Hepoxilin Analogs Inhibit Bleomycin-Induced Pulmonary Fibrosis in the Mouse

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ABSTRACT

Bleomycin has been suggested to incite plasma extravasation and influx of inflammatory cells leading to pulmonary fibrosis. We hypothesized that stable analogs of the 12-lipoxygenase product, hepoxilin, may attenuate these effects. We initially investigated the effects of the four hepoxilin analogs (PBT-1 to -4) coadministered intradermally with bleomycin and found that PBT-1 and -2 significantly opposed the vascular permeability effects of bleomycin in rat skin. We subsequently tested the hepoxilin analogs for their actions in opposing the intratracheal bleomycin-evoked acute inflammatory phase of lung fibrosis in the mouse, characterized by a marked accumulation of macrophages and an increase in the rate of collagen synthesis and deposition. We found that the bleomycin-evoked effects on macrophage influx were inhibited by all the hepoxilin analogs (PBT-1, -3, and -4 > PBT-2) administered i.p. for 8 days. Increased total lung collagen was completely abrogated by PBT-1 and -2, whereas PBT-3 and -4 had little effect. A dose-response study with PBT-1 indicated that the effective dose for inhibition of bleomycin-induced inflammatory and histological changes was below 10 μg/day. These studies demonstrate an in vivo action of stable analogs of hepoxilin and support an effect on inflammation and vascular permeability from these novel compounds, especially for PBT-1.

Idiopathic pulmonary fibrosis is a devastating disorder that is poorly understood and resistant to treatment (Cooper, 2000). The observation that the antibiotic bleomycin sulfate (BL), a potent cancer chemotherapeutic agent (Adamson, 1984; Nici et al., 1998), may cause interstitial lung fibrosis in humans (Yagoda et al., 1972) led to the development of animal models in which a single dose of BL administered intratracheally induced changes resembling human idiopathic pulmonary fibrosis histopathologically (Kelley et al., 1980). The acute phase of this response is characterized by a marked accumulation of inflammatory cells and an increase in the rate of collagen synthesis and deposition. We found that the bleomycin-evoked effects on macrophage influx were inhibited by all the hepoxilin analogs (PBT-1, -3, and -4 > PBT-2) administered i.p. for 8 days. Increased total lung collagen was completely abrogated by PBT-1 and -2, whereas PBT-3 and -4 had little effect. A dose-response study with PBT-1 indicated that the effective dose for inhibition of bleomycin-induced inflammatory and histological changes was below 10 μg/day. These studies demonstrate an in vivo action of stable analogs of hepoxilin and support an effect on inflammation and vascular permeability from these novel compounds, especially for PBT-1.

Previous animal studies have demonstrated that BL-evoked lung fibrosis is exacerbated with nordihydroguaiaretic acid, a lipooxygenase inhibitor, suggesting that a lipooxygenase product may be involved in endogenous mechanisms controlling lung fibrosis (Giri and Hollinger, 1996). The hepoxilins (HXs) may be candidates for the control of lung fibrosis as they are formed through the 12-lipoxygenase pathway of metabolism of arachidonic acid (Pace-Asciak et al., 1983; Pace-Asciak, 1984; Pace-Asciak and Martin, 1984). These compounds have previously been shown to have significant biological actions (Pace-Asciak, 1984; Dho et al., 1990; Laneuville et al., 1992; Pace-Asciak et al., 1995; Reynaud et al., 1996, 1999; Sutherland et al., 2000). HXs raise free intracellular calcium in human neutrophils ex vivo through the release from stores and vascular tissue in vitro and block the intracellular calcium rise evoked by inflammatory mediators (Laneuville et al., 1993). HX formation is stimulated by inflammatory mediators in the skin (Wang et al., 1999a,b) and in psoriasis (Anton and Vila, 2000), suggesting that they may be involved in anti-inflammatory mecha-

ABBREVIATIONS: BL, bleomycin sulfate; HX, hepoxilin; ANOVA, analysis of variance; PBT-1, 8(S)-hydroxy-11,12-cyclopropyl-eicosa-5Z,9E,14Z-trienoic acid methyl ester; PBT-2, 8(R)-hydroxy-11,12-cyclopropyl-eicosa-5Z,9E,14Z-trienoic acid methyl ester; PBT-3, 10(S)-hydroxy-11,12-cyclopropyl-eicosa-5Z,8Z,14Z-trienoic acid methyl ester; PBT-4, 10(R)-hydroxy-11,12-cyclopropyl-eicosa-5Z,8Z,14Z-trienoic acid methyl ester; i.t., intratracheal.
Effects of HX analogs on bleomycin-evoked Evans blue dye leakage from skin as an index of vascular plasma leakage. Comparative quantitative effects on vascular permeability in the skin are shown for saline alone (control; open bar), saline + bleomycin (saline; filled bar), and saline + bleomycin + PBT-1 to -4 (filled bars). Bars represent mean ± S.E.M. (n = 6 per group). *p < 0.05, by one-way ANOVA, for PBT-1 and -2 when coadministered with bleomycin and saline compared with bleomycin and saline alone.

Experimental Procedures

Materials. HX analogs (PBT-1–PBT-4) were prepared as previously described (Demin and Pace-Aciak, 1993). BL, formamide, hematoxylin, collagen type I, eosin, Evana blue, Fast Green FCF, and picric acid-saturated solution were purchased from Sigma-Aldrich (St. Louis, MO); Direct Red 80 was obtained from Aldrich (Milwaukee, WI); Sirius Red from Pfaltz and Bauer (St. Louis, MO); and Direct Red 80 was obtained from Aldrich (St. Louis, MO). Formamide was used as a precipitant of collagen with 0.5 M acetic acid. The mixture was left at room temperature for 30 min and then centrifuged at 3000 rpm for 30 min. Absorbance of samples was measured at 540 nm and plotted against standards with known concentration of collagen type I.

Histology and Immunohistochemistry. Histological analysis was performed on paraffin-embedded sections using an avidin-biotin-peroxidase complex method (Hsu et al., 1981) and then counterstained with Carazzi hematoxylin, dehydrated, cleared in xylene, and mounted. Pulmonary fibrosis was assessed in paraffin sections from all animals in each group stained with the Sirius Red F3B (0.1%) and Fast Green FCF (0.1%) in a saturated aqueous solution of picric acid (Gascon-Barre et al., 1989; Nagler et al., 1999). Alveolar and interstitial pulmonary macrophages were identified and counted by immunohistochemistry on paraffin-embedded sections using an avidin-biotin-peroxidase complex method (Hsu et al., 1981) and then counterstained with Carazzi hematoxylin, dehydrated, cleared in xylene, and mounted. Dilutions of the primary and secondary antisera were 1:50 (4 µg/ml) and 1:250, respectively. Antibody specificity was verified by omitting the primary antisera. Macrophages were quantified by counting positively stained cells per random high-power microscopic field (four animals per group and average of four fields per animal).

Quantification of Lung Collagen. Collagen content in lung tissue was quantified by a spectrophotometric method (Marotta and Martino, 1985) utilizing precipitation of collagen with Sirius Red in acetic acid solution. Absorbance values of the dye solution after precipitation with collagen decrease linearly with increasing collagen concentration. After sacrifice, the thoracic contents were removed en bloc. The right lung was separated from the heart, airways, and major pulmonary vessels and homogenized in 0.5 M acetic acid. The homogenate was sonicated at 40 W for 30 s, left on ice for 30 min, and then centrifuged at 1000g for 10 min to remove insoluble debris. Samples (20 µl in 1 ml of 0.5 M acetic acid) and collagen type I standards (0–500 µg/ml in 0.5 M acetic acid) were added to an equal volume of Sirius Red (120 µg/ml) in 0.5 M acetic acid. The mixture was left at room temperature for 30 min and then centrifuged at 3000 rpm for 30 min. Absorbance of samples was measured at 540 nm and plotted against standards with known concentration of collagen type I.

Analysis of Data. Values are expressed as the mean ± S.E.M. Statistical significance (p < 0.05) was determined using Student’s t test or one-way ANOVA with assessment of differences using Duncan’s multiple range test (Snedecor and Cochran, 1980).

Results

Evans blue dye leakage, as a marker of vascular permeability, was quantified and compared between saline alone,
BL in saline, and BL + HX analogs (PBT-1–PBT-4) in saline, as shown in Fig. 1. A significant inhibition \((p < 0.05)\) of the BL-evoked plasma leakage was observed with PBT-1 and PBT-2, which were comparable to saline alone, whereas PBT-3 and PBT-4 had no effect relative to BL alone \((p > 0.05)\).

The histopathological changes from representative mice lung sections stained with H&E are shown in Fig. 2. The lung sections from BL-exposed animals showed severe lesions (Fig. 2B), including diffuse alveolar hemorrhage, edema, and cellular infiltrate in the alveolar space and interstitium, compared with saline-exposed controls (Fig. 2A). Treatment with PBT-1 (Fig. 2, C, D, and E) and PBT-3 (Fig. 2G) markedly attenuated the BL-induced changes. The BL effects were only partially improved in the lungs from groups treated with the other HX analogs (Fig. 2, F and H).

Lung sections stained with Sirius Red for collagen are shown in Fig. 3. The sections from the BL-exposed, saline-treated group (Fig. 3B) showed a marked increase in collagen deposition around vessels and airways (stained red) compared with saline-exposed controls (Fig. 3A). Similar to the improvements seen on H&E staining, treatment with PBT-1 (Fig. 3, C, D, and E) significantly opposed the BL effect, resulting in markedly reduced collagen content. The attenuation of BL-induced collagen deposition increased with increasing dose of PBT-1 (Fig. 3, C, D, and E). PBT-2 (Fig. 3F) and PBT-3 (Fig. 3G) showed some inhibitory effects on BL-induced collagen deposition in the lungs, whereas PBT-4 (Fig. 3H) showed little effect.

Collagen content in the right lungs of animals was greatly increased by BL exposure, compared with saline-exposed controls \((p < 0.05; \text{Fig. } 4)\). PBT-1 and -2 significantly \((p < 0.05)\) opposed the BL effect, whereas PBT-3 and -4 had little effect.
0.05) reduced total collagen compared with BL-exposed, saline-treated controls. PBT-3 and -4 were not effective in preventing lung collagen deposition, which is significantly increased relative to non-BL-exposed, saline-treated animals (p < 0.05).

Alveolar and interstitial macrophage content in the lung was greatly increased by BL exposure, compared with saline-exposed controls (Fig. 5). All the HX analogs significantly (p < 0.05) reduced the macrophage number compared with BL-exposed, saline-treated controls. PBT-1 was also efficacious at lower doses of 10 and 50 µg/day (p < 0.05 compared with BL-exposed saline-treated animals; Fig. 5). PBT-2 was not as effective as the other HX analogs in reducing macrophage number, which are significantly increased relative to non-BL-exposed, saline-treated animals (p < 0.05).

Discussion

Bleomycin, in a single dose administered intratracheally, induces highly reproducible inflammatory and fibrotic pulmonary changes in mice (Cooper et al., 1988), which has been useful in directing research toward treatment strategies for pulmonary fibrosis in humans (Cooper, 2000). The early lesions include diffuse alveolar hemorrhage and marked accumulation of numerous lymphocytes, plasma cells, and macrophages. Subsequently, increased numbers of fibroblasts secrete a collagenous extracellular matrix that progressively thickens the alveolar interstitium and severely compromises pulmonary function.

The mechanisms of HX protection against lung fibrosis may include the following. First, previous studies have dem-
PBT-2 to -4 (100 μg/day i.p.) were evident at a dose as little as 10 μg/day i.p. PBT-2, but not PBT-3 or -4, was also a potent inhibitor of collagen deposition when measured in the total lung. Of note, we found that the effects of PBT-2 on macrophage accumulation were reduced in comparison to its high potency against BL-induced vascular permeability in the skin and lung collagen deposition. Since pulmonary collagen deposition induced by BL has been suggested to involve plasma extravasation (Thrall et al., 1979), we believe that PBT-1 and -2, may have inhibited collagen deposition by attenuating BL effects on vascular permeability. This may also explain the lack of efficacy of PBT-3 and -4, which we found to be ineffective in preventing BL-induced increased vascular permeability in the skin. Our finding that all of the HX analogs were effective (PBT-2) less than the others) inhibitors of macrophage influx indicate that macrophages may not be directly related to increased vascular permeability and collagen deposition induced by BL. The precise mechanisms by which the HX analogs exert their effects in vivo and an understanding of structure-activity relationships of the different analogs require further elucidation. We conclude that these novel compounds warrant further investigation as potential therapeutic agents for disorders involving increased vascular permeability, inflammatory cell influx, and fibrosis.

**References**


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