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Original Contribution

ENHANCED BENZENE-INDUCED DNA DAMAGE IN PMA-STIMULATED CELLS IN VITRO AND IN LPS-TREATED ANIMALS

JINGSHENG TUO,* STEFFEN LOFT,* and HENRIK E. POULSEN*†

*Department of Pharmacology, Panum Institute, University of Copenhagen, DK-2200, Copenhagen, Denmark; and †Department of Clinical Pharmacology Q7642, Rigshospitalet, 20 Tagensvej DK-2200 N, Copenhagen N, Denmark

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Abstract—The present study investigated the interaction between inflammatory reactions and benzene in vitro and in vivo with respect to oxidative DNA damage. In the in vitro models the oxidative burst of cells was induced by the pretreatment with phorbol myristate acetate (PMA) and in the in vivo models of inflammation mice were pretreated with lipopolysaccharide (LPS). The oxidative DNA damage was indicated by 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and strand breaks as assessed by alkaline single cell gel electrophoresis (SCGE, Comet assay). The results showed that combination of PMA and benzene enhanced the level of 8-oxodG in DNA from mouse bone marrow cells by 197%, from human lymphocytes by 188% and from human neutrophils by 205% ($p < .05$). Pretreatment of mice with LPS and benzene resulted in an enhanced Comet score formation in bone marrow cells by 98% and in lymphocytes by 39% in Comet score ($p < .05$) and in an enhanced 8-oxodG level in bone marrow cells by 290%. The effects of the combined treatment with PMA/LPS and benzene exceeded the sum of the effects induced by PMA/LPS or benzene alone. The production of nitrate/nitrite showed a two fold increase in the supernatant from incubation of benzene and PMA-pretreated neutrophils. The increase in the 8-oxodG level in the human neutrophil incubation system demonstrated a correlation with nitrate/nitrite production, indicating a possible relationship with the generation of reactive nitrogen species.

Keywords—Benzene, Inflammation, Lipopolysaccharide, Phorbol myristate acetate, 8-Oxo-7,8-dihydro-2′-deoxyguanosine, Comet assay, Free radical

INTRODUCTION

The toxic effects of benzene on the haemopoietic system have been well documented both in vivo and in vitro [1]. However, the underlying mechanism is still not fully known, although it is accepted that benzene metabolism is required for the toxicity. The most current theory considers cytochrome P4502E1 (CYP2E1) as the dominant metabolic route producing active compounds [2–5]. This pathway alone is insufficient to fully elucidate the mechanism due to the fact that the primary metabolites of benzene via CYP2E1 do not induce toxic reactions as benzene per se does [6–9].

Although a significant proportion of bioactivation of xenobiotics occurs via P450 enzymes, other cellular processes can lead to bioactivation [10,11]. Thus, the metabolic activation of xenobiotics can occur during the oxidative burst of polymorphonuclear leukocytes [12]. A 2-3-fold increase of generation of hydroxylated reactive intermediates after incubation of phorbol myristate acetate (PMA)-stimulated human leukocytes with benzo-[a]pyrene 7,8-dihydrodiol in the presence of nitrite implied the possibility for hydroxylation of aromatic rings in a non-CYP pathway [13].

Benzene metabolites can stimulate the generation of nitric oxide and superoxide anions both in vivo and in vitro [14–21]. Chronic inflammation is associated with the production of a number of factors including nitric oxide and superoxide, that can react to form peroxynitrite. The involvement of inflammation in benzene toxicity is supported by the blockade of myelotoxicity with nonsteroidal anti-inflammatory drugs [22].

Although peroxynitrite can damage DNA directly [23], recent data indicate that peroxynitrite can modify benzene non-enzymatically to hydroxylated as well as

Address correspondence to: Prof. Henrik E. Poulsen, Department of Clinical Pharmacology Q7642, Rigshospitalet, 20 Tagensvej DK-2200 N, Copenhagen N, Denmark; E-Mail: henrikep@rh.dk

nitrated aromatic compounds, including phenol, *p*-nitrophenol, *o*-nitrophenol, *m*-nitrophenol and nitrobenzene in a non-biological incubation system; phenol and *p*-nitrophenol were also generated by incubation of benzene with human neutrophil pretreated with PMA [24].

The present study investigated the interaction between the inflammatory reaction and benzene-induced oxidative DNA damage both in vitro and in vivo and the possible involvement of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the mechanism. DNA damage was measured by 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in mouse bone marrow cells, human neutrophils and lymphocytes in the in vitro experiment, and 8-oxodG in mouse bone marrow cell and DNA breaks in mouse bone marrow cells and lymphocytes in an in vivo experiment. The effect of exogenous phenol and *p*-nitrophenol, proposed as the metabolites of benzene generated from its interaction with ROS and RNS on the formation of 8-oxodG in neutrophils, was also studied.

MATERIALS AND METHOD

Chemicals

Benzen (BZ) (Riedel-deHaen Co. Seelze, Germany. purity: 99.7%, CAS No. 71-43-2), corn oil (Sigma, St. Louis, MO, USA), Lymphoprep™-1.077 (Nycomed pharma AS Oslo, Norway), Eagle medium (GibcoBRL, Scotland), Mg^{++} and Ca^{++} free PBS (0.1 M, PH 7.4) (GibcoBRL, Scotland), fully frosted microscope slides (Labcraft, Houston, TX, USA), dextran wt: 500,000 (Pharmacia Fine Chemical Lot No:11648) and YOYO-1 (Molecular Probes, Netherland), alkaline phosphatase (Bohringer Mannheim, Mannheim, Germany) were purchased from the sources indicated. Histopaque-1.083, agarose type 1-A and type VII, tritonX-100, phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS) from *escherichia coli serotype* 026:B6, 2-deoxyguanosine (dG), nuclease P1, 8-oxodG, phenol and *p*-nitrophenol were purchased from Sigma, St. Louis, MO. The nitrate/nitrite colorimetric assay kit was purchased from Alexis Corporation (Århus, Denmark) and 30 kDa mol.wt. cut-off filters were purchased from Whatman, Denmark. 2′-7′-dichlorofluorescin diacetate (DCFH-DA) and 2′-7′-dichlorofluorescin (DCF) was purchased from Molecular Probes, Eugene, USA.

In vitro study

Separation of cells. The bone marrow cells from normal mice were obtained by the same methods as in the in vivo study. The separation of human neutrophils and lymphocytes was performed with the Ficoll-paque method. Twenty ml of heparinized 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 mins to sediment red blood cell. The upper layer was then moved to another tube and 5 ml of lymphoprep™ was layered at the bottom. After centrifugation for 30 mins at 400g, the interface layer containing lymphocytes and the pellet containing neutrophils were collected. The cells were washed twice and diluted to 2.5×10^6 cells/ml in PBS (0.01 M sodium phosphate, 0.01 M potassium chloride, 0.14 M sodium chloride and 5 mM glucose, pH 7.3).

Cell incubation with benzene. Cells were pretreated with PMA (500 nM in final concentration) in 37°C. After 5 min, benzene (final concentration: 10 mM) was directly pipetted to 2 ml of cell suspension (2.5×10^6 cells/ml). After another 5 min, the second 500 nM PMA in final concentration was added to the incubation system. The incubation lasted for 25 mins after benzene addition. Vehicle control was carried out with an identical amount of DMSO. The whole suspension was then centrifuged for 15 mins at $800 \times g$ and the nitrate/nitrite in the supernatants of human neutrophil incubation system was determined spectrophotomically [25]. 8-OxodG in nuclear DNA from the cell pellet was quantitated by HPLC [26,27].

Assay for reactive oxygen species. Two ml of neutrophil suspension was preloaded with 10 μM DCFH-DA for 15 min. After centrifugation at $800 \times g$ for 10 min, the supernatant was discharged and the pellet was resuspended in PBS. PMA stimulation was performed as above. The fluorescence of unstimulated and stimulated neutrophils was recorded for 30 min on a Perkin-Elmer LS-5 luminescence spectrometer with excitation wavelength at 448 nm (band width 5 nm) and emission wavelength 526 nm (band width 20 nm) [28]. Phenol and *p*-nitrophenol (final concentration: 5 μM each) were added directly to 2 ml of human neutrophils suspension (2.5×10^6 cells/ml in PBS) in 37°C for 25 min. The whole suspension was then centrifuged for 15 min at $800 \times g$ and 8-oxodG in the nuclear DNA was analysed as above.

In vivo studies

Male NMRI mice (20–22 g b.wt.) from the animal center, Panum Institute, Copenhagen, were housed in an environmentally controlled facility operating on a 12 h light/dark cycle at 22–24°C with free access to a standard diet and tap water. The vehicle + agents volumes given were kept constant at 10–15 μl/g BW for gavage and IP administrations.

Thirty-two mice were randomly assigned to 4 groups of 8 animals. Two groups were pretreated with LPS, 5 mg/kg in saline IP. Fifteen min later, one of the pretreated groups received 200 mg/kg of benzene in corn oil by gavage whereas the other group received coin oil by gavage. The other two groups served as the vehicle control (saline IP and coin oil by gavage) and benzene alone treatment (200 mg/kg benzene by gavage plus saline IP), respectively. Six hours after benzene or corn oil administration, blood samples were collected from the orbital vessels. Lymphocytes were separated for the Comet assay as described previously [2] . The animals were killed and bone marrow was collected from femurs. Due to insufficient bone marrow material for both Comet assay and 8-oxodG determination, an additional experiment was carried out for determination of 8-oxodG in the bone marrow after the four treatments.

The epiphysial plates of the femurs were removed. The bone marrow was flushed out with 1 ml of cold Eagle medium supplemented with 10 units/ml heparin. An aliquot of 100 μl was mixed with 1 ml Eagle medium. Two hundred μl Histopaque-1083 was underlaid. The samples were centrifugated at 200 × *g*, 3 min, 4°C. The nucleated cells were collected at the interface and washed twice with 1 ml of Eagle medium. The cell density was adjusted to about 10^5 per 1 ml for the Comet assay.

Analyses

The Comet assay was conducted as described elsewhere with a modification using 85 μl of 0.5 μM YOYO-1 in PBS (0.1 M, pH 7.4) as the DNA stain [29,30]. DNA breaks were evaluated with an Olympus Imagine Analysis System after coding the slides. One hundred randomly selected cells (images) were classified visually as belonging to one of five classes of damage according to the ratio of the head and the tail of the Comet, from score 1 (no visible tail, i.e., zero detectable damage) to score 5 (very small head in Comet, most of DNA in tail), and given a score of 1, 2, 3, 4, or 5, respectively [31]. The evaluation was processed with standard images with 1-5 cells on the screen (Fig. 1). The results were presented by the average distribution of the Comet classes and as the total score for each slide calculated as:

$$\text{Total score} = (\text{number of cells with score1} \times 1 + \text{number of cells with score 2} \times 2 + \text{number of cells with score 5} \times 5).$$

Thus, the score will range from 100 to 500 for each slide.

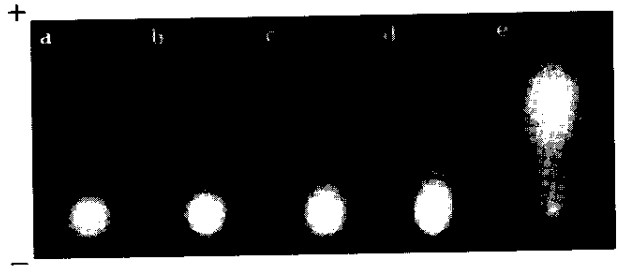

Fig. 1. Representative of Comet images; a) class 1, score 1; b) class 2, score 2; c) class 3, score 3; d) class 4, score 4; e) class 5, score 5; + −: directions of electrophoresis.

The level of nuclear 8-oxodG was analysed as previously described [26,27]. The nitrate/nitrite determination was based on the Griess reaction using a commercial kit [25]. Plasma was filtered through a 30 kDa molecular weight cut-off filter. Fifty μl ultrafiltrate of plasma was used in the assay.

Statistics

Groups were compared by means of one-way ANOVA. Duncan's multiple range test was used for post hoc comparison of means. Linear regression was done by the least square method. Differences were considered significant when $p < .05$.

RESULTS

After in vitro incubation with benzene, the 8-oxodG level was not significantly changed, although it was 37% and 59% higher ($p > .05$) in mouse bone marrow cells and human lymphocytes and unchanged in human neutrophils, respectively (Table 1). After pretreatment with PMA the 8-oxodG levels were non-significantly higher in mouse bone marrow cells (63%) and in human lymphocytes (63%) whereas the increase by 66% was significant in human neutrophils ($p < .05$). The combination of pretreatment with PMA and incubation with benzene increased 8-oxodG by 197%, 188% and 205% in mouse bone marrow cells, human lymphocytes and neutrophils respectively (all p values $< .05$ vs. all the other groups) (Table 1). The effects of the combined treatment with PMA and benzene exceeded the sum of the effects induced by PMA or benzene alone.

PMA-stimulated neutrophils had a higher ROS generation as measured by oxidised DCFH (DCF formation 50.7 ± 15.8 pmol/10^6 cells/min, $n = 4$) compared with unstimulated cells (DCF formation 19.4 ± 1.2 pmol/10^6 cells/min, $n = 4$) ($p < .01$).

In the neutrophil incubation system benzene did not change the nitrate/nitrite level in the supernatant. PMA

Table 1.

	n	Control	BZ	PMA	PMA + BZ
8-oxodG/10^5dG					
BM cell	5	0.62 ± 0.13	0.85 ± 0.17	1.02 ± 0.20	1.84 ± 0.90[a]
Lymphocytes	5	0.70 ± 0.11	1.12 ± 0.22	1.15 ± 0.64	2.03 ± 0.83[a]
Neutrophil	5	1.21 ± 0.56	1.11 ± 0.15	2.00 ± 0.55[b]	3.68 ± 0.75[a]
Nitrate/nitrite (nM/10^6 cells)					
Supernatant from neutrophil incubation	4	44.0 ± 10.9	35.0 ± 19.2	78.5 ± 14.4	99.9 ± 39.4[a]

The nuclear (8-oxodG (8-oxodG/10^5dG) from mouse bone marrow (BM), human lymphocytes and neutrophils after incubation with benzene (BZ), with or without PMA. The nitrate/nitrite level in supernatant from neutrophil incubation. The nuclear 8-oxodG (8-oxodG/10^5dG) from mouse bone marrow, human lymphocytes and neutrophils after incubation with benzene (BZ), with or without PMA. The values are given as mean ± SD.

[a] $p < .05$ in comparison with the control, benzene (BZ) and phorbol acetate (PMA) groups.

[b] $p < .05$ in comparison with the Control and BZ group.

pretreatment induced a slight increase in the nitrate/nitrite level ($p > .05$). Incubation with benzene after pretreatment with PMA caused a two-fold increase in the nitrate/nitrite in the supernatant of neutrophil incubation (Table 1). The nitrate/nitrite in the supernatant and 8-oxodG level in the DNA of the neutrophils showed a positive linear correlation ($r = .7182, p < .05$); (Fig. 2).

The incubation of neutrophils with phenol and *p*-nitrophenol did not result in significant changes in the 8-oxodG level in the nuclear DNA. The values (8-oxodG/10^5dG, mean ± SD) were 1.12 ± 0.12 in control, 0.92 ± 0.60 in phenol, 1.09 ± 0.31 in *p*-nitrophenol and 1.11 ± 0.45 in phenol plus *p*-nitrophenol treated cells (all *p* values > .05).

Pretreatment of mice with LPS resulted in a slight increase in the number of cells with higher Comet classification (more DNA breaks) both in the bone marrow cells and lymphocytes. Treatment with benzene alone also slightly shifted the distribution pattern in the same direction in the two cell types. Pretreatment with both

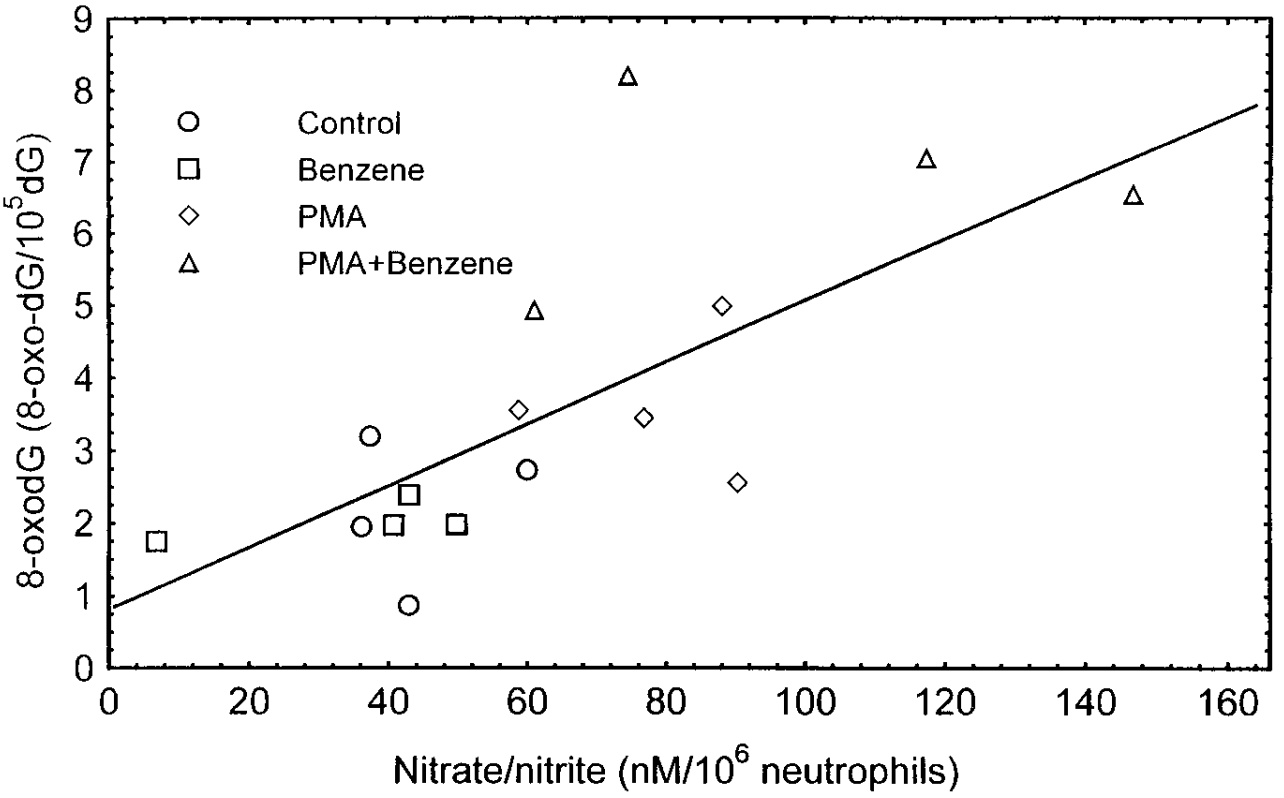

Fig. 2. Correlation between nitrate/nitrite in the supernatant of incubation and nuclear 8-oxodG level of phorbol myristate acetate (PMA) pretreated neutrophils incubated with benzene; $r = 0.7182, p < .05$.

Table 2. The Effect of Lipopolysaccharide (LPS) on the Formation of DNA Breaks Induced by Benzene (BZ) in Mice

	Lymphocytes				Bone Marrow Nucleated Cells			
	Control	BZ	LPS	LPS + BZ	Control	BZ	LPS	LPS + BZ
Class 1	92.2 ± 8.6	90.5 ± 1.7	84.0 ± 2.6	71.5 ± 3.9	89.4 ± 6.1	85.2 ± 2.8	67.2 ± 13.5	45.1 ± 8.0
Class 2	4.4 ± 2.2	4.0 ± 0.8	7.0 ± 2.0	11.8 ± 3.1	6.6 ± 5.3	6.7 ± 3.3	16.2 ± 7.6	18.1 ± 7.9
Class 3	1.9 ± 2.1	2.8 ± 0.5	3.6 ± 0.6	6.5 ± 3.1	1.3 ± 1.8	4.1 ± 2.7	5.8 ± 4.6	12.6 ± 5.8
Class 4	0.7 ± 0.4	1.0 ± 0.8	2.7 ± 0.6	4.5 ± 2.7	1.4 ± 1.1	2.3 ± 1.2	4.2 ± 3.2	10.1 ± 4.3
Class 5	0.7 ± 0.7	1.8 ± 1.5	2.7 ± 1.2	5.0 ± 2.4	0.8 ± 0.6	1.7 ± 0.5	5.0 ± 3.7	14.0 ± 7.7
Comet scores	113.2 ± 9.5	133.0 ± 9.8[a]	119.5 ± 15.3	157.7 ± 13.9[a]	116.1 ± 12.8	158.7 ± 25.1[a]	128.7 ± 9.1	229.7 ± 26.9[a]

DNA breaks were evaluated with the Comet assay by recording the distribution in 5 Comet classes among 100 cells from each sample. The total score was obtained by summing the different class numbers. The data are the average distribution of 8 samples and SD from 8 mice/group.
[a] $p < .01$ in comparison with the other three treatments.

LPS and benzene induced a considerable increase in the number of cells with higher Comet classification in the two cell types (Table 2). If evaluating the results from the Comet assay by the total score based on each slide, the scores were 11% and 6% higher in LPS treated mice in bone marrow cells and lymphocytes respectively ($p > .05$); (Table 2). The treatments with benzene increased the score by 37% in bone marrow cells and 17% in lymphocytes ($p < .05$), whereas after the treatment with both LPS and benzene the scores were enhanced by 98% in bone marrow cells and 39% in lymphocytes ($p < .05$); (Table. 2). BZ treatment enhanced the formation of the nuclear 8-oxodG in the bone marrow cells by 180% in comparison with the control group ($p < .05$). LPS treatment by itself tended to increase the 8-oxodG level but not significantly ($p > .05$). In contrast, LPS + BZ increased the 8-oxodG level by 290% as compared with the control ($p < .05$). (Fig. 3). The effects from the

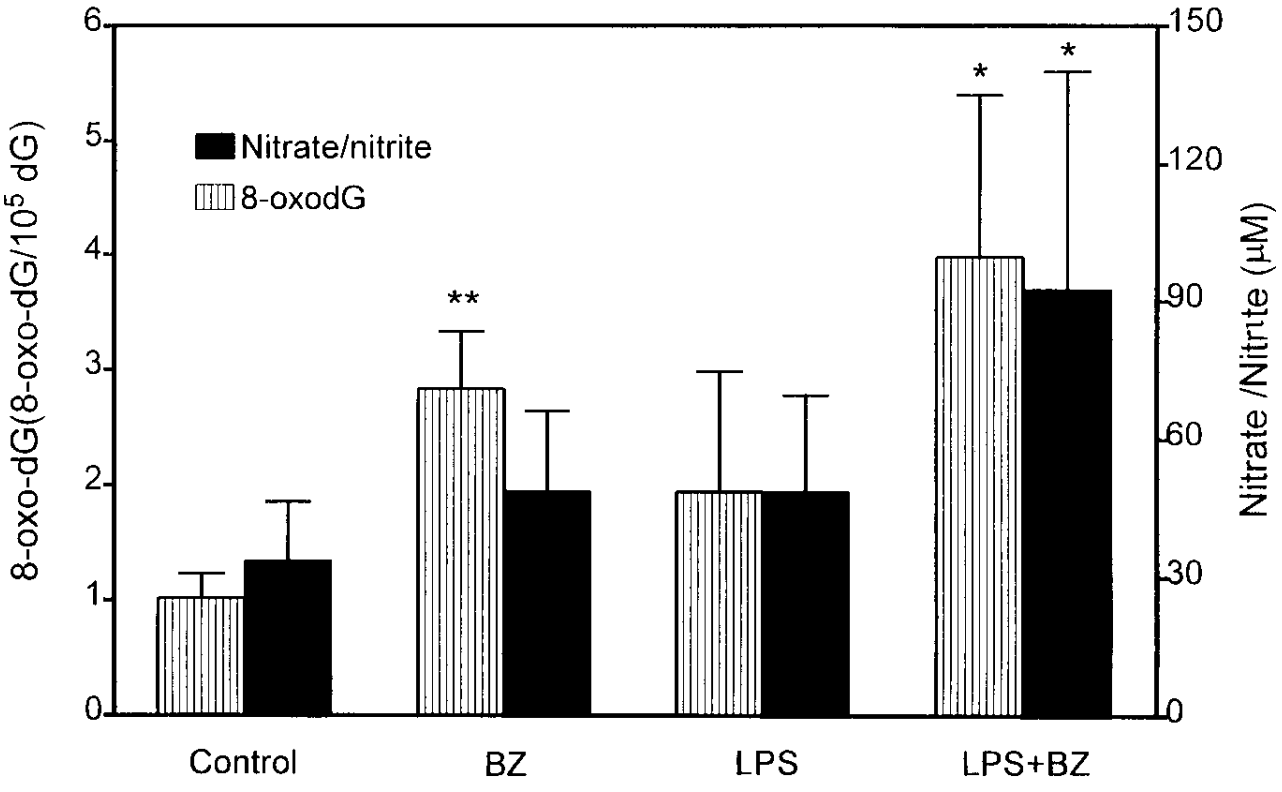

Fig. 3. The nuclear 8-oxodG from mouse bone marrow cells and nitrate/nitrite levels in serum after in vivo treatment of BZ, LPS and their combinations. The columns and bars of 8-oxodG indicate the means and SD from 6 samples in LPS group, 5 samples in control and BZ groups and 4 samples in control groups. The columns and bars of nitrate/nitrite level indicate means and SD of 5 samples from LPS+DEX group, 6 samples from control, LPS group and BZ group and 7 samples from the other groups. *$p < .05$ in comparison with all other group. **$p < .05$ in comparison with control.

combined treatment with LPS and benzene exceeded the sum of the effects (8-oxodG) induced by LPS or benzene alone.

DISCUSSION

This study demonstrated that benzene exposure to PMA-stimulated cells and LPS-treated mice resulted in substantial oxidative damage to their DNA in terms of strand breaks and 8-oxodG formation. In fact, the effect of the combined treatment with PMA/LPS and benzene exceeded the sum of the effects. Moreover, the 8-oxodG level correlated with the nitrate/nitrite formation in the neutrophil incubation system, indicating a possible relationship with the formation of RNS.

We have previously demonstrated that benzene administration leads to increased formation of DNA breaks in lymphocytes and bone marrow cells in mice in agreement with earlier observations [30,32]. Benzene exposure alone in vivo can induce the formation of 8-oxodG and hydroxylated benzene metabolites produced oxidative DNA damage in HL-60 cells [33]. Exposure of human neutrophils to PMA can result in formation of DNA strand breaks and nuclear 8-oxodG [34,35]. LPS-induced ROS release and DNA breaks have also been reported [36–38].

In the present study, we pretreated human peripheral blood cells and mouse bone marrow cells with PMA to mimic an inflammatory state in vitro. Increased generation of ROS from neutrophils indicated by DCF formation from DCFH confirmed the efficacy of the model under our experimental condition. Benzene exposure to such cells, particularly human neutrophils, lead to a considerably increased formation of 8-oxodG, indicating increased oxidative stress. To further test a similar situation in vivo using the Comet assay and 8-oxodG to estimate DNA modification, we stimulated mice with LPS, known to result in an increased oxidative burst in neutrophils [39]. In this situation we found that benzene increased the formation of DNA breaks in mouse lymphocytes and bone marrow cells and 8-oxodG formation in bone marrow cells, and that the LPS pretreatment with subsequent benzene exposure increased the DNA breaks and 8-oxodG level considerably.

ROS can hydroxylate deoxyguanosine residues in DNA resulting in the formation of 8-oxodG. Evidence of 8-oxodG in tissues has been suggested important pathogenetic events in carcinogenesis and tumour promotion [40]. The Comet assay is a promising tool to study chemical-induced DNA damage. Although there is no convincing evidence for the relation of Comet formation and carcinogenesis, a correlation has been reported between the Comet formation and the 8-oxodG level or the mutation frequency during some carcinogen exposures [27,41]. Increased 8-oxodG after treatment of neutrophils with PMA and enhanced Comet scores and 8-oxodG after administration of benzene to mice in our study are in agreement with those studies. Furthermore, our data also showed that co-administration of benzene with PMA in vitro or with LPS in vivo lead to an enhanced effect on both the formation of DNA breaks and 8-oxodG.

The mechanism of the combined effect of benzene and PMA/LPS is still unknown. One hypothesis might be bioactivation of benzene by peroxynitrite and other forms of ROS/RNS formed during the burst of inflammatory cells. It is known that there are many pathways to trigger the generation of ROS/RNS by benzene or PMA/LPS. Benzene has been shown in vitro to activate protein kinase C [42]. PKC activation is involved in the PMA/LPS-stimulated oxidative burst in neutrophils, lymphocyte and macrophage from bone marrow cells [43–46], that could be the initial signal pathway and then lead to the formation of ROS/RNS by up-regulating NADPH oxidase and nitric oxide synthase [44,46,47]. The higher 8-oxodG levels in the incubation system with human neutrophils than in the bone marrow cells is consistent with the fact that human leukocytes have a stronger potential to produce reactive species than the cells from mice [48]. The interaction of the ROS/RNS with their stimulator, e.g., benzene, could probably further activate benzene and contribute to DNA damage. The previous study in our laboratory demonstrated that peroxynitrite interacts with benzene to form phenol, nitrophenols and nitrobenzene [24]. The similar findings were reported in the nitration and hydroxylation of aromatic ring in tyrosine, salicylate and phenylalanine [49–52]. In general, compounds containing nitro-group have higher initial oxidation potentials and dipole moments(mu) than their non-nitro-containing counterparts [53–55], that means that the compounds with nitro-group could show higher mutagenic activity than their non-nitro-containing counterparts. The unchanged 8-oxodG level after the exogenous addition of phenol and nitrophenol in the neutrophils incubation system seems to be in disagreement with our hypothesis. The further investigation is needed to test the toxicological significance of this interaction by elucidating the possible difference of the fate between the exogenously added metabolites and the endogenously formed ones.

We further estimated the nitrate/nitrite concentration in the medium of the neutrophils incubation. The results showed a convincing correlation of nitrate/nitrite with 8-oxodG levels. This implies that such challenges, especially the combination of PMA and benzene, to cells respond with an increased production of RNS. This could parallel the formation of oxygen radicals that are capable of modifying DNA. However, it could also induce per-

oxynitrite-mediated DNA modification that due to spontaneous rearrangement could contribute to the formation of 8-oxodG [56].

In summary, an inflammatory state both in cell incubation systems (mouse bone marrow cells, human lymphocytes and neutrophils) stimulated by PMA and in mice treated by LPS enhanced the benzene-induced DNA damage showed by the Comet assay and 8-oxodG formation. The increase of 8-oxodG in human neutrophil incubation system demonstrated a correlation with nitrate/nitrite production, indicating a possible relationship with the generation of RNS.

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ABBREVIATIONS

BZ—benzene
LPS—lipopolysaccharide
PMA—phorbol myristate acetate
8-oxodG—8-oxo-7,8-dihydro-2′-deoxyguanosine
SCGE—single cell gel electrophoresis
DCFH-DA—2′-7′-dichlorofluorescin diacetate
DCF—2′-7′-dichlorofluorescin