposing peroxynitrite first with 66 µl of 1 M HCl before adding it to the benzene solution. The reaction mixtures were scanned spectrophotometrically between 190 and 400 nm. The curve was compared with the relevant control's to find the new peaks. The quantity was initially evaluated by comparing

the shifts of peaks and the peaks of standards. The further identification and quantification were carried out by HPLC.

Experiment in Neutrophil Incubation System

Separation of human neutrophils was performed with the Ficoll-paque method.[22,23] In brief, 20 ml of heparized blood from healthy donors was carefully mixed with 13 ml of 2% T-500 dextran in saline. The solution was left at room temperature for 20 min to sediment red blood cells. The upper layer of the solution was then moved to another tube and 5 ml of Lymphoprep™-1.077 was layered at the bottom. After centrifugation for 30 min at 400*g*, the pellet containing neutrophils was collected. The cells were washed twice and diluted to around 2.5×10^7 cells/ml in PBS (10 mM sodium phosphate, 10 mM potassium chloride, 140 mM sodium chloride and 5 mM glucose, pH 7.3). PMA pretreatment was carried out twice in 5 min interval at a final concentration of 500 nM each time. Benzene was added to 2 ml of neutrophil suspension (final concentration of benzene: 10 mM) and incubated at 37°C for 25 min with or without PMA pretreatment. The whole mixture was centrifuged at 800*g* for 15 min. The benzene derivatives after the incubation in the supernatant were determined with HPLC as described.

HPLC Analysis

The following Merch-Hitachi instruments were used: AS-2000A autosampler, L-6200A intelligent pump and L-4000 UV detector with a model D-6000 chromatography data station software (Version 2). Twenty-five µl of the reaction mixtures was injected directly onto a reverse-phase Nucleoside C18 column (4.6 mm × 25 cm, 3 µM, Saülentechnik Knauer, Berlin, Germany). The mobile phase consisted of 50% methanol in water. The series of standards were made in mobile phase. The samples were eluted at a flow rate of 1 ml/min and the inlet pressure of the system was approximately 200 bar. The yields of the reaction were detected at an ultraviolet absorbance of 278 nm. The retention times of phenol, *p*-nitrophenol, *o*-nitrophenol, *m*-nitrophenol and nitrobenzene were around 4.6, 5.6, 7.8, 9.0 and 13.6 min respectively and minimum quantifiable concentrations were 0.88 µM for each of the five compounds.

The HPLC conditions for assaying the supernatants from experiments in the neutrophil incubation system were the same as above except for the change of mobile phase to 25% methanol. The retention times of phenol, *p*-nitrophenol, *o*-nitrophenol, *m*-nitrophenol and nitrobenzene were 10.3, 14.4, 17.8, 24.5 and 31.9 min respectively. The quantification was performed by comparing the area of the peaks with that of the relevant standards.

To verify the identification, the ratios of the shifts of the retention time of each peak were measured by changing the mobile phase and from analysis by HPLC with diode array detection.

The HPLC/diode array analysis was performed using a Hewlett Packard, *HP 1090* liquid chromatography system, with conditions as above including a mobile phase of 25% methanol. The temperature of the system was set to 40°C. Ultraviolet/visible spectra of eluting peaks were obtained by scanning from 210 to 400 nm.

RESULTS

By comparing the UV spectrum of reaction mixture with that of relevant controls, an increase at 269.9 nm in UV scanning was found in the mixture after the benzene–peroxynitrite reaction. The further identification by both HPLC (Figure 1) and HPLC/diode array (not shown) showed the presence of phenol, *p*-nitrophenol, *o*-nitrophenol, *m*-nitrophenol and nitrobenzene in the reaction mixture.

The dose–effect relationship of the generation of phenol and nitrated aromatic compounds (*p*-nitrophenol, *o*-nitrophenol, *m*-nitrophenol and nitrobenzene) from benzene with increasing amounts of peroxynitrite added appears

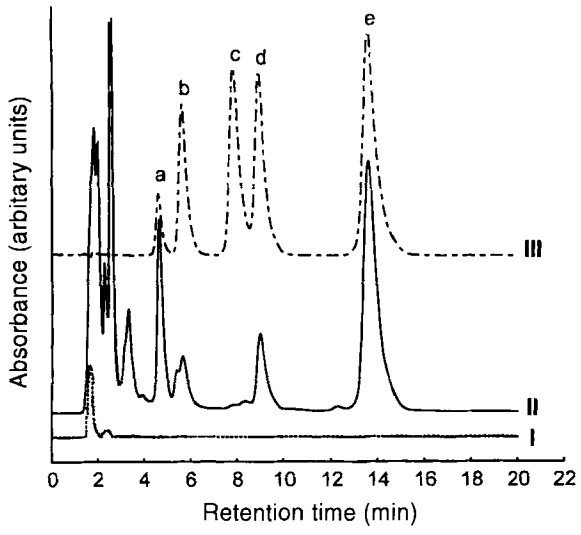

FIGURE 1 HPLC chromatograms (UV detector at 278 nm) from the reaction mixtures of benzene and peroxynitrite. (a) phenol; (b) *p*-nitrophenol; (c) *o*-nitrophenol; (d) *m*-nitrophenol; (e) nitrobenzene. I: Reaction mixture of 10 mM benzene with decomposed peroxynitrite. II: Reaction mixture of 10 mM benzene with 3 mM peroxynitrite. III: 50 μM of standards.

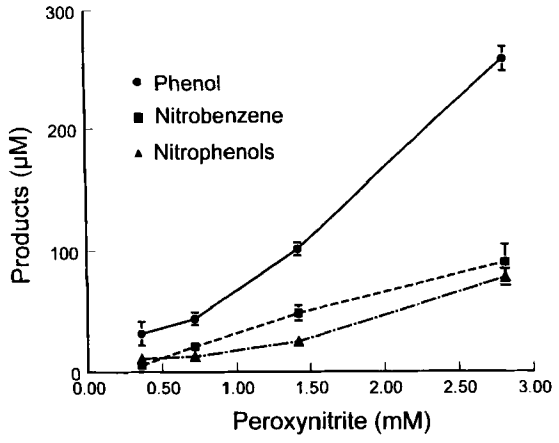

FIGURE 2 Benzene (10 mM) in 5 ml of 100 mM potassium phosphate buffer (pH 4.6) was exposed to 100 μl of peroxynitrite synthesised by the quenched reaction of nitrite with hydrogen peroxide. The series of peroxynitrite was obtained by spontaneous decomposition with variable storage time. The yields of phenol and nitrated compounds were detected by HPLC. The symbols and bars represent the means and SD from 5 experiments.

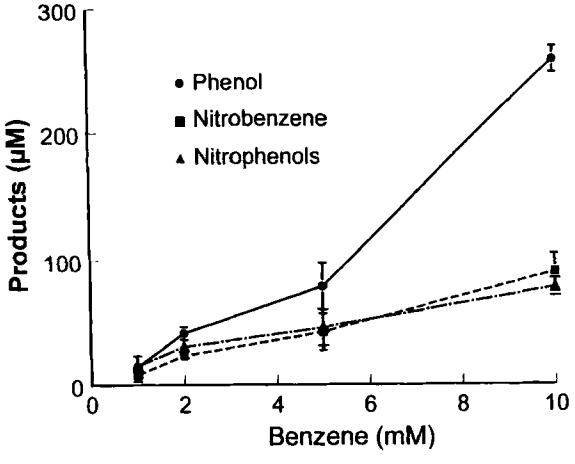

FIGURE 3 Different concentrations of benzene in 5 ml of 100 mM potassium phosphate buffer (pH 4.6) were exposed to the peroxynitrite (final concentration of peroxynitrite: 2.8 mM). The yields of phenol and nitrated compounds were detected by HPLC. The symbols and bars represent the means and SD from 5 experiments.

from Figure 2. In the range of experimental concentrations, the molar yield of phenol accounted for about 9% of the molar concentration of peroxynitrite, while that of nitrated aromatic compounds was about 5% of the molar concentration of peroxynitrite.

With a fixed concentration of 2.8 mM of peroxynitrite, the yield of phenol and nitrated aromatic compounds increased linearly with benzene increments in the reaction system (Figure 3).

As shown in Figure 4, there were detectable amounts of phenol and *p*-nitrophenol generated after incubation of 10 mM of benzene with neutrophils (2.5×10^7/ml) pretreated with PMA. The concentrations of phenol and *p*-nitrophenol were 1.29 ± 0.22 and 1.56 ± 0.61 μM (mean ± SD) from 3 experiments, respectively, whereas the samples without pretreatment of neutrophils with PMA did not yield detectable peaks.

The analysis of the neutrophil incubation medium with diode array detector showed that the patterns of phenol and *p*-nitrophenol peaks were the same as standards (not shown). The change of the HPLC mobile phase from 25% of methanol to 20% methanol resulted in the same ratio of peak shift in samples as in the standards (not shown).

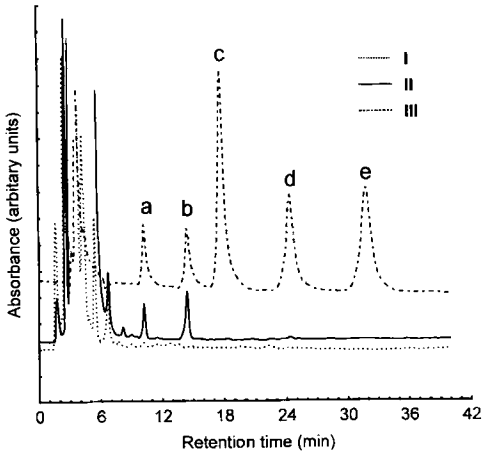

FIGURE 4 HPLC chromatograms from incubation supernatants of human neutrophil and benzene. I: supernatant from benzene and neutrophils incubation system without pretreatment by PMA. II: supernatant from benzene and neutrophils incubation system pretreated by PMA. III: 1.75 μM of standards: (a) phenol (10.3 min); (b) *p*-nitrophenol (14.4 min); (c) *o*-nitrophenol (17.8 min); (d) *m*-nitrophenol (24.5 min); (e) nitrobenzene (31.9 min).

DISCUSSION

This study indicated that hydroxylated and nitrated metabolites of benzene can be formed in reaction with synthesised peroxynitrite and in incubation with neutrophils activated by PMA that primes the cells to peroxynitrite production.[24]

It is clear that peroxynitrite played a main role in the hydroxylation and nitration of benzene in the pure chemical reaction experiments because no hydroxylated or nitrated compounds were found in the control with same amounts of residual H_2O_2 and nitrite, and the same final pH as in experimental samples. In the neutrophils incubation experiment, it is uncertain whether the hydroxylation and nitration of benzene were carried out only by peroxynitrite. The reactive intermediates from the reaction of nitrite with hypochlorous acid are also capable of nitrating the agents with aromatic compounds.[25,26] After stimulating neutrophils with PMA, induced myeloperoxidase is capable of aromatic hydroxylation of salicylate.[27,28] The same mechanism might be applied in the hydroxylation of benzene by stimulated neutrophils.

The molar yield of compounds with hydroxyl-groups was higher than compounds with nitro-groups both in the chemical reaction and neutrophils incubation experiments. It is speculated that the attack on the benzene ring by hydroxyl ($^{\bullet}OH$) is more effective than free radical nitrogen dioxide ($NO_2^{\bullet}$) during the decomposition of peroxynitrite. In the neutrophil incubation system, the different ratio of yields from that obtained in the chemical might be attributed to the presence of trapping agents such as heme with different affinity to the $^{\bullet}OH$ and $NO_2^{\bullet}$. Again, in neutrophil incubation experiment, only phenol and *p*-nitrophenol instead of all five products generated in chemical reaction experiment were identified. As for *o*-nitrophenol and *m*-nitrophenol, smaller amount than others was generated also in the chemical reaction system. The even less yield in the neutrophil incubation system might have been below the limit of quantitation. As for nitrobenzene, the higher organic solubility and volatility might cause difficulty for detection after stirring under 37°C for 25 min.

Previous studies have demonstrated that exposure to benzene together with inflammatory mediators can increase the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) including $O_2^{\bullet-}$ and $NO^{\bullet}$ from neutrophils and macrophages.[29–31] Redox cycling of certain metabolites such as hydroquinone-*p*-benzoquinone would also be likely to generate $O_2^{\bullet-}$,[32] which reacts with $NO_2^{\bullet}$ to form a more powerful oxidative species, peroxynitrite. The nitration and hydroxylation of aromatic rings by peroxynitrite have been proved by the interaction of peroxynitrite and the aromatic amino acid tyrosine, salicylate and phenylalanine.[33–36] In general, compounds containing the nitro-group have higher initial oxidation potentials and dipole moments (μ) than their non-nitro-containing counterparts and could be more mutagenic.[37–41] Although there is no report of

nitrated aromatic compounds *in vivo* related to benzene exposure so far, our study indicated that peroxynitrite can react with benzene to form nitrated and hydroxylated aromatic compounds which may cause toxic effects.

In summary, this study indicated that peroxynitrite can modify benzene to hydroxylated and nitrated compounds. These compounds are indeed formed by intact cells activated to peroxynitrite formation. We propose the peroxynitrite mediated hydroxylation and nitration of benzene to be associated with immune activation as a novel pathway for the *in vivo* generation of proximal carcinogens.

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