The benzene metabolite para-benzoquinone is genotoxic in human, phorbol-12-acetate-13-myristate induced, peripheral blood mononuclear cells at low concentrations

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Abstract Benzene is one of the most prominent occupational and environmental pollutants. The substance is a proven human carcinogen that induces hematologic malignancies in humans, probably at even low doses. Yet knowledge of the mechanisms leading to benzene-induced carcinogenesis is still incomplete. Benzene itself is not genotoxic. The generation of carcinogenic metabolites involves the production of oxidized intermediates such as catechol, hydroquinone and para-benzoquinone (p-BQ) in the liver. Further activation to the ultimate carcinogenic intermediates is most probably catalyzed by myeloperoxidase (MPO). Yet the products of the MPO pathway have not been identified. If an oxidized benzene metabolite such as p-BQ was actually the precursor for the ultimate carcinogenic benzene metabolite and further activation proceeds via MPO mediated reactions, it should be possible to activate p-BQ to a genotoxic compound in vitro. We tested this hypothesis with phorbol-12-acetate-13-myristate (PMA) activated peripheral blood cells exposed to p-BQ, using the cytokinesis-block micronucleus test. Addition of 20–28 ng/ml PMA caused a significant increase of micronuclei at low and non-cytotoxic p-BQ concentrations between 0.04 and 0.2 μg/ml (0.37–1.85 μM). Thus with PMA or p-BQ alone no reproducible elevation of micronuclei was seen up to toxic concentrations. PMA and p-BQ induce micronuclei when administered jointly. Our results add further support to the hypothesis that MPO is a key enzyme in the activation of benzene.

Keywords Benzene · para-Benzoquinone · Phorbol-12-acetate-13-myristate

Introduction

Benzene [CAS 71-43-2] is widely used as a solvent and an intermediate in chemical syntheses. In addition, occupational exposure occurs in oil refineries and coke oven plants. Environmental exposure is nearly exclusively caused by benzene contained in gasoline. Benzene is a proven human carcinogen probably even at low doses. Particular concern has been aroused by two independent studies that showed a possible contribution of environmental benzene exposure to childhood leukemia (Crosignani et al. 2004; Steffen et al. 2004). Furthermore, acute hematotoxicity in adults can occur at atmospheric concentrations below 1 ppm benzene (Lan et al. 2004). Despite its outstanding importance as an ubiquitous, highly toxic...
pollutant, knowledge of the mechanisms of benzene toxicity is still incomplete. Although a variety of effects on bone marrow can be demonstrated it is not yet clear that which metabolites are most important in either benzene-induced aplastic anemia or leukemia. Benzene metabolism alone is insufficient to fully describe benzene toxicity. The impact of benzene metabolites on bone marrow cells must be fully explored to determine how benzene exposure can result in decreased viability or genetic toxicity to cells in the bone marrow (Snyder 2007). Moreover, the reason for the observed species differences in benzene toxicity between experimental animals and humans is unclear: whereas hematopoietic malignancies occur in humans, benzene produces a variety of tumors in rats and mice (Maltoni et al. 1985; National Toxicology Program 1986). Furthermore, it is disputed if benzene induces only leukemia in humans (Lamm et al. 2005; Seidler et al. 2007) or a broader spectrum of hematologic malignancies (Hayes et al. 1997; Mehlman 2004; Smith et al. 2007).

The answers for these open questions are strongly hampered by the complex and not yet fully clarified metabolism of benzene. Initially, benzene is oxidized to metabolites such as 1,2-benzenediol [CAS No. 120-80-9] (catechol), hydroquinone [CAS 123-31-9] and p-BQ [CAS 106-51-4] in the liver, primarily by cytochrome P450 2E1. Since benzene does not induce liver tumors and human exposure is predominantly associated with disorders of the hematopoietic system, it was concluded that myeloperoxidase (MPO) mediated reactions are involved in the generation of the ultimate carcinogenic metabolites (Hayes et al. 1997; Smith et al. 2007; Environmental Protection Agency USA 1998). Actually, benzene causes malignancies in experimental animals preferentially in peroxidase rich tissues (Maltoni et al. 1985; National Toxicology Program 1986).

Benzene-induced oxidative DNA damage is enhanced in PMA-stimulated cells in vitro and in LPS-treated animals (Tuo et al. 1999). Consequently it was proposed that “peroxidative metabolism of benzene’s phenolic metabolites may be responsible for the increased free radical activity and toxicity produced by benzene in bone marrow, and that free radicals contribute, at least in part, to the toxic and leukemogenic effects of benzene” (Subrahmanym et al. 1991). However, direct evidence for MPO mediated generation of genotoxic metabolites does not exist and benzene adducts which were detected in humans and experimental animals could not be identified (Bechtold et al. 1992; Krewet et al. 1993; McDonald et al. 1994; Creek et al. 1997).

Human MPO is expressed at high levels in the bone marrow and in peripheral blood cells such as neutrophils and monocytes. The enzyme is stored in “large dense azurophil granules” (Bainton et al. 1971) and is released during host defence and inflammatory reactions, parallel to the generation of H$_2$O$_2$ and HOCI. MPO activation can be experimentally induced by PMA (phorbol-12-acetate-13-myristate) or LPS (bacterial lipopolysaccharide). MPO is proposed to play a major role in the generation of tissue damage caused by chronic inflammation (Winterbourn 2002). Reactions accompanying MPO activation are suggested to take part in the toxification of oxidizable substances (Winterbourn 2002; Trush 1984; Eastmond and Smith 1990) or may act as co-factor in chemical carcinogenesis (Cousens and Werb 2002).

Benzene is one of the few strong carcinogens that yield negative results in in vitro mutagenicity tests (Zarani et al. 1999), whereas genotoxic effects are observed in vivo (Tweats et al. 2007). In addition, genotoxicity of benzene metabolites such as hydroquinone, catechol and p-BQ is absent or weak at toxic concentrations (Ludewig et al. 1989; Yager et al. 1990; Léavy and Bodell 1992). This most probably reflects the inability of in vitro systems to model a complex metabolism and interactions of different metabolizing compartments.

However, if an intermediate such as p-BQ is actually the precursor for the ultimate carcinogenic benzene metabolite and further activation proceeds via MPO mediated reactions, it should be possible to activate p-BQ to a genotoxic compound in vitro. Since benzene exposure leads to clastogenic effects (Smith et al. 2007; Tough et al. 1970; Forni 1971; Picciano 1979; Popp et al. 1992; Tompa et al. 1994; Rothman et al. 1995, 1996; Smith et al. 1998; Zhang et al. 1998, 2005), micronuclei should be suitable endpoints to monitor such effects.

Treatment of HL-60 cells with 3 μM p-BQ caused a threefold reactive oxygen species (ROS) production, cell proliferation and prolonged phosphorylation of ERK1/2 proteins. Catalase or the MEK protein inhibitor PD98059 reduced cell recruitment into the S-phase and ERK1/2 protein phosphorylation. Thus, p-BQ is able to induce ROS and cell proliferation. This would theoretically enhance the vulnerability of the exposed cells (Ruiz-Ramos et al. 2005).

Therefore, we proposed that the combination of p-BQ and PMA might be especially effective to investigate p-BQ activation by PMA and investigated if p-BQ can be activated by MPO mediated reactions by using the cytokinesis-block micronucleus technique and PMA activated peripheral human blood mononuclear cells.

**Materials and methods**

The study was approved by the Ethics committee of the Medical Faculty of the Georg-August-University, Göttingen. Written informed consent was obtained from all participants.
Micronucleus test

The cytokinesis-block micronucleus assay was carried out according to Fenech (1993) using primary human lymphocytes from three different donors (donor 1 was a 45-year-old male smoker; donor 2 a 47-year-old female non-smoker and donor 3 a 38-year-old male non-smoker). Blood was collected by venipuncture in Sodium-heparin tubes (Sarstedt AG & Co, Nümbrecht, Germany), diluted 1:1 in phosphate buffered saline (PBS) and separated by a FICOLL gradient (Biochrom KG, Berlin, Germany) for isolation of lymphocytes. Approximately $1.2 \times 10^6$ cells were seeded in 2.5 ml RPMI medium each (ICN, Eschwege, Germany) supplemented with 5% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). Lymphocytes were stimulated by adding 5 $\mu$g/ml phytohemagglutinin (Biochrom KG, Berlin, Germany) and cultivated at 37°C, 100% humidity and 5% CO$_2$ for 70 h. PMA and p-BQ were added simultaneously 24 after having started the cultures. Cell division was blocked 44 h after starting the cultures by adding 4.5 $\mu$g/ml cytochalasin-B (Sigma-Aldrich, Steinheim, Germany).

Without addition of PMA, up to 5 $\mu$g/ml p-BQ (minimum 97% purity, Sigma-Aldrich, Steinheim, Germany) were applied to the cultures (2.5 ml). Co-incubation with PMA was initially done using 24–80 ng PMA and up to 1 $\mu$g/ml p-BQ. PMA was processed as follows: PMA was dissolved in DMSO (1 mg/ml) and stored in 10 $\mu$l aliquots at $-18^\circ$C. Prior to the addition to the cultures PMA was mixed with 990 $\mu$l medium. Mitomycin C (Sigma-Aldrich) served as positive control. One sample without addition of p-BQ and PMA served as negative control as did another sample with addition of PMA only.

Lymphocytes were sampled 68 h after having started the cultures and 28 h after addition of cytochalasin-B by centrifugation for 5 min at 175 x g and 20°C. Hypotonic treatment was performed by careful resuspension of the cells in 5 ml hypotonic saline (0.07 M KCl/0.15 M NaCl). Cells were collected for 5 min at 175 x g and 20°C immediately after the addition of hypotonic solution. The cell pellet was resuspended in 3 ml ice cold methanol/acetic acid (4:1). Cell fixation was performed twice. Finally the cells were pelleted for 5 min at 4°C and 175 x g and transferred onto ice cold degreased slides. The slides were randomized and subsequently stained with 3% Giemsa R66 ‘Gurr’ (BDH, Promochem GmbH, Wesel, Germany) for 7 min.

Six independent experiments were performed for donor 1 and four experiments each for donors 2 and 3. For each experiment blood was freshly collected and each single experiment was done on a different date. At least five concentrations were examined. Cell viability was determined by trypan blue exclusion test (Merck, Darmstadt, Germany) and additionally by evaluation of the nuclear division index (NDI, Eastmond and Tucker 1989). Cell scoring was done according to the criteria outlined by Fenech (1993). Binucleated cells and cells not containing more than four micronuclei with preserved cytoplasm were counted.

Additional acceptance criteria for a genotoxic effect were: positive- and solvent control within the historical range, significantly elevated frequency of micronuclei exceeding the respective control (0 $\mu$g/ml p-BQ with or without PMA) at non-toxic concentrations and unchanged pH. More than one succeeding concentration yielding total cell counts below 1,000 binucleated cells, a NDI < 1.4 and viability below 60% were regarded as toxic.

Statistics

The micronucleus rates of selected experiments were compared to the rates of negative controls with PMA and without p-BQ, and additionally to the rates of negative controls without PMA and without p-BQ using Fisher’s exact test. The Cochrane-Armitage test was applied to selected dose samples to test positive trends. SAS 9.2 software was used for all calculations (SAS Institute Inc, Cary, NC).

Results

The concentration range for the tests was initially determined using between 24 and 80 ng/ml PMA in combination with up to 8.0 $\mu$g/ml p-BQ. At and above 32 ng/ml PMA (70 ng/2.5 ml medium) toxicity and strong variations of the effects occurred (data not shown). In single concentration range finding experiments PMA concentrations at and above 32 ng/ml caused a significant increase of micronuclei (example shown in Table 1, experiment 2). However, these effects were poorly reproducible and most probably attributable to toxicity.

In some concentration range finding experiments significantly elevated micronuclei counts occurred with addition of p-BQ alone at and above 4 $\mu$g/ml (example shown in Table 1, experiment 11). Yet these effects were as well poorly reproducible and attributable to toxicity.

In single concentration range finding experiments in which PMA concentrations at and above 32 ng/ml were used, elevation of micronuclei showed a bimodal distribution. This appeared without noticeable effects on the NDI. In case of bimodal distribution of elevated micronuclei, the second elevation occurred at about 60% cytotoxicity (example shown in Table 1, experiments 1 and 2). In another set of experiments weak PMA genotoxicity occurred and the combination with p-BQ yielded a strong synergistic elevation of micronuclei.

With the aim to improve reproducibility, we performed a more detailed analysis of the p-BQ concentration range...
### Table 1 Toxic effects of *para*-benzoquinone in the in vitro micronucleus test

| Exp. no. | PMA (ng/ml) | *p*-BQ (µg/ml) | NDI | Viability (%) | MN | *P* | **P** |
|----------|-------------|----------------|-----|--------------|----|-----|-------|
| **Donor 1** |
| 1        | 0           | 0              | 1.53| 98           | 2  |     |       |
|          | 0           | 2.5            | 1.41| 40           | 2  |     |       |
|          | 0           | 5              | 1.21| 40           | Tox|     |       |
|          | 32          | 0              | 1.62| 80           | 1  |     |       |
|          | 32          | 0.05           | NE  | 80           | NE |     |       |
|          | 32          | 0.2            | 1.62| 80           | 9  | 0.0212 |       |
|          | 32          | 0.3            | 1.76| 60           | 2  |     |       |
|          | 32          | 0.4            | 1.49| 60           | 12 | 0.0033 | 0.0126 |
|          | 32          | 0.5            | 1.67| 60           | 1  |     |       |
| 2        | 0           | 0              | 1.75| 90           | 1  |     |       |
|          | 0           | 2              | 1.59| 80           | 1  |     |       |
|          | 0           | 4              | 1.45| 40           | 1  |     |       |
|          | 32          | 0              | 1.62| 80           | 18 | <0.0001 |       |
|          | 32          | 0.04           | 1.63| 80           | 9  | 0.0212 |       |
|          | 32          | 0.08           | 1.56| 60           | 30 | <0.0001 |       |
|          | 32          | 0.12           | 1.45| 60           | 28 | <0.0001 |       |
|          | 32          | 0.16           | 1.64| 60           | 12 | 0.0033 |       |
|          | 32          | 0.20           | 1.55| 60           | 24 | <0.0001 |       |
| 3        | 0           | 0              | 1.79| 90           | 1  |     |       |
|          | 0           | 2              | 1.63| 90           | 1  |     |       |
|          | 0           | 4              | Tox | 60           | Tox|     |       |
|          | 32          | 0              | 1.55| 90           | 1  |     |       |
|          | 32          | 0.04           | 1.57| 90           | 2  |     |       |
|          | 32          | 0.08           | 1.55| 80           | 1  |     |       |
|          | 32          | 0.12           | 1.48| 80           | 14 | 0.0009 | 0.0009 |
|          | 32          | 0.16           | 1.60| 70           | 6  |     |       |
|          | 32          | 0.20           | NE  | 70           | NE |     |       |
| 4        | 0           | 0              | 1.73| 98           | 1  |     |       |
|          | 0           | 4              | Tox | 40           | Tox|     |       |
|          | 0           | 8              | Tox | 20           | Tox|     |       |
|          | 28          | 0              | 1.51| 80           | 2  |     |       |
|          | 28          | 0.1            | 1.60| 80           | 1  |     |       |
|          | 28          | 0.2            | 1.44| 80           | 10 | 0.0380 | 0.0115 |
|          | 28          | 0.3            | 1.56| 70           | 1  |     |       |
|          | 28          | 0.4            | 1.61| 60           | 2  |     |       |
|          | 28          | 0.5            | 1.64| 60           | 2  |     |       |
| 5        | 0           | 0              | 1.75| 98           | 1  |     |       |
|          | 0           | 2              | 1.68| 80           | 3  |     |       |
|          | 0           | 4              | 1.51| 70           | 1  |     |       |
|          | 28          | 0              | 1.70| 80           | 2  |     |       |
|          | 28          | 0.04           | 1.68| 80           | 3  |     |       |
|          | 28          | 0.08           | 1.70| 70           | 1  |     |       |
|          | 28          | 0.12           | 1.65| 70           | 20 | 0.0001 | <0.0001 |
|          | 28          | 0.16           | 1.68| 70           | 20 | 0.0001 | <0.0001 |
|          | 28          | 0.20           | 1.67| 70           | 12 | 0.0126 | 0.0033 |
| Exp. no. | PMA (ng/ml) | p-BQ (µg/ml) | NDI | Viability (%) | MN | P   | P* | P** |
|----------|-------------|--------------|-----|--------------|----|-----|----|-----|
| 6        | 0           | 0            | 1.6 | 95           | 3  | 3   |    |     |
|          | 0           | 2            | 1.46| 80           | 2  |     |    |     |
|          | 0           | 4            | 1.30| 60           | 2  |     |    |     |
|          | 24          | 0            | 1.50| 80           | 1  |     |    |     |
|          | 24          | 0.04         | 1.47| 80           | 18 | <0.0001 | 0.0014 |
|          | 24          | 0.08         | 1.39| 80           | 4  |     |    |     |
|          | 24          | 0.12         | 1.41| 80           | 12 | 0.0033 | 0.0345 |
|          | 24          | 0.16         | 1.41| 80           | 12 | 0.0033 | 0.0345 |
|          | 24          | 0.20         | 1.28| 80           | 3  |     |    |     |

Positive control: 0.08–0.1 µmol/ml mitomycin induced 11–27 MN

Donor 2

| Exp. no. | PMA (ng/ml) | p-BQ (µg/ml) | NDI | Viability (%) | MN | P   | P* | P** |
|----------|-------------|--------------|-----|--------------|----|-----|----|-----|
| 7        | 0           | 0            | 1.69| 98           | 2  |     |    |     |
|          | 0           | 2            | 1.68| 98           | 6  |     |    |     |
|          | 0           | 4            | 1.65| 90           | 1  |     |    |     |
|          | 28          | 0            | 1.41| 98           | 3  |     |    |     |
|          | 28          | 0.04         | 1.54| 80           | 11 | <0.0001 | 0.0220 |
|          | 28          | 0.08         | 1.53| 80           | 22 | 0.0001 | <0.0001 |
|          | 28          | 0.12         | 1.61| 80           | 12 | 0.0345 | 0.0126 |
|          | 28          | 0.16         | 1.57| 80           | 2  |     |    |     |
|          | 28          | 0.20         | 1.59| 80           | 9  | 0.0647 |    |     |

| Exp. no. | PMA (ng/ml) | p-BQ (µg/ml) | NDI | Viability (%) | MN | P   | P* | P** |
|----------|-------------|--------------|-----|--------------|----|-----|----|-----|
| 8        | 0           | 0            | 1.89| 90           | 1  |     |    |     |
|          | 0           | 2            | 1.88| 70           | 1  |     |    |     |
|          | 0           | 4            | 1.44| 40           | 3  |     |    |     |
|          | 28          | 0            | 1.78| 80           | 2  |     |    |     |
|          | 28          | 0.04         | 1.80| 80           | 1  |     |    |     |
|          | 28          | 0.08         | 1.77| 80           | 10 | 0.0380 | 0.0115 |
|          | 28          | 0.12         | 1.77| 80           | 2  |     |    |     |
|          | 28          | 0.16         | 1.83| 80           | 1  |     |    |     |
|          | 28          | 0.20         | 1.78| 80           | 2  |     |    |     |

| Exp. no. | PMA (ng/ml) | p-BQ (µg/ml) | NDI | Viability (%) | MN | P   | P* | P** |
|----------|-------------|--------------|-----|--------------|----|-----|----|-----|
| 9        | 0           | 0            | 1.84| 90           | 3  |     |    |     |
|          | 0           | 2            | 1.76| 70           | 1  |     |    |     |
|          | 0           | 4            | 1.50| 40           | 1  |     |    |     |
|          | 24          | 0            | 1.58| 80           | 3  |     |    |     |
|          | 24          | 0.04         | 1.74| 80           | 3  |     |    |     |
|          | 24          | 0.08         | 1.47| 80           | 5  | 0.0023 |    |     |
|          | 24          | 0.12         | 1.79| 80           | 8  |     |    |     |
|          | 24          | 0.16         | 1.73| 80           | 10 |     |    |     |
|          | 24          | 0.20         | 1.70| 80           | 1  |     |    |     |

| Exp. no. | PMA (ng/ml) | p-BQ (µg/ml) | NDI | Viability (%) | MN | P   | P* | P** |
|----------|-------------|--------------|-----|--------------|----|-----|----|-----|
| 10       | 0           | 0            | 1.79| 90           | 1  |     |    |     |
|          | 0           | 2            | 1.88| 60           | 2  |     |    |     |
|          | 0           | 4            | Tox | 50           | Tox|     |    |     |
|          | 20          | 0            | 1.76| 90           | 1  |     |    |     |
|          | 20          | 0.04         | 1.75| 90           | 7  |     |    |     |
|          | 20          | 0.08         | 1.84| 90           | 5  |     |    |     |
|          | 20          | 0.12         | 1.79| 90           | 2  |     |    |     |
|          | 20          | 0.16         | 1.79| 90           | 1  |     |    |     |
|          | 20          | 0.20         | 1.72| 90           | 2  |     |    |     |

Positive control: 0.08–0.1 µmol/ml mitomycin induced 11–27 MN
by applying 20–28 ng/ml (0.37–1.85 μM) PMA. Under these conditions, a significant increase of micronuclei occurred between 0.04 and 0.2 μg p-BQ: In 4 out of 11 experiments using 20–28 ng/ml PMA, significance was reached in terms of a positive trend test and in two experiments with addition of 20 ng/ml PMA neither a positive trend test nor a significant elevation was seen (experiments 10 and 13). Viability—according to the trypane exclusion test—constituted 70–90% and only minor changes in the NDI were seen (Table 1).

| Exp. no. | PMA (ng/ml) | p-BQ (μg/ml) | NDI | Viability (%) | MN  | P* | P** |
|----------|-------------|--------------|-----|---------------|-----|----|-----|
| Donor 3  |             |              |     |               |     |    |     |
| 11       | 0           | 0            | 1.84| 95            | 1   |    |     |
|          | 0           | 2            | 1.75| 50            | 3   |    |     |
|          | 0           | 4            | 1.63| 40            | 8   | 0.0386 |     |
|          | 24          | 0            | 1.48| 80            | 3   |    |     |
|          | 24          | 0.04         | 1.58| 80            | 16  | 0.0042 | 0.0003 |
|          | 24          | 0.08         | 1.41| 90            | 12  | 0.0345 | 0.0033 |
|          | 24          | 0.12         | 1.58| 80            | 12  | 0.0345 | 0.0033 |
|          | 24          | 0.16         | 1.52| 80            | 2   |    |     |
|          | 24          | 0.20         | 1.59| 80            | 1   |    |     |
| 12       | 0           | 0            | 1.87| 99            | 1   |    |     |
|          | 0           | 2            |     | Tox 60        |     | Tox |     |
|          | 0           | 4            |     | Tox 40        |     | Tox |     |
|          | 24          | 0            | 1.32| 90            | 5   |    |     |
|          | 24          | 0.02         | 1.57| 90            | 9   | 0.0229 | 0.4223 |
|          | 24          | 0.03         | 1.53| 90            | 14  | 0.0623 |     |
|          | 24          | 0.04         | 1.48| 90            | 13  | 0.0948 |     |
|          | 24          | 0.08         | 1.43| 90            | 2   |    |     |
|          | 24          | 0.12         |     | NE NE NE      |     |     |     |
| 13       | 0           | 0            | 1.87| 90            | 1   |    |     |
|          | 0           | 1            | 1.75| 80            | 5   |    |     |
|          | 0           | 2            |     | Tox 60        |     | Tox |     |
|          | 20          | 0            | 1.42| 90            | 1   |    |     |
|          | 20          | 0.02         | 1.54| 90            | 4   |    |     |
|          | 20          | 0.04         | 1.68| 90            | 4   |    |     |
|          | 20          | 0.08         | 1.62| 85            | 3   |    |     |
|          | 20          | 0.12         | 1.55| 80            | 7   |    |     |
|          | 20          | 0.16         | 1.55| 80            | 4   |    |     |
| 14       | 0           | 0            | 1.80| 90            | 1   |    |     |
|          | 0           | 1            |     | Tox 80        |     | Tox |     |
|          | 0           | 2            |     | Tox 50        |     | Tox |     |
|          | 20          | 0            | 1.59| 90            | 1   |    |     |
|          | 20          | 0.02         | 1.47| 90            | 4   | 0.0473 |     |
|          | 20          | 0.04         | 1.50| 90            | 6   |    |     |
|          | 20          | 0.08         | 1.51| 90            | 2   |    |     |
|          | 20          | 0.12         | 1.46| 80            | 5   |    |     |
|          | 20          | 0.16         | 1.45| 80            | 1   |    |     |

Results from the in vitro micronucleus test using isolated lymphocytes from three different donors. Results of 14 independent experiments are displayed.

PMA Phorbol-12-acetat-13-myristat, p-BQ para-benzoquinone, NDI nuclear division index, MI mitotic index, MN micronuclei, NE not evaluable, tox toxic, P one-sided P value of Cochran-Armitage trend test (experiments shown in italics), P* two-sided P value of Fisher’s exact test based on the corresponding controls with PMA and without p-BQ, P** two-sided P value of Fisher’s exact test based on the samples without PMA and without p-BQ.

Positive control: 0.08–0.1 μmol/ml mitomycin induced 12–20 MN

Table 1

by applying 20–28 ng/ml (0.37–1.85 μM) PMA. Under these conditions, a significant increase of micronuclei occurred between 0.04 and 0.2 μg p-BQ: In 4 out of 11 experiments using 20–28 ng/ml PMA, significance was reached in terms of a positive trend test and in two experiments with addition of 20 ng/ml PMA neither a positive trend test nor a significant elevation was seen (experiments 10 and 13). Viability—according to the trypane exclusion test—constituted 70–90% and only minor changes in the NDI were seen (Table 1).
Discussion

Central questions concerning benzene toxicity are still unanswered: why is benzene such a potent carcinogen whereas compounds with a similar chemical structure such as phenol, hydroquinone or p-BQ are not (although they are metabolites of benzene)? Does benzene cause the formation of mutagenic DNA-adducts or rather act by a clastogenic effect? Why are only hematopoietic malignancies seen in humans whereas a broader tumor spectrum occurs in experimental animals? Are only myeloid stem cells targets of benzene toxicity in humans? Since no convincing evidence for genotoxic effects of p-BQ, phenol, 1,2-benzenediol or hydroquinone exists, do other benzene metabolites contribute to benzene carcinogenicity instead? An appropriate in vitro system could help to answer these open questions.

We observed a significant elevation of micronuclei at very low, non-cytotoxic concentrations between 0.04 and 0.2 µg/ml p-BQ using PMA activated peripheral blood cells. This is the first report that shows genotoxic effects of p-BQ in peripheral blood cells at non-toxic concentrations and the first direct evidence for in vitro activation of a benzene metabolite by PMA inducible reactions. Notably, we achieved positive results at very low p-BQ concentrations between 0.37 and 1.85 µM, whereas in formerly published investigations p-BQ alone induced an elevation of micronuclei only at toxic concentrations above 35 µM in V79 cells (Ludewig et al. 1989) and at and above 2.5 µM in human lymphocytes (Yager et al. 1990). Treatment of HL-60 cells with cytotoxic concentrations of 500 µM hydroquinone and 250 µM 1,2,4-benzenetriole resulted in the formation of a single DNA adduct in HL-60 cells which was detected by 32P-postlabelling. We could not reproduce former positive results using the cytokinesis-block micronucleus technique, human lymphocytes and up to 46.3 µM p-BQ without addition of PMA. Only addition of PMA at relatively low concentrations resulted in reproducible p-BQ genotoxicity. These effects occurred at a narrow concentration range between 20 and 28 ng/ml PMA. Toxicity at and above 32 ng/ml PMA masked genotoxic effects of p-BQ; whereas addition of less than 24 ng/ml PMA yielded only weak effects.

Cytotoxic artifacts are often misinterpreted as genotoxic effects when using in vitro assays and eukaryotic cells (Kirkland et al. 2007). The in vitro micronucleus test however allows the parallel investigation of the nuclear division index or the mitotic index to monitor cytotoxicity, and evaluation of binucleated (vital) cells can avoid the inclusion of damaged cells (Fenech 1993). Admittedly in our experiments concentrations at and above 32 ng/ml PMA yielded inconclusive results and poor reproducibility, even at concentrations which did not appreciably affect the NDI. Some of these experiments showed a bimodal elevation of micronuclei, first between 0.04–0.2 µg/ml p-BQ and 28–32 ng/ml PMA; second at and above 0.4 µg/ml p-BQ and, according to the typane blue exclusion test, at about 60% cytotoxicity (Table 1). This second elevation might be caused by cytotoxic artefacts.

Acceptance criteria for a positive in vitro micronucleus test are not consistently used. A previous publication of the “Gesellschaft für Umwelt-Mutationsforschung” defined a dose–effect relationship as follows: if there were at least two consecutive concentrations having increased aberration frequencies and the effect of the higher concentration was more pronounced than that of the lower, this was regarded as dose–effect relationship. A doubling over control was not necessarily considered adequate by itself (Miller et al. 1998). According to a more recent publication a dose-related increase in micronuclei frequencies (e.g. trend test) which exceeds statistically significantly the upper limit of the historical and concurrent negative control (chi-square or Fisher’s exact test) or at least a twofold increase over mean control micronuclei frequencies are required for a positive call. If a chemical meets all these criteria, it is proposed to be considered clearly positive. If a chemical satisfies none of these criteria, it should be considered clearly negative (Corvi et al. 2008).

In 2 out of 11 of our experiments in which 20 and 28 ng/ml PMA were applied, we achieved a significant dose–response according to the Cochrane-Armitage test and a statistically significant deviation from the concurrent negative control according to the Fisher’s exact test. Two experiments were clearly negative, in both we had applied 20 ng/ml PMA. Accordingly the minimal effective PMA dose concerning p-BQ activation was between 20 and 24 ng/ml PMA. The remaining experiments showed a significant elevation according to a positive trend test or a positive Fisher’s exact test.

The concentration range for a genotoxic effect is very narrow, most probably because the dose–response could be influenced by cytotoxic effects of both PMA and p-BQ. In addition, the concentration range can vary from donor to donor and test to test because the amount of inducible cells may be different and the PMA response may be modified by genetic- or life style factors. In fact, donor 1 showed the strongest variations in the results and a dose–response relationship could not be established. Interestingly donor 1 was the only smoker. However, we did not systematically evaluate a possible smoking effect on the results.

Phorbol-12-acetate-13-myristate induced peripheral blood cells are routinely used as a model for the investigation of inflammation and might enable the investigation of the role of inflammation in chemically induced carcinogenesis (Eastmond and Smith 1990). PMA induces oxidative bursts (Walrand et al. 2003) and oxidative DNA damage (Dzarlieva-Petrusevska and Füsenig 1985; Petrusevska et al. 1988). An influence of PMA on the generation of
benzene-induced oxidative DNA damage in rats was shown (Subrahmanyan et al. 1991). Toxic effects of PMA are mediated by MPO induction. MPO and other peroxidases catalyze the generation of hypochloric acid from H₂O₂ and Cl⁻ (Aucherè and Capeillé-Blandin 1999), reactions of phenolic compounds (Eastmond et al. 1986) and 1,2-benzenediul to highly reactive intermediates. The binding of 1,2-benzenediul peroxidation products to N-7 guanine and N-3 adenine results in DNA depurination (Cavalieri et al. 2002). Accordingly, benzene-induced oxidative DNA damage is enhanced in PMA-stimulated cells in vitro and in LPS-treated animals (Tuo et al. 1999). Altogether evidence points towards peroxidase mediated benzene toxicity.

Physiologically MPO is released during host defense and inflammatory reactions, parallel to the generation of H₂O₂. The enzyme was proposed to be involved in the generation of tissue damage caused by chronic inflammation (Winterbourn 2002). Reactions accompanying MPO activation were suggested to take part in the toxification of oxygen reactive substances (Winterbourn 2002; Trush 1984; Eastmond and Smith 1990) or to act as co-factor in chemical carcinogenesis (Coussens and Werb 2002). Activation of p-BQ via PMA-induced reactions which is shown in our experiments, may provide an experimental approach to test this hypothesis in vitro. Furthermore our results will enable the comparative investigation of other benzene metabolites such as HQ or catechol.

In conclusion, p-BQ can be activated to genotoxic intermediates when administered jointly with PMA. Our results confirm the hypothesis that MPO is a key enzyme in the activation of benzene.

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