p-Benzoinnone, a Reactive Metabolite of Benzene, Prevents the Processing of Pre-interleukins-1α and -1β to Active Cytokines by Inhibition of the Processing Enzymes, Calpain, and Interleukin-1β Converting Enzyme

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Chronic exposure of humans to benzene affects hematopoietic stem and progenitor cells and leads to aplastic anemia. The stromal macrophage, a target of benzene toxicity, secretes interleukin-1 (IL-1), which induces the stromal fibroblast to synthesize hematopoietic colony-stimulating factors. In a mouse model, benzene causes an acute marrow hypopcellularity that can be prevented by the concomitant administration of Il-1α. The ability of benzene to interfere with the production and secretion of Il-1α was tested. Stromal macrophages from benzene-treated mice were capable of the transcription of the Il-1α gene and the translation of the message but showed an inability to process the 34-Kd pre-I1α precursor to the 17-Kd biologically active cytokine. Treatment of normal murine stromal macrophages in culture with benzoinnone (HQ) also showed an inhibition in processing of pre-IL-1α. Hydroquinone is oxidized by a peroxidase-mediated reaction in the stromal macrophage to p-benzoquinone, which interacts with the sulfhydryl (SH) groups of proteins and was shown to completely inhibit the activity of calpain, the SH-dependent protease that cleaves pre-IL-1α. In a similar manner, HQ, via peroxidase oxidation to p-benzoquinone, was capable of preventing the IL-1β autocine stimulation of growth of human B1 myeloid tumor cells by preventing the processing of pre-IL-1β to mature cytokines. Benzoquinone was also shown to completely inhibit the ability of the SH-dependent IL-1β converting enzyme. Thus benzene-induced bone marrow hypopcellularity may result from apoptosis of hematopoietic progenitor cells brought about by lack of essential cytokines and deficient IL-1α production subsequent to the inhibition of calpain by p-benzoquinone and the prevention of pre-IL-1 processing. — Environ Health Perspect 104(Suppl 6):1251–1256 (1996)

Key words: benzene, p-benzoquinone, pre-interleukin-1 processing, calpain, interleukin-1β processing enzyme

Introduction

Benzene (BZ) is a hematotoxin that causes bone marrow cell depression in experimental animals and aplastic anemia in humans that are chronically exposed (1–3). The bone marrow stromal macrophage (SØ) is a target of BZ hematotoxicity (4–6). Hydroquinone (HQ), a hepatic metabolite of BZ, accumulates in the bone marrow (7) where it undergoes peroxidase-mediated oxidation in the SØ to p-benzoquinone (BQ) (8), a direct-acting biologically reactive electrophile that interacts with the sulphydryl (SH) group of cysteine residues in cellular proteins (8).

The SØ is involved in hematopoietic regulation (9–11) through the synthesis of several cytokines including interleukin-1 (IL-1), which synergizes with IL-3 to promote the development of the pluripotent stem cell to myeloid and lymphoid stem cells (12). IL-1 is also involved in lymphocyte development and in the induction of cytokine production by stromal fibroblasts (11,13,14). Inhibition of the production of active IL-1 in SØ could result in a lack of cytokines, increased physiological cell death (apoptosis) of hematopoietic progenitor cells, and thus bone marrow cell depression. BZ-induced bone marrow cell depression might result from interference by BQ in IL-1 production, processing, and/or secretion in SØ. Lipopolysaccharide (LPS)-induced secretion of IL-1 by murine SØ (15) and P388D1 macrophagelike cells (16) was decreased in vitro after exposure to HQ and a decrease in chemotrapsinlike activity in extracts of P388D1 cells was observed concomitant with a decrease in the release of IL-1 into the conditioned medium (16), leading the authors (16) to suggest that HQ decreased IL-1 release by inhibiting proteolytic conversion of the molecule from its membrane-bound precursor form.

Two IL-1 cytokines, IL-1α and IL-1β, are the products of distinct genes located on chromosome 2 (17). The transcript of each gene is translated as a precursor protein of approximately 34 kDa that is converted by a specific protease to a biologically active cytokine of 17 kDa (18). The processing of pre-IL-1α to mature cytokine is catalyzed by the SH-dependent protease, calpain (19,20). The release of IL-1β from the cell is also associated with the cleavage of its precursor form by a sulphydryl (SH)-dependent protease referred to as IL-1β converting enzyme (ICE) (21–23).

We report here that HQ, via peroxidation to BQ, has no effect on the transcription or translation of mRNAs for the 34-kDa pre-IL-1α or pre-IL-1β but prevents the proteolytic conversion of the pre-IL-1 forms to the 17-kDa active cytokines in murine SØ or human B1 myeloid cells, respectively. In addition, SØ from BZ-treated mice produce the 34-kDa pre-IL-1α when stimulated in culture with LPS (24) but cannot convert the precursor to IL-1α. These results suggest that BQ also prevents the conversion in vitro and thus may be responsible for BZ-induced bone marrow cell depression. In this connection,
BZ-induced bone marrow cell depression can be prevented by the concomitant administration with BZ of native but not heat-inactivated, recombinant IL-1α, thus bypassing the inability of the HQ-inhibited SØ to produce IL-1α (24). We also show that BQ is an excellent inhibitor of the proteolytic activity of calpain and ICE.

Methods
Isolation of resident SØ, preparation of an adherent stromal layer from BZ-treated animals, exposure to HQ, and determination of intracellular and membrane-bound IL-1α were carried out as previously described (24). Treatment of mice with BZ and the effect of IL-1α on BZ-induced bone marrow cell depression were previously described (25). The B1 cell line was established from the bone marrow of a patient with a relapse of acute lymphocytic leukemia (ALL) characterized by a 4;11 chromosomal translocation, the expression of the stem cell marker CD34, and an immature pre-B/myeloid phenotype (26,27). The cells secrete IL-1β, which supports the autocrine growth of the cells (27). The cells were seeded at a density of 5 x 10^5 in α minimal essential medium (αMEM) containing 10% fetal bovine serum (FBS) and incubated at 37°C with 5% CO₂. The cells were fed every 7 days and diluted to the initial seeding density. Cells growing at a density of 4 x 10^6/ml secrete approximately 25 pg/ml IL-1β in 24 hr. Cells (4.5 x 10^6/ml) were exposed to 2 μM HQ at zero time and allowed to incubate for 4 days. Cell viability after HQ exposure was 95% as measured by Trypan Blue exclusion. IL-1β was added to the HQ-treated cells 4 hr after the addition of HQ. Control cultures received phosphate-buffered saline, the vehicle in which HQ was dissolved. All treatments were carried out in triplicate. At the end of 4 days, cells from each treatment group were collected by centrifugation and the culture medium saved for determination of the presence of IL-1β by Western blot analysis.

Assay for BQ Inhibition of Calpain and ICE. Calpain, with a specific proteolytic activity against casein of 53 U/ml, was purified from fresh-washed human platelet concentrates (28) and was assayed by measuring the hydrolysis of a specific peptide substrate, 3-carboxypropionyl-leu-tyr-NH-7-(4-methyl)coumarylamide, which liberates a fluorescent product (29). The doped enzyme and buffer (or inhibitor) were mixed and immediately added to a rectangular quartz cuvette at 25°C containing 1 mM peptide substrate in 60 mM Tris/HCl buffer, pH 7.5, 2.5% (v/v) dimethylsulfoxide (DMSO) and 5 mM CaCl₂. The rate of hydrolysis of peptide was continuously recorded by a fluorescence spectrophotometer connected to a chart recorder. The absorbance excitation maximum of substrate occurred at 380 nm (5-nm slit width) and emission response at 440 nm (10-nm slit width). Proteolytic activity of recombinant ICE, in the presence or absence of BQ, was monitored by Western immunoblot analysis of the proteolytic product, the mature 17-kDa IL-1β, using recombinant pre-IL-1β as the substrate and a monoclonal anti-IL-1β antibody to identify the both the pre-IL-1β and the mature IL-1β.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting. Sodium dodecyl sulfate (SDS)-PAGE was carried out using a precast 4 to 15% gradient polyacrylamide minigel run at 200 volts for 45 min. The gel was placed in transfer buffer (200 mM glycine, 3 mM SDS, 30 mM Tris–HCl, pH 8.3, 20% v/v methanol) and electrobotted at 25 mAmp overnight onto a BA-38 nitrocellulose membrane. The membrane was equilibrated for 30 min in phosphate-buffered saline (PBS)-A-1% Tween and then blocked for 1 hr in a solution of 5% bovine serum albumin (BSA)/PBS-A-Tween. After washing, the blot was probed with mouse monoclonal anti-human IL-1β (5 mg/ml) for 1 hr at room temperature. The blot was then washed and incubated for 1 hr with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (IgG) diluted 1:1000. After washing, the blot was incubated for 1 min with ECL horseradish peroxidase developing reagent and exposed to X-ray film for 5 sec.

Statistical Analysis. Data points represent the mean of the number of cells of three replicates ± SD. No error bars are shown, the SD was too small to plot.

Results and Discussion
Ability of Recombinant Murine IL-1α to Prevent Benzene-induced Depression of Bone Marrow Cellularity
If our premise is correct that BZ-induced bone marrow cell depression in a mouse model results from an inhibition of the processing of pre-IL-1α by calpain, then BZ-induced bone marrow hypocellularity should be prevented by the coadministration of IL-1α with BZ. Figure 1 presents a representative of five experiments, all of which showed similar results. Seven groups (n = 4) of C57Bl/6 mice were treated as

![Figure 1. Benzene-induced depression of bone marrow cellularity and its prevention by IL-1α. Seven groups (n = 4) of male C57Bl/6 mice were established. One group served as the control and received only corn oil and PBS. Five groups were injected ip with BZ (600 mg/kg bw) in corn oil twice per day, 7 hr apart, for 2 days. One of these groups received BZ only, the others were also injected with rmull-1α (500, 1000, or 2000 U/animal) or heat-inactivated rmull-1α administered in PBS containing 0.2% BSA. A seventh group of animals received only rmull-1α (2000 U/animal). Fourteen hours after the final injection, the animals were killed by cervical dislocation, their femurs removed, and the nucleated bone marrow cells obtained and counted. Data are expressed as the mean ± SD. Where there were differences between groups, they were significant at the p < 0.01 level. From Niculescu and Kalf (25), with permission.](image-url)
follows: One group was treated with vehicle (corn oil) only and served as control. One group received BZ (600 mg/kg body weight, ip, twice/day for 2 days) and another group received only recombinant murine IL-1α(rMuIL-1α) (2000 U/ml). Three groups were given rMuIL-1α (500, 1000, or 2000 U/animal) 18 hr prior to the first daily BZ injection. The remaining group was administered heat-inactivated rMuIL-1α (2000 U/animal) prior to BZ treatment. Administration of BZ to mice decreased the nucleated cells in the bone marrow (Figure 1) to 25% of control, measured 17 hr after the last BZ injection (on day 3). Native rMuIL-1α provided a dose-dependent protection against the depressive effects of BZ (Figure 1, bars 3–5), with complete protection occurring at 2000 U/animal (100 U/g bw). Heat-inactivated rMuIL-1α did not protect (bar 6) and pretreatment with rMuIL-1α alone (bar 7) did not affect bone marrow cellularity in 3 days. rMuIL-1α provided similar protection when the dose of BZ administered was 800 mg/kg bw. Taken together, these results suggest that the depression of marrow cellularity in mice may result from an inability of the SO to process pre-IL-1α, the major form in the mouse, to biologically active cytokine required for the induction in stromal fibroblasts of colony-stimulating factors essential for the survival of hematopoietic progenitor cells. Additional support for this hypothesis is the demonstration that BZ-induced myelotoxicity is completely prevented when exogenous IL-1α obviates the lack of IL-1α in the SO of BZ- or HQ-treated animals.

Inhibition of the Conversion of Pre-IL-1α to Mature Cytokine in SO from Benzene-treated Mice and in SO Treated with Hydroquinone in Culture. The inhibition of conversion of precursor to cytokine was demonstrated in SO of mice treated with BZ under conditions that cause myelotoxicity measured as severe depression of bone marrow cellularity. Three groups (n = 4) of C57Bl/6j mice were established. One group received the vehicle only. The second was treated with 800 mg/kg BZ ip twice daily for 2 days. Indomethacin (2 mg/kg), a prostaglandin H synthase-peroxidase inhibitor was concomitantly administered to group three. Indomethacin has been shown to prevent BZ-induced bone marrow cell depression and genotoxicity (30). It has no effect on the hepatic metabolism of BZ at this dosage but does prevent oxidation of HQ to reactive species in the SO. Eighteen hours after the final BZ administration, femoral bone marrow was obtained and the number of nucleated marrow cells determined. Bone marrow cellularity of BZ-treated mice was depressed by 45% of control, while coadministration of indomethacin prevented depression (data not shown). The ability of SO from BZ-treated animals to convert 34-kDa pre-IL-1α to 17-kDa cytokine was assessed by placing bone marrow cells from the three experimental groups into culture to establish an adherent layer consisting predominately of SO and fibroblasts. IL-1α production by the SO was stimulated by incubation with LPS for 18 hr. Conversion of the precursor to the 17-kDa cytokine was analyzed in cell lysate protein by Western immunoblotting. As can be seen in Figure 2, SO from control animals stimulated with LPS produced both a 34-kDa pre-IL-1α and a 17-kDa mature cytokine (lane 2). The precursor was produced in LPS-treated SO from animals administered BZ, indicating that transcription and translation were not affected, but the precursor was not converted to the 17-kDa mature cytokine (lane 1). Indomethacin administered concomitantly with BZ prevented the inhibition of precursor processing (lane 3) that results from the oxidation of HQ to BQ by peroxidase.

The inhibition of conversion of pre-IL-1α to cytokine by HQ in normal mouse SO was demonstrated by culturing femoral bone marrow cells with recombinant macrophage colony-stimulating factor for 7 days, which resulted in a population of greater than 95% SO, as determined by morphological and biochemical analysis (data not shown). Confluent SO were treated with HQ (0.5–10.0 µM) for 6 hr, followed by stimulation with LPS for 24 hr. Immunoblot analysis of cell lysate protein indicated that SO did not express IL-1α in the absence of LPS stimulation (Figure 3, lane 1), whereas in the presence of LPS, precursor was produced and converted to mature cytokine (lane 2). Exposure to HQ did not affect transcription or translation of pre-IL-1α at 10 µM, but 0.5 µM HQ completely inhibited the conversion of pre-IL-1α to cytokine (Figure 3, lanes 3–6). Thus, SO from BZ-treated mice and from those treated in culture with HQ are incapable of producing active cytokine, most probably because BQ has inhibited the protease responsible for cleaving the 34-kDa precursor to the 17-kDa active molecule. HQ has been reported to both inhibit calpain II activity and selectively decrease calpain II content in mouse bone marrow SO as measured by immunoblot analysis (31); the inhibition and decrease in calpain activity may occur because of an increased turnover of the inactive, damaged BQ-adducted calpain.
Inhibition of Calpain, the Pre-IL-1α Processing Enzyme, by p-Benzoinoquione

Processing of the membrane-associated 34-kDa pre-IL-1α to the 17-kDa biologically active cytokine is catalyzed by the membrane-bound SH-dependent protease, calpain (19,20). BQ, produced from HQ in the cytosol of the SØ, may inactivate calpain by forming a covalent adduct with the SH group of an essential cysteine residue at the active site (32). We have demonstrated previously that macrophages oxidize radiolabeled HQ to BQ, which covalently binds to proteins (8), and in the presence of excess cysteine to compete with protein SH groups, BQ is trapped as the BQ-Cysteine monoaduct (8). As can be seen in Figure 4, BQ causes a concentration-dependent inhibition of the activity of purified human platelet calpain with a 50% inhibitory concentration of 3 μM, making BQ one of the most potent inhibitors of this protease. For these reasons we postulate that BQ inhibits calpain by covalently binding to the essential SH group of cysteine 108 at the active site (32).

HQ Inhibition of Pre-IL-1β Conversion to Mature Secreted IL-1β

In the human cell, IL-1β represents the major isoform. IL-1β undergoes slow release from the cell associated with cleavage of pre-IL-1β by the cysteine protease, ICE. To determine the effects of HQ on the processing of pre-IL-1β, we turned to the B1 cell line as a model. This cell line exhibits density-dependent growth and secretes an autostimulatory growth factor that has been identified as IL-1β. B1 cells constitutively express mRNA for pre-IL-1β and the IL-1 receptor and secrete the active cytokine. An inhibition of ICE by treatment of the cells with HQ should prevent conversion of pre-IL-1β, secretion of IL-1β into the growth medium, and autocinucine stimulation of the cells. As can be seen from a representative (Figure 5) of three experiments, all of which gave virtually identical results, untreated B1 cells increase approximately 7-fold over the 5-day test period due to autocinucine stimulation by IL-1β. HQ (2 μM) significantly inhibited the proliferation of B1 cells at day 4 (p≤0.001) (Figure 5), presumably because the HQ-inactivated ICE is incapable of converting pre-IL-1β to secretable, biologically active cytokine. The failure of HQ-treated cells to increase in number because of loss of autocinucine stimulation by IL-1β is supported by the fact that the growth curve of HQ-treated cells supplemented with recombinant human interleukin-1β (rHuIL-1β), approached that of control cells. The lack of IL-1β secretion by the HQ-treated cells was confirmed by the failure to demonstrate the presence of the 17-kDa IL-1β in the conditioned medium on day 4 by Western immunoblotting with monoclonal anti-IL-1β antibody (Figure 6, lane 3). A distinct band indicative of a protein of 17 kDa, which migrated to the same position as the IL-1β standard (lane 1), was observed in the culture media of control cells (Figure 6, lane 2) and cells treated with HQ+IL-1β (lane 4). However, in the presence of HQ, there was no observable band at 17 kDa (lane 3) indicative of the inability of HQ-treated cells to convert pre-IL-1β to the secretable cytokine.

Taken together, these results indicate that B1 cells, whose proliferation in culture is dependent on the autocinucine secretion of IL-1β, are prevented by HQ from processing pre-IL-1β to active secreted cytokine and thus from autocinucine growth.

Effect of Indomethacin on the Ability of Hydroquinone to Inhibit Growth of B1 Cells and Pre-IL-1β Processing

To demonstrate that a peroxidase-mediated conversion of HQ to BQ is required for inhibition of ICE and the subsequent lack of conversion of pre-IL-1β to mature cytokine, B1 cells were treated with HQ as in Figure 5 in the presence or absence of indomethacin, a peroxidase inhibitor. The presence of 20 μM indomethacin concomitantly with HQ significantly prevented HQ from inhibiting ICE (Figure 7, bar graph), and thus from reducing the cell number at day

**Figure 4.** Inhibition of calpain by BQ. Homogeneous human platelet calpain (53 U/ml, casein assay) was assayed in the presence of 0.1 to 100 μM BQ by following the hydrolysis of a calpain-specific fluorescent peptide substrate (1 mM) in 60 mM Tris/HCl, pH 7.5, 2.5% OMSO and 5 mM CaCl<sub>2</sub> using an absorbance excitation maximum of 380 nm and measuring an emission response at 440 nm.

**Figure 5.** HQ inhibition of IL-1β autocinucine stimulation of B1 cell growth. A culture of B1 cells (4.5×10<sup>6</sup>/ml) was divided into three treatment groups, each set up in triplicate. (○-○) control (untreated), (●-●) treated with a final concentration of 2 μM HQ, (▲-▲) treated with 2 μM HQ and 25 μg/ml of rHuIL-1β. IL-1β was added to the HQ-treated cells 4 hr after the addition of HQ, to allow time for HQ to enter the cells and be metabolized to BQ, which must then bind ICE. Control cultures received PBS. The cells were cultured in aMEM/10% FBS for 5 days; cell counts and viability studies were performed daily. Viability after treatment was always greater than 95%. Data points represent the mean number of cells from three replicates ± SD. Where no error bars are seen, the SD was too small to plot. The experiment presented is representative of three experiments that gave virtually identical results. HQ-treated sample at day 4 was significantly different from the control group at p≤0.001.

**Figure 6.** Western immunoblot analysis for the presence or absence of IL-1β in culture supernatants of B1 cells after treatment with HQ and HQ/IL-1β. Supernatant samples from day 4 cultures (Figure 5) were mixed with an equal volume of SDS sample solvent and boiled for 5 min. Equal amounts of protein (2 μg) were added to each well of a polyacrylamide minigel. PAGE analysis was carried out using a precast 4 to 15% gradient polyacrylamide minigel run at 200 V for 45 min. Western immunoblotting for the identification of IL-1β was carried out by electroblotting into a nitrocellulose membrane, blocking and probing the blot with mouse monoclonal antihuman IL-1β (5 μg/ml) for 1 hr. The blot was washed, incubated for 1 hr with horseradish peroxidase-conjugated sheep antimouse IgG (1:1000), and developed by incubation with peroxidase developing reagent and exposed to X-ray film. Lane 1, rHuIL-1β (300 ng); lane 2, control B1 supernatant; lane 3, HQ-treated B1 cell supernatant; lane 4, HQ + IL-1β- treated B1 cell supernatant. The experiment was repeated two additional times with identical results.
4. This result suggests that the peroxidase-mediated oxidation of HQ to BQ, the more active compound, is occurring in B1 cells. Indomethacin, which by itself showed some effect on cell growth, restored the cell number in the presence of HQ to the number seen with indomethacin alone. In addition, it can be seen from a Western blot analysis of pre-IL-1β in the cell lysate (Figure 7, insert) and mature IL-1β in the conditioned medium that neither HQ nor indomethacin have an effect on the transcription or translation of the 31-kDa pre-IL-1β (cell lysate) but that HQ does inhibit the formation of the mature 17-kDa IL-1β and thereby its secretion into the conditioned medium, and this inhibition is prevented by indomethacin.

Inhibition of IL-1β Converting Enzyme by BQ

The ability of BQ to inactivate ICE was assayed by monitoring the conversion of the 31-kDa recombinant pre-IL-1β to the 17 kDa IL-1β by BQ-treated recombinant ICE using Western immunoblot analysis and a specific IL-1β monoclonal antibody (Figure 8). Pretreatment of ICE for 10 min with 3 μM BQ prior to incubation with the precursor resulted in complete inhibition of the conversion of pre-IL-1β to the mature 17-kDa cytokine (Figure 8, lane 3 compared with lane 2). This experiment was repeated three times. Treatment of pre-IL-1β or IL-1β with BQ does not affect mobility of the cytokine in the gel, its reaction with antibody, or its use as a substrate for ICE (data not presented). The inhibition of ICE protease activity by BQ was similar to the inhibition by iodoacetic acid, which is known to alkylate free SH groups and to inhibit ICE (data not presented).

In summary, BZ induces a severe bone marrow cell depression in mice that can be prevented by IL-1α. Marrow S0 from BZ-treated mice can affect the transcription and translation of the 34-kDa pre-IL-1α but cannot proteolytically cleave the pre-cursor to the mature, biologically active cytokine. Murine S0 treated with as little as 5 x 10^-7 M HQ cannot process pre-IL-1α because of the peroxidase-mediated oxidation of HQ to BQ that inhibits the SH-dependent protease, calpain. HQ, by oxidation to BQ in the human myeloid-type B1 cell, also inactivates ICE, the SH-dependent protease responsible for converting pre-interleukin-1β to biologically active cytokine, which results in the inhibition of IL-1β-dependent autocrine growth of B1 cells. The results indicate that BZ-induced bone marrow hypopcellularity in the mouse results from apoptosis of hematopoietic progenitor cells brought about by a lack of essential cytokines subsequent to S0 dysfunction and deficient IL-1 production caused by BQ inactivation of the protease responsible for processing pre-IL-1α. In view of the ability of BZ to cause aplastic anemia, it is of interest that in two large studies of aplastic anemia, a high percentage of patients showed stomal S0 dysfunction and deficient IL-1 production in comparison with controls (33,34).

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