Lucigenin chemiluminescence assay as an adjunctive tool for assessment of various stages of inflammation: A study of quiescent inflammatory cells

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A simple, fast, precise and biologically relevant toxicity assay for screening cytotoxicity of minerals would have distinct advantages due to its cost benefits and relative savings in time. Furthermore, a bioassay to differentiate acute and chronic *in vivo* pulmonary reactions could have potential value as predictors of fibrogenicity and pathogenicity. In this study we examined the potential use of lucigenin as a probe to evaluate the correlation between chemiluminescence (CL) generated by alveolar macrophages with the known cytotoxicity and pathogenicity by conventional bioassays. In this study, we used small doses of dust (20 μg) to minimize cellular overload and to maintain homeostasis. Crystalline silica a highly fibrogenic dust was used as positive control and results are compared with those for bentonite, kaolin and talc. Among the three minerals compared with silica, bentonite was more reactive (27%) in CL assay and declined sharply compared to other minerals. This sudden decline in bentonite CL is caused by cytotoxicity leading to cell death. CL-induced by talc was comparable to silica and declines slowly. Kaolin on the other hand produced relatively a weaker (25%) CL compared to silica. Our data using relatively low doses of dust suggest that the CL assay may have a better predictive value in cytotoxicity evaluations compared to conventional toxicity assays.

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1. Introduction

Several decades of intensive research have produced a valuable and complex body of investigation showing that some toxic occupational and/or environmental dusts are either fibroproliferative or carcinogenic agents. However, toxicological data available to assess potential risk to humans is often controversial or require extrapolations. Furthermore, a large number of extensive toxicity evaluations are required to determine the value of bioassays as predictors of their potential fibroproliferative or carcinogenic nature. In addition, *in vitro* toxicity data often do not correlate with *in vivo* responses in different species and epidemiologic studies in man. This may be partly due to the relatively simple interactions of particles with isolated single cells vs the more complex interactions (lipid mediators, chemokines, cytokines and enzymes) that can occur with particles in tissues over time.

However, a simple, fast and precise, chemically-based assay for screening dusts would have a distinct advantage due to its relative cost benefit and time-saving characteristics. Luminescence is a widely used investigative tool

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Abbreviations used: CL, Chemiluminescence; HBSS, Hank's balanced salt solution; $^{\bullet}O_2^-$; oxidants superoxide; TNF-α, tumour necrosis factor-alpha.

in toxicological, clinical and diagnostic studies (Antonini *et al* 1994; Van Dyke *et al* 1994, 2002). A luminescence-based assay could be used with single cells, tissues or cells which are exposed *in vivo* and assayed *in vitro* a day or more later. The assay is straight forward, and excellent quantitation and reproducibility can be obtained is short time.

Current concepts involved in the development of occupational and environmental dust-associated pulmonary disease suggest that a crucial initial step is particle-based activation and/or injury of pulmonary alveloar macrophages. We have shown that exposure of alveolar macrophages to silica *in vitro* or *in vivo* can result in toxic reactions resulting in leakage or production of cellular enzymes, cytokines, chemokines, and major increases in the production of oxidative and nitrative substances (Vallyathan *et al* 1988a; Castranova *et al* 2002). The exact mechanisms of disease development are unclear, but our studies have shown generation of oxidants by the phagocytosis of minerals play an important role in triggering the secretion of mediators of inflammation, and signalling events and activation of transcription factors important in disease development. Therefore stimulation of phagocytes leading to the production of reactive oxygen species (ROS) measured by chemiluminescence (CL) may provide rewarding insights into the initial molecular basis of development which may assist in the design of prevention strategies.

A key to understanding and preventing the main toxicity of silica, leading to fibrosis, is control of the three stages of the inflammatory process caused by silica (figure 1). The first is a subacute or immediate phase leading is linked to vanillin receptors which bind capsaicin. The second is an acute stage in which inflammatory proteins are induced by nuclear induction factors like activator protein (AP-1) (Ding *et al* 1999) and nuclear factor kappa B (NF-*k*B) (Sacks *et al* 1998). Inhalation of silica stimulates the release of a variety of substances, including lipid mediators, proteins, enzymes, cytokines, chemokines, and the oxidants superoxide ($^{\bullet}O_2^-$) and nitro compounds (peroxynitrite). The oxidants and nitrative compounds produce oxidative and nitrosative stress. During this process, antioxidants are consumed. If steroidal antiinflammatory drugs are administered to an exposed animal (Van Dyke *et al* 1994) or to man (Goodman *et al* 1992) during an 8 h therapeutic window, they are effective in partially blocking silica-based inflammation, and fibrosis. However, they must be continuously administered after silica exposure and this can produce a variety of untoward side-effects. Non-steroidal anti-inflammatory drugs can be used as well, but although they reduce pain-associated inflammation, they do not block induction of inflammatory secretions and proteins. A most effective method to block the inflammatory cascade is to use antagonists of tumour necrosis factor alpha (TNF-*a*), e.g. antibodies against TNF-*a* or using the TNF-*a* receptor protein called Etanercept (Shanahan and St Clair 2002). This is an injectable-protein substance which when combined with methotrexate therapy produces a disease-modifying process which greatly alleviates this form of inflammation. There is information available which suggests that antibodies to TNF-*a* can partially block fibrosis from silica particles which occurs in the lung (Piguet *et al* 1990). The third portion of the inflammatory process produced by silica is the chronic stage which produces fibrosis. This is much more difficult to treat, and there are no effective approved drugs for treatment. There is literature which suggests that the Chinese drug tetrandrine is partially effective as an antifibrotic agent (Li 1981). It is believed that a major factor in the development of fibrosis is production of copious amounts of peroxynitrite by nitric oxide (NO) (Sacks *et al* 1998; Castranova *et al* 2002). Therefore, anything which interferes with production of peroxynitrite might be partially effective against fibrosis.

Lucigenin is used commonly to increase the sensitivity of cellular CL assays. It has been reported to react almost exclusively with $^{\bullet}O_2^-$ if reactions are accomplished under

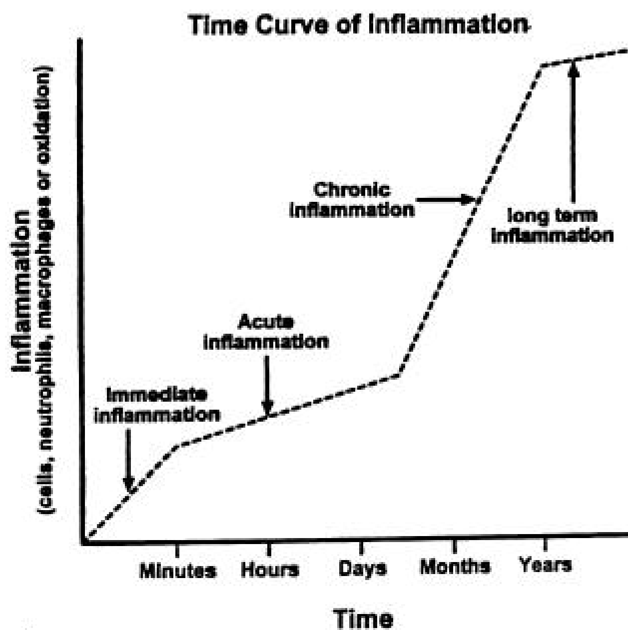

Figure 1. Time curves of the various states of inflammation. It can be observed that there are three major states of inflammation: (i) Immediate inflammation, (ii) acute inflammation and (iii) chronic inflammation (fibrosis). These states are marked in time as well as in the extent in which cells generate metabolites. The first two states are amenable to control by drugs, as indicated in the text. The chronic state is more difficult to treat and no approved drugs are available. This is a composite drawing taken from Castranova *et al* (2002).

the correct conditions (Trush and Li 2002). Since lucigenin reacts mostly with $^{\bullet}O_2^-$, it represents an important portion of peroxynitrite (OONO–). The source of $^{\bullet}O_2^-$ from macrophages in the subacute inflammatory phase is mitochondria (Trush and Li 2002). The source of the $^{\bullet}O_2^-$ in the acute phase is both from mitochondria and membrane-associated NADPH-enzyme complex (figure 2). However, it is reported that in the chronic stage of inflammation, the major source of $^{\bullet}O_2^-$ is the mitochondria of the macrophages, and that the NADPH oxidase system can be shut down due to the toxicity of peroxynitrite formed in the chronic stage. In figure 2 it is illustrated that mitochondria during the chronic stage of inflammation is actually a much greater source of $^{\bullet}O_2^-$ than is the NADPH-oxidase complex. An advantage of lucigenin is that not only does it cross the plasma membrane of macrophages to measure both internal and external oxidants, it also penetrates the mitochondria to measure $^{\bullet}O_2^-$ generated within this organelle. Therefore, lucigenin is a valuable probe in all three states of inflammation. Since toxic particles like silica cause all three stages of inflammation over time, lucigenin luminescence is particularly useful adjunct tool.

2. Materials and methods

2.1 *Silica and other minerals*

Crystalline silica (Min-U-Sil) was obtained from Pennsylvania Sand and Glass Corporation, Pittsburgh, PA, USA. The other minerals were obtained in native form from primary producers. All the minerals were size fractionated to < 5 μm using a particle classifier. Purity of all

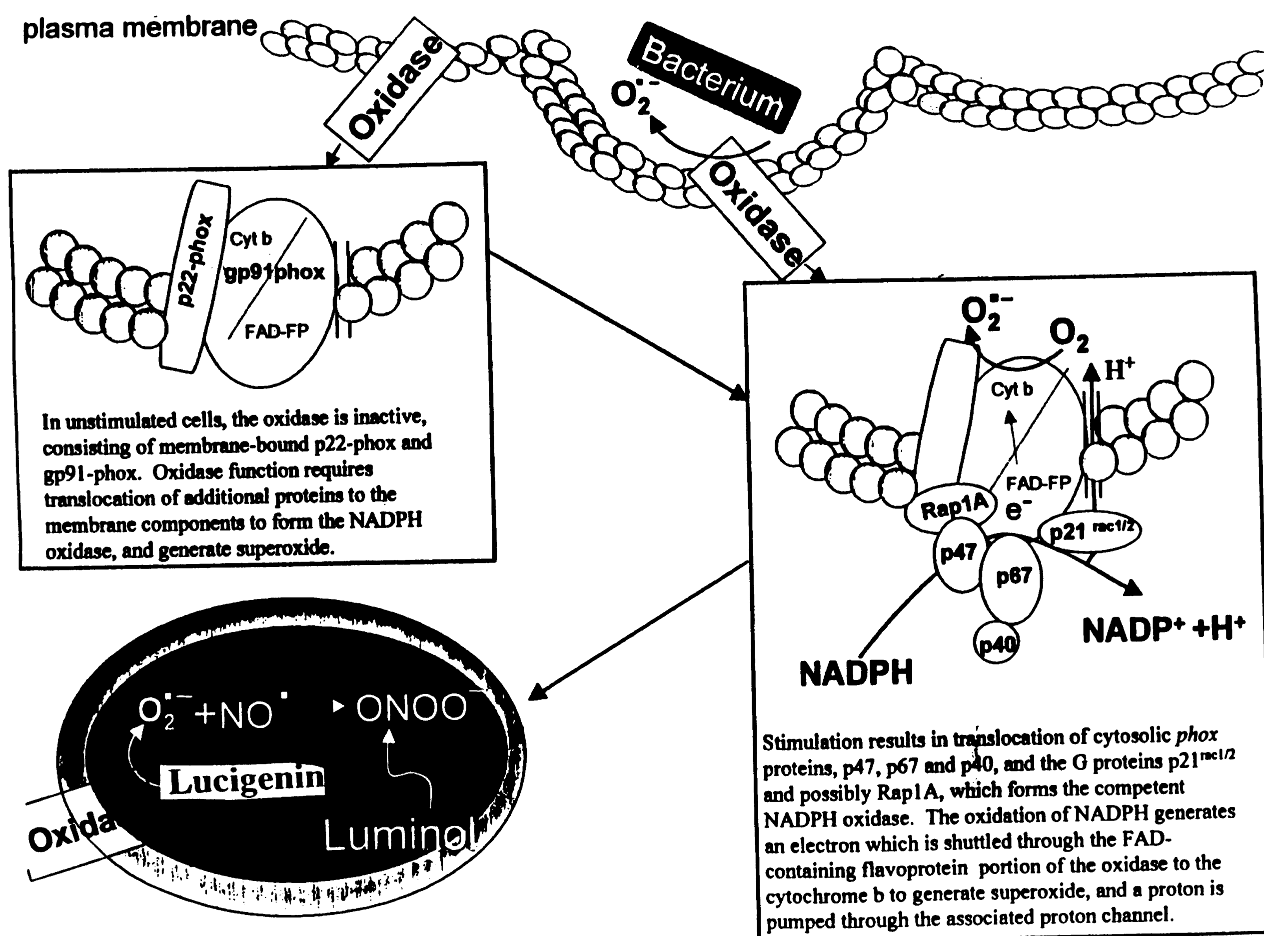

Figure 2. The different states of NADPH oxidase which are involved in the generation of superoxide in the acute stages of inflammation. In the quiescent or dormant cells, this system produces little or no superoxide. In the active, state there is translocation of proteins, generation of an electron from oxidized NADPH and a proton is pumped from inside the cell to the outside via an associated proton channel. To produce luminescence lucigenin reacts with superoxide and luminol reacts with peroxynitrite. Luminol produces blue light, while light from lucigenin is yellow-green.

the minerals was determined by elemental analysis using X-ray spectrometry and photon-induced X-ray emission analysis (PIXE) or atomic absorption spectrometry. All the minerals used were relatively pure (crystalline silica 98·7%, bentonite 96·0%, kaolin 99·6% and talc 98·3%).

2.2 *Macrophage isolation*

Sprague-Dawley pathogen free male rats alveolar macrophages were harvested by bronchoalveolar lavage using calcium-free and magnesium-free Hank's balanced salt solution (HBSS). Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip; Butler, OH, USA) and euthanized by exanguination of the abdominal aorta. The trachea was cannulated and the lungs were lavaged 10 times with 8 ml of HBSS and were combined and centrifuged at 500 *g* for 5 min at 2°C. Cells were washed carefully discarding medium, and were resuspended and centrifuged as before. Cells were resuspended in HEPES containing 145 mM NaCl, 5 mM KCl, and 10 mM HEPES and 5 mM glucose (pH 7·4). Cell counts and viability were determined using microscopy. Viability was determined by trypan blue exclusion (90–95% viability). These were quiescent macrophages, i.e. they were not activated *in vivo* by an inflammatory stimulant.

2.3 *CL assay*

Samples for CL studies contained the following ingredients in a 500 μl total volume: 100 μl of HEPES buffer pH 7·4 with 1×10^6 alveolar macrophages, 100 μl of $2{\cdot}5 \times 10^{-6}$ M lucigenin in HEPES buffered solution, 100 μl of HEPES-buffered solution containing 20 μg of particles, e.g. silica in HEPES buffer, and 200 μl of HEPES-buffered solution. Data are expressed as counts per minute. The CL was monitored for 30 min in a Berthold model 9505 C luminometer with temperature control set at 37°C. The counts are expressed as counts per min over a 30 min period.

3. Results

In figure 3, the lucigenin CL of quiescent rat alveolar macrophages or macrophages treated with different dusts, e.g. silica, kaolin, bentonite, or talc, is illustrated. Bentonite appears to react avidly with alveolar macrophages, but luminescence decreases almost to zero level in 20 min. This may indicate that bentonite is directly toxic to cells since the rapid decline of luminescence is usually linked to cell death. In previous studies, it has been shown that bentonite is the most hemolytic and cytotoxic of all the dusts tested (Vallyathan *et al* 1988a). Silica and talc exert similar kinetics with a 10 min peak around 14,000 cpm, decreasing to 12,000 cpm at 30 min. Kaolin appeared to produce the least CL, at 10, 20, or 30 min. This pattern of CL for the interaction of dusts and quiescent macrophages shown in figure 3 is a representative of four separate experiments.

4. Discussion

Since lucigenin has been shown to react with mitochondrial-based $^{\bullet}O_2^-$, it may reflect upon the ability of the different dusts to stimulate scavenger receptors on the cells which produce a series of events which disturb the ability of mitochondria to produce $^{\cdot}O_2^-$. In some cases (e.g. with bentonite), the interaction with the macrophages can lead to cell death. In other cases, the macrophages can continue to produce elevated amounts of $^{\bullet}O_2^-$ for some time. Note that control macrophages produce a very low-base line CL, probably reflecting minimal low-generation of $^{\bullet}O_2^-$.

In previous studies, we have shown that surface characteristics of dusts are key factors in the toxicity of dusts

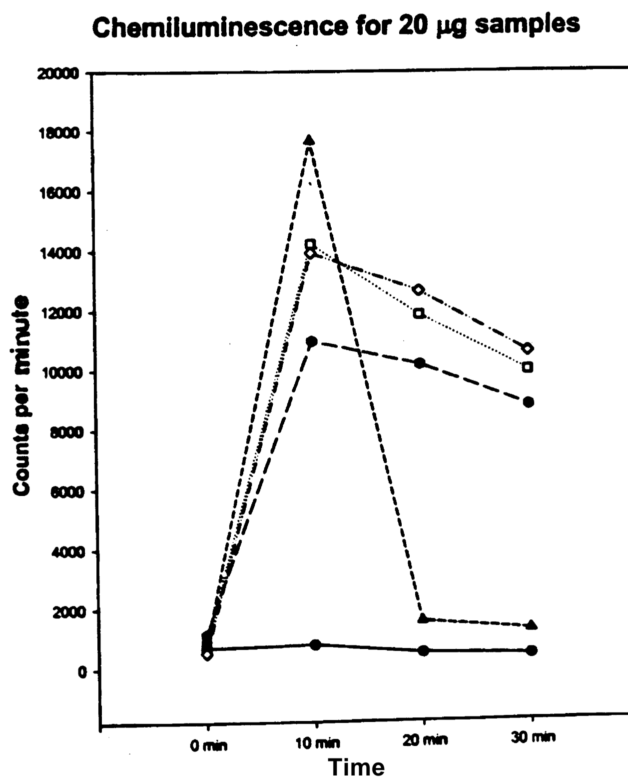

Figure 3. Time curve of CL produced from 10^6 alveolar macrophages with or without particles (20 μg of the different particles were used in tubes containing particles). Particles used were silica, bentonite, talc, or kaolin. CL is expressed as counts per min, and assays are accomplished for 30 min. Data presented is from one single representative experiment of four. (●), Control; (□), silica; (▲), bentonite; (Δ), talc; (- -●- -), kaolin.

(Vallyathan *et al* 1988b; Castranova *et al* 1989). When dusts are freshly fractured, they are even more toxic to cells than aged dusts. This is probably due to the fact that the physical fracture of the dust actually cleaves bonds at the fracture plane of the dust producing siloxyl free radicals. The siloxyl radicals generate highly toxic hydroxyl radicals in aqueous solution (Vallyathan *et al* 1988b; Castranova *et al* 1989). In addition, freshly cleaved silica avidly binds calcium (Van Dyke *et al* 1993) and this high amount of calcium may be released inside the phagocytic macrophage once the particle has been engulfed. We have shown that this is very toxic to macrophages and by chelating excessive calcium a marked decrease in the toxicity of silica can be observed (Van Dyke *et al* 1993).

If the macrophages are exposed to particles *in vivo*, a totally different scenario occurs. Upon contact of the particles with the macrophage surface, transcription factors are activated leading to induction of inflammatory mediators, growth factors, enzymes, cytokines, chemokines, and reactive oxygen and nitrogen species. Certainly, one would not observe chronic inflammation which by definition takes weeks to occur inside an animal (Castranova *et al* 2002). In this CL bioassay using small doses of dust (20 μg) to minimize cellular overload and retaining homeostasis, our results demonstrate bentonite is relatively more toxic compared to kaolin and talc. These results suggest that the CL assay may have a better predictive potential value in evaluating the toxic effects of minerals that alter pulmonary inflammation and integrity.

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