Fluoromicroscopic Studies of Bleomycin-induced Intracellular Oxidation in Alveolar Macrophages and Its Inhibition by Taurine

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The mechanism of bleomycin-induced pulmonary fibrosis is not yet clear. Recent studies have shown that alveolar macrophages (AM) can be stimulated by bleomycin in vitro releasing inflammatory cytokines, suggesting that the interaction of bleomycin with AM is an important step in the drug-induced fibrotic process. Bleomycin is known to bind DNA and generate oxygen radicals through complexation with Fe²⁺ and oxygen. To provide more insight into the cellular oxidative property of bleomycin, we have developed a fluoromicroscopic method using 2,7-dichlorofluorescin diacetate (DCFHDA) as an oxidative fluorescence probe to study the bleomycin-induced intracellular oxidation in rat AM and the inhibition of the oxidation by taurine, a compound known to inhibit the bleomycin-induced fibrosis. Bleomycin at 5 to 20 µg/ml has a moderate stimulatory effect (1.87 to 2.66-fold) on the secretion of superoxide anion. A high concentration of bleomycin (20 µg/ml), however, inhibits cell response to zymosan-induced secretion of superoxide anion. At 4 µg/ml, bleomycin has no effect on cell membrane integrity or morphology but results in a significant increase in intracellular oxidation. This oxidative process is Fe²⁺-dependent and is accompanied by an increase in intracellular calcium (35 nM). Both the intracellular oxidation and calcium rise induced by internalized bleomycin are inhibited by pretreatment of cells with varying concentrations of taurine (25, 125, and 187.5 µM). The inhibitory effect on intracellular oxidation was found to be 36, 57, and 60%, respectively. These results demonstrate a stimulatin-inhibition relationship between bleomycin and taurine on the cellular oxidation at a subcytotoxic dose of bleomycin, suggesting that the oxidative effect of the intracellular bleomycin-Fe²⁺ complex is important in the initiation of the fibrotic process. — Environ Health Perspect 102(Suppl 10):91–96 (1994)

Key words: taurine, bleomycin, DCFHDA, intracellular oxidation, alveolar macrophages

Introduction

The pulmonary system is most susceptible to oxygen radical-induced diseases due to its constant exposure to the highest oxygen tension, and the potential encounters with oxidant gases such as ozone, nitrogen oxides, and toxic doses of dust particles. The lung is also a well-perfused organ and thereby vulnerable to the effect of circulating xenobiotics such as parquat, and, bleomycin, which is known to cause pulmonary injuries (1). Alveolar macrophages (AM), which act as a means of defense by phagocytizing inhaled dust particles and microorganisms while increasing production of reactive oxidative species, have been shown to play an important role in mechanisms leading to oxidative lung injury and fibrosis (2–4).

Bleomycin is a glycopeptide antineoplastic agent that acts by forming intracellular bleomycin-Fe²⁺ complexes capable of producing oxygen free radicals and causing DNA damage (5,6). The therapeutic value of this drug is greatly limited by its association with the development of pulmonary fibrosis. Although the mechanism by which bleomycin causes fibrosis is not known, the oxidative property of the drug cannot be overlooked, particularly in light of the fact that other fibrotic stimulants such as silica dust (SiO₂) also generates oxygen radicals (7). Recent studies by Scheule et al. (8) showed that bleomycin at subcytotoxic doses induced macrophage production of interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) in dose and time-dependent manners, suggesting that the bleomycin-induced fibrosis involves the fibrogenic potentiating activities of alveolar macrophages. It is possible that the cellular secretion of elevated levels of cytokines may be related to the drug’s intracellular oxidative activity.

Several studies have shown that the bleomycin-induced pulmonary fibrosis in experimental animals may be blocked by treatment with exogenous taurine (9,10), a sulfonic acid naturally occurring in high concentrations in most mammalian tissues (11). Although not clear, the action of taurine has been attributed to its antioxidant and membrane stabilizing properties (11). Recent studies by Banks et al. (12,13) indicate that there is an active uptake of taurine by alveolar macrophages that results in an inhibition of oxidative cell injury caused by ozone exposure. These results suggest that taurine may be used to study the potential relationships between intracellular oxidative events and the fibrotic process.

The most significant and deleterious consequences of oxidation, such as DNA strand breakage (5), damage to the cytoskeleton (14,15), and a rise in intracellular calcium (16), occur intracellularly and can lead to a cascade of cellular events resulting in cell death. Detection of intracellular oxidative radicals is difficult as they are highly reactive and extracellular modes of detection such as luminol-derived chemilu-
mines or electron spin resonance signals rely on detection of extracellularly generated radicals (17) or leakage from a compromised cell membrane. These methods may detect injury at a relatively late stage in its evolution. Alternatively, intracellular oxidation can be monitored using 2',7'-dichlorofluorescin diacetate (DCFDA) as a fluorescence probe to provide information on the initial mechanism of the oxidative process (18). DCFHDA is a membrane permanent molecule that can be hydrolyzed by nonspecific esterases to yield 2',7'-dichlorofluorescin (DCFH), a polar compound that is retained in intact cells. Upon oxidation, DCFH is converted to the highly fluorescent 2',7'-chlorofluorescein (DCF), which remains in intact cells and can be measured for quantitative assessment of intracellular oxidation (19,20). Using this fluorescence probe, we report in the present study a fluorimicroscopic method for the determination of bleomycin-induced intracellular oxidation in AM and the inhibition of the bleomycin effect by taurine.

Materials and Methods

Alveolar macrophages were harvested from male Sprague-Dawley rats (225–250 g, Hilltop Labs, Scottsdale, PA) by bronchoalveolar lavage. Rats were anesthetized by ip injection of sodium pentobarbital (0.2 g/kg bw). The trachea was cannulated and the lungs were lavaged 10 times with 8-ml aliquots of phosphate-buffered saline (145 mM NaCl, 5 mM KCl, 1.9 mM NaH₂PO₄, 9.35 mM Na₂HPO₄, 5.5 mM glucose, pH 7.4). Lavage cell suspensions were centrifuged at 500g for 5 min at 4°C. The cell pellets were then washed twice by alternate resuspension and centrifugation in HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 5.5 mM glucose, 1 mM CaCl₂, pH 7.4). Cell viability was measured by trypan blue exclusion and was typically found to be >95%. The cell density was adjusted to 3 x 10⁵ cells/ml.

One hundred microliter aliquots of cell suspensions obtained as described above were incubated with 5 μM DCFHDA (Molecular Probes, Eugene, OR), 2% fetal bovine serum in HEPES buffer (heat inactivated) (Sigma Chemicals, St. Louis, MO) for 1 hr on 25-mm glass coverslips. Measurement of the intracellular DCF was made in the cell monolayer regions with a density of about 120 cells per observing area. The use of DCFHDA as an intracellular probe for oxygen radical-mediated oxidation has been previously reported (20). Taurine (Sigma) in doses of 25, 125, or 187.5 μM was added to the cells at this time. Care was taken to ensure that taurine or serum added did not affect fluorescence probe uptake. At the end of 1 hr the excess probe and nonadherent cells were washed away gently and cells were placed in a well with the glass coverslips as the bottom of the well. The oxidation of fluorescent probe was monitored fluorimicroscopically by focusing on the cells under a Nikon Diaphot fluorescence microscope using the Spex-CM system, and recording the signal obtained in the single wavelength mode with the excitation filter set at 450 to 490 nm and emission set at 520 to 560 nm. Fluorescence signals were obtained at 5-min intervals. After measurements of basal fluorescence for 15 min, treatments of control and taurine-pretreated cells with oxidative agents such as 10 μM hydrogen peroxide, 5 μM A23187 (Molecular Probes, Eugene, OR), or bleomycin (4 μg/ml) were made and the fluorescence monitored as a function of time. Cell viability was monitored during and at the end of each experiment using propidium iodide (PI, 1 μg/ml) as a membrane-damage fluorescence probe (21).

For the measurement of intracellular free calcium (16), AM cultures were incubated with 5 μM Fura-2 AM in HEPES buffer with 2% FBS for 1 hr at 37°C and washed with the same buffer to remove free dye and nonadherent cells. (Stock solution of Fura-2 AM was made by dispersing 50 μg Fura-2 AM in 100 μl of 20% w/v of Pluronic F-127 in dimethyl sulfoxide.) The fluorimicroscopic measurement of Fura-2 was carried out using the Spex-CM system in the dual wavelength mode. Excitation light was provided by two monochromators preset at 340 and 380 nm and selected in rapid alteration (100 Hz) by a rotating chopper mirror. Emitted light was collected through a 510-nm interference filter and photon counted in synchrony with the chopper so that the signals from the two excitation wavelengths were stored in separate memories of a Spex Datamate microcomputer. The ratio of fluorescence excited at 340 nm to that at 380 nm was used as an indicator of the intracellular calcium rise in response to stimulation by 4 μg/ml bleomycin in the control and taurine-treated cells. At the end of each experiment, 2 μg/ml digitonin and 10 mM EGTA were added to ensure the responsiveness of the system. Determination of intracellular calcium concentrations was made using the fluorescence ratio according to the method previously reported (22). Oxygen consumption was measured with an oxygraph equipped with a Clark electrode (23). Rat AM (4 x 10⁶) were preincubated at 37°C in 1.75 ml of HEPES-buffered medium for 10 min. After preincubation, the cell suspension was transferred to a temperature-controlled chamber (37°C) and resting oxygen consumption was monitored. Then 125 μM taurine was added to determine the effects of taurine on oxygen consumption. Particle-stimulated oxygen consumption was determined by addition of zymosan (2 mg/ml) to cells preincubated for 5 min at 37°C in the absence or presence of taurine. The oxygraph was calibrated using media equilibrated with gases of known oxygen content.

Superoxide anion release was determined spectrophotometrically at 550 nm as the reduction of cytochrome c as reported by Sweeney et al. (24). Briefly, AM (4 x 10⁶) were preincubated at 37°C for 10 min in 6 ml of HEPES-buffered medium containing 0.12 mM cytochrome c. After preincubation, cells were treated with bleomycin (1–20 μg/ml) and/or zymosan (2 mg/ml) and superoxide release measured over the next 30 min. Absorbance changes were converted to nmol of reduced cytochrome c using an extinction coefficient of 18.7 mM⁻¹ cm⁻¹.

Results

Effect of Bleomycin on Macrophage Oxidative Burst

A common response of AM to particle and molecular stimulants, such as silica, zymosan, and phorbol esters, is the generation of reactive oxygen species with increased oxygen consumption. Since hyperoxic conditions have been shown to potentiate bleomycin-induced fibrosis (25), the potential effect of bleomycin on the secretion of superoxide anion by AM was investigated. Table 1 shows that 5 to 20 μg/ml bleomycin has a moderate stimulatory effect (1.87–2.66 times the control) on the secretion of superoxide anion by otherwise nonchallenged (resting) AM. The drug

| Table 1. Effect of bleomycin on the secretion of superoxide anion from alveolar macrophages. |
|-----------------------------------------------|
| Concentration of bleomycin (μg/ml) | Superoxide release, % control | Zymosan-stimulated |
|-----------------------------------------------|
| 0 | 100 | 100 |
| 1 μg/ml | 183 ± 33 | 102 ± 8 |
| 5 μg/ml | 266 ± 26b | 95 ± 8 |
| 10 μg/ml | 187 ± 15b | 88 ± 9 |
| 20 μg/ml | 157 ± 18b | 70 ± 21b |

*Mean of seven experiments. **Statistically different from control.
show that AM stimulation.

Determination and Characterization of Intracellular Oxidation

Because bleomycin acts by binding with Fe²⁺ of intracellular sources in the generation of oxygen free radicals, studies were carried out to examine whether intracellular oxidation may be induced by subcytotoxic dose of bleomycin, and if such events can be correlated with mechanisms related to cellular injury such as the intracellular calcium homeostasis, or inhibited by pretreatment of cells with taurine. Table 1 shows that bleomycin concentrations of 5 µg/ml or lower exhibit only a moderate effect on the zymosan-stimulated oxidative burst activity of AM, while on resting AM, a dose of 5 µg/ml exhibited maximum stimulation. At these concentrations, cells remain viable and show no morphologic changes as indicated by scanning electron micrographs (data not shown). Based on these results, the measurement of intracellular oxidation was carried out using a concentration of 4 µg/ml bleomycin.

AM monolayers preloaded with DCFHDA were prepared for fluoromicroscopic examinations. The use of DCFHDA as an intracellular probe for oxygen radical-mediated oxidation has been previously reported (20). This probe undergoes deacetylation to form DCFH, which can be oxidized to yield the fluorescent DCF. Both DCFH and DCF remain in intracellular spaces in intact cells due to their relatively low lipophilicity. The reactivity of DCFH with hydrogen peroxide has been well studied (26). It has also been shown to react with superoxide anion and hydroxyl free radicals (19) but is resistant to autoxidation (18). Hence, DCFHDA is a useful probe for the study of bleomycin-induced intracellular oxidation. Figure 1 shows the generation of intracellular fluorescence in macrophages by hydrogen peroxide in the absence and presence of 200 µM FeSO₄. Hydrogen peroxide can permeate the cell membrane, thereby causing intracellular oxidation. Visual observation through the fluorescence microscope revealed fluorescence trapped within the cytosol. The hydrogen peroxide effect at the concentrations used did not cause cell membrane damage as indicated by absence of PI fluorescence. In the presence of Fe²⁺, intracellular DCF fluorescence reached a maximum, indicating the generation of intracellular hydroxyl free radicals through the Fenton reaction. These results indicate that the fluoromicroscopic method is sensitive for the determination of intracellular oxidation caused by oxygen radicals. Figure 2 shows that alveolar macrophages exhibit a significant increase in their intracellular DCF fluorescence when treated with 5 µM of the Ca²⁺ ionophore A23187. The Ca²⁺ ionophore is known to initiate a calcium influx and to result in the activation of the protein kinase C-dependent cytochrome b₂₄₅ kinase (27). The results shown in Figure 2 suggest that the oxidative process may be induced by a rise of intracellular free calcium.

Effect of Bleomycin and Taurine on Intracellular Oxidation

Figure 3 shows that treatment of AM with 4 µg/ml bleomycin resulted in an appreciable increase in the fluorescence generated. At this concentration, bleomycin did not affect cell viability or morphology as confirmed by the PI exclusion and scanning electron micrographs (data not shown). The bleomycin-induced increase

Figure 1. Effect of H₂O₂ and Fe²⁺ on the intracellular DCF fluorescence in AM as a function of time. (A) effect of 10 µM H₂O₂. (B) effect of 10 µM H₂O₂ in cells preincubated with 200 µM FeSO₄. Arrow indicates point of addition of H₂O₂. Y-axis denotes change in fluorescence from the initial time point (cps x 10⁴).

Figure 2. Induction of intracellular fluorescence in AM by Ca²⁺ ionophore A23187. (A) control, (B) 5 µM A23187. Arrow indicates time of addition of the ionophore.

Figure 3. Effect of bleomycin (4 µg/ml) on the intracellular DCF fluorescence in AM. (A) control, (B) bleomycin-treated. Arrow indicates addition of bleomycin or sham treatment by addition of an equal volume of HEPES buffer.
in intracellular fluorescence appears to follow a time-delayed sigmoidal curve, suggesting events such as internalization and subsequent activation of drug may precede the increase in DCF fluorescence. This is likely, since AM have receptors for bleomycin allowing for the internalization of the drug molecule (28). Other studies have shown that bleomycin possesses a bithiazole ring that can intercalate into the DNA helix, and Fe$^{2+}$ binding sites involving the pyrimidine and imidazole moieties that are capable of generating oxygen radicals in the vicinity of the DNA molecule (5,6). The role of Fe$^{2+}$ in bleomycin-induced intracellular oxidation is shown in Figure 4. When AM were concurrently treated with bleomycin and 200 μM FeSO$_4$ (curve C) the intracellular fluorescence reached a maximum in a fashion similar to that of the Fe$^{2+}$-H$_2$O$_2$ effect. This indicates that the internalization of bleomycin, already complexed with Fe$^{2+}$ and possibly oxygen, has a strong and immediate effect on intracellular oxidation. The complexation of bleomycin with Fe$^{2+}$ may also facilitate the internalization of the drug as well as Fe$^{2+}$. As shown in Figure 4, curve B, when Fe$^{2+}$ was added after the bleomycin effect, there is a significant but relatively moderate increase in the Fe$^{2+}$-related intracellular fluorescence. These results show that Fe$^{2+}$ plays a key role in the cellular oxidative activation of bleomycin, and that the measurement of intracellular DCF fluorescence is a direct indication of the drug’s pharmacologic and toxicologic activity.

The effect of taurine on bleomycin-induced intracellular oxidation was studied using AM pretreated with 25, 125, or 187.5 μM taurine. Figure 5 shows the typical fluorescence-time curves measured in control and taurine-treated cells in response to bleomycin stimulation. Since cells exhibit varying basal levels of intracellular fluorescence due to their phagocytic property, quantitative evaluations of the effects of taurine and/or bleomycin on intracellular fluorescence were made by measuring the slope changes in response to bleomycin, expressed as the ratio of drug-treated slope versus the initial slope of the fluorescence-time curve, and by measuring the area under the curve (AUC) corrected from the basal fluorescence of nontreated cells. The results are summarized in Table 2. Taurine did not have any appreciable effect on the oxygen consumption or the intracellular DCF fluorescence change of resting AM (data not shown). However, as can be seen from Table 2, this aminosulfonic acid has significant inhibitory effect on bleomycin-induced intracellular oxidation. In cells pretreated with 25 μM taurine, the inhibitory effect was found to be 35%. At higher doses (125 and 187.5 μM), about 60% of the bleomycin-induced intracellular oxidation was blocked by internalized taurine.

The antioxidative effect of cytoplasmic levels of taurine has been observed in lungs of hamsters treated with tracheal instillates of bleomycin (9,10) and in rat lungs exposed to ozone (12). Taurine is normally found in millimolar concentrations, especially in tissues that are excitable and generate oxidants. Active uptake of taurine by AM is sodium- and energy-dependent and can achieve an intracellular concentration of 4.27 mM (13). Studies have shown that intracellular taurine exists in a rapidly exchangeable free pool and in a slowly exchangeable bound pool (29). It is proposed that the antioxidant effect may be due to the ability of free taurine to react with hypochlorous acid to form taurine chloramine (11), and the ability of bound taurine to exert a stabilizing effect on the plasma membrane and cause formation of gap junctions in alveolar epithelia (30). Both of these effects may contribute to its inhibitory action on bleomycin-induced intracellular oxidation.

### Effects of Bleomycin and Taurine on Intracellular Calcium

Figure 6 shows that bleomycin-induced intracellular oxidation is accompanied or preceded by a rapid rise in cytosolic calcium (curve A). This effect, which represents a moderate increase in calcium concentration of 35 nM, appears to be transient and reversible and is inhibited by pretreatment of cells with 125 and 187.5 μM taurine (curves B and C). The concentration of bleomycin used in these experiments was 4 μg/ml, which did not cause any membrane damage as indicated by PI

### Table 2. Effect of taurine on bleomycin-induced intracellular oxidation in alveolar macrophages as indicated by the formation of DCF from DCFHDA*

| Taurine, μM | Bleomycin, μg/ml | Intracellular fluorescence of DCF | Yield, % ± |
|------------|------------------|----------------------------------|------------|
| 0          | 0                | 1.02 ± 0.60                      | –          |
| 0          | 4                | 3.00 ± 0.74                      | 100        |
| 25         | 4                | 2.71 ± 0.63                      | 64.2 ± 10.8|
| 125        | 4                | 1.41 ± 0.74                      | 43.2 ± 9.2 |
| 187.5      | 4                | 1.78 ± 0.46                      | 39.7 ± 5.4 |

*Mean of four experiments. *R* is the ratio of the slopes of fluorescence curves denoting bleomycin-induced increase over the control. *C* Calculated as the area under the fluorescence curves corrected from control to indicate bleomycin stimulation.
Discussion

The mechanism of bleomycin-induced fibrosis is not yet clear, but studies suggest that the generation of oxygen radicals by the bleomycin-Fe$^{2+}$ complex may play an important role in the initiation of the fibrotic process. There are, however, few studies regarding the cellular oxidative events caused by bleomycin. It is also not clear whether direct oxidant cell injury is linked to fibrosis. This lack of information is due, at least in part, to the need of a sensitive method for the detection of cellular oxidation at subcytotoxic drug concentrations. The present study reports a fluorimicroscopic method using DCFHDA as a fluorescence probe for the quantitation of oxygen radical-induced intracellular oxidation. This method is sensitive and allows one to measure oxidative events in cells that remain intact and viable. It further offers the advantage that other events in relation to the oxidative process such as intracellular calcium mobilization and membrane integrity can be concurrently determined by fluorimicroscopy for mechanistic studies.

Studies have shown that bleomycin stimulates the macrophage release of IL-1β and TNF-α in vitro, suggesting that the interaction of bleomycin with AM is an important step in the mechanism of bleomycin-induced pulmonary fibrosis (8). AM have been shown to exhibit high- and low-affinity binding sites for bleomycin (28). The results of this study show that bleomycin has a moderate stimulatory effect on macrophage production of superoxide anion but inhibits the stimulation of superoxide anion release by zymosan. These results are consistent with those of Scheule et al. (8) that show that bleomycin also inhibits the phorbol ester-mediated macrophage release of superoxide anion.

These studies indicate that the fibrogenic activity of bleomycin is probably not due to the excessive stimulation of macrophage respiratory burst. However, it is clear that bleomycin interacts with AM and alters the macrophage function. Given the DNA-binding and oxidative properties of this drug, it is possible that the oxidative action brought upon by the internalized bleomycin is responsible for the cellular production of fibrogenic cytokines.

The bleomycin-mediated intracellular oxidation is Fe$^{2+}$-dependent and occurs in the absence of extracellular Fe$^{2+}$. This suggests that the internalization of bleomycin is required for the observed oxidation. The bleomycin effect is also associated with a rapid increase in intracellular calcium that appears to precede the oxidation, suggesting that intracellular calcium mobilization may play a role in the internalization of the drug. This is possible since calcium mobilization is required for the cytoskeletal control of plasma membrane transport and a compound such as taurine, known to decrease cytosolic calcium, can serve to stabilize the plasma membrane. The fact that cellular loading of taurine prior to the bleomycin treatment can inhibit both the intracellular calcium rise and oxidation indicates that these two events are sequentially related. Taurine is a naturally occurring sulfur-containing amino acid that has been shown to have a marked in vivo inhibitory effect on bleomycin-induced fibrosis (9,10). The results of the present study demonstrate a stimulatory and inhibitory relationship of bleomycin and taurine on macrophage activity even at a low dose of bleomycin that does not cause membrane damage or a change in cell morphology. This suggests that the oxidative effect of bleomycin may play an important role in the initiation of the bleomycin-mediated fibrotic process.

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