Chapter 23

Inhibition by Antilipoxygenase Drugs of Cellular Chemiluminescence in Silica Activated Phagocytic cells -- Alveolar Macrophages, Human Neutrophils and Human Leukemia (HL-60) Cells+

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ABSTRACT

Since it has been demonstrated that silica caused phagocytic cells to activate lipoxygenase metabolism of unsaturated fatty acids we thought selective inhibition of this metabolic pathway might be helpful in treatment of silicosis. It is known that lipoxygenase metabolism plays a role in the pathogenesis of a variety of inflammatory and hypersensitivity conditions. Further, it is believed that the alveolar phagocytic cells which engulf silica begin the inflammatory cycle which produces the metabolic products, cytokines and growth factors which causes fibrosis in the lung. There have been indications that drugs which inhibit lipoxygenase can inhibit fibrosis from other causes.

Previously, we developed a method which detects the activity of lipoxygenase metabolism by measuring free radical oxidation via light production or chemiluminescence (CL). We decided to apply this method to phagocytic cells that would be found in the lung in the presence of silica with and without newly developed inhibitors of the lipoxygenase reaction.

We used drugs which inhibit lipoxygenase directly including AA861 (TAKEDA), A63162 (Abbott), and an inhibitor that scavenges free radicals produced from the lipoxygenase reaction, nordihydroguairetic acid. In addition we used an indirect inhibitor, tetrandrine.

We found drug inhibition of silica activated CL from alveolar macrophages, neutrophils and human leukemia (HL-60) cells differentiated with DMSO. Nordihydroguairetic acid $2x10^{-5}M$ produced almost complete inhibition in all three cell types. In human leukemia cells (HL-60) A63162 and tetrandrine were equally inhibitory at 42% while in human neutrophils A63162 and tetrandrine were less active 22 and 10% respectively. In macrophages A63162 and AA861 were less than 50% inhibitory to cellular CL at $2x10^{-5}M$. Direct assay of tetrandrine ($2x10^{-5}M$) with sodium arachidonate and soybean lipoxygenase reveals no inhibition.

All antilipoxygenase drugs regardless of mechanism were inhibitory to cellular chemiluminescence from all three types of phagocytic cells. These drugs alone or in mixtures could prove useful in treatment of silicosis.

INTRODUCTION

The exact etiologic mechanism(s) that control the inflammation and fibrogenic consequences of silicosis are not clearly understood. However there have been a variety of studies that pinpoint important steps involving the cells and secretions important in this environmentally induced disease. First, respirable silica particles (0.1-5 microns) are inhaled and find their way into the deep spaces of the lung (1). The particles are engulfed by macrophages and neutrophils which reside in the lung (2). This interaction between the particles and the cells begins the inflammatory response and is known as phagocytosis. This phagocytic mechanism occurs in a series of steps outlined in Figure 1. Phagocytosis triggers the oxidative burst, depolarization of the cell membrane and the release of enzymes and hormones known as cytokines (3). In addition there is breakdown of phospholipids containing arachidonic acid which produces either prostaglandin products via the cyclooxygenase mechanism or the HPETE, HETEs or leukotrienes (4) from the lipoxygenase system (Figure 2). Englen et al. (5) have shown that as the dose of silica to be engulfed increases, the metabolism of alveolar macrophages switches from cyclooxygenase to lipoxygenase metabolism. The controlling mechanism for the switch in the metabolic pathway may be controlled by the level of internal calcium available (6).

We know from the studies of others that silica-activated alveolar macrophages release interleukin I (7), tumor necrosis factor (8) and macrophage derived fibroblast growth factor (9). The release of these cytokines or growth factors may somehow be linked to the lipoxygenase system. Maybe by inhibiting lipoxygenase the cytokines will be similarly affected (10).

5 lipoxygenase is a non-heme, iron-containing enzyme that catalyzes the reaction of molecular oxygen with arachidonate and other unsaturated fatty acids. The amino acid sequence of human 5' lipoxygenase (H5L) is known (11) and the gene for the enzyme has been sequenced. Further, the activation of this enzyme requires a membrane bound protein known as five lipoxygenase activating protein (FLAP) as well as adenosine 5' triphosphate (ATP) and calcium (12,13). When the

mechanism is activated, the cytoplasmic H5L moves to the membrane and binds to the FLAP protein in the presence of ATP and calcium. Additional factors may be important for the arachidonate metabolism by H5L (14). There is controversy regarding whether arachidonate is released from the phospholipid before lipoxygenase metabolism or whether the 5HL enzyme can react directly with the phospholipid containing arachidonate (15). Maybe both reactions occur.

Since we developed a method (16) to measure the free or cellular 5' lipoxygenase using luminol chemiluminescence (CL), we decided to measure the effects of various known lipoxygenase inhibitors against the cell activation by silica. However, with human neutrophils the silica should be opsonized (coating of particles with antibodies and complement) before reaction. In the case of macrophages or differentiated HL60 cells silica opsonization was not required.

The idea we researched in this paper was to see if various antilipoxygenase drugs could be effective inhibitors of silica activated cellular chemiluminescence from alveolar phagocytic cells linked to lipoxygenase. Ultimately we would like to determine whether these inhibitors could effectively block the inflammation and fibrosis caused by silica in vivo.

MATERIALS AND METHODS

MATERIALS

CELLS AND CULTURE CONDITIONS. The human promyelocytic HL-60 leukemic cells were provided by Dr. John Durham, Medical Center, West Virginia University (this cell line was originally isolated from a leukemic patient by Robert C. Gallo, National Cancer Institute, Bethesda, MD). The cells were cultured in corning polystyrene tissue culture flasks. Every 100 ml of the medium for the cultures was prepared mixing: RPMI-1640 medium without glutamine (Whittaker Bioproducts, Walkersville, MD) 87.5 ml; glutamine (Whittaker Bioproducts, Walkersville, MD) 2 mM; fetal bovine serum (Whittaker Bioproducts, Walkersville, MD) was heat-inactivated at (57°C for 30 minutes) 10 ml; gentamicin sulfate (Whittaker Bioproducts, Walkersville, MD) 5 mg; and amphotericin-B (Gibco laboratories, Grand Island, NY) 250 ug. The HL-60 cells were maintained in suspension culture at 37°C in a 100% humidified atmosphere containing 5% CO_2.

Human neutrophils were isolated using fresh heparinized human blood and Mono-Poly resolving media (Flow Labs, McLean, VA).

BUFFERS. HEPES buffer with and without calcium was used throughout this study. HEPES buffer with calcium contained 40mM HEPES (Sigma Chem Co., St. Louis, MO) 22 mM glucose, 20 mM KcL, 580 mM NaCl, and 4mM CaCl2. HEPES buffer without calcium contained 10 mM HEPES, 4.5 mM glucose, 5 mM KCl, 145 mM NaCl and contained no added calcium. The buffers were adjusted to a pH 7.4

STOCK LUMINOL SOLUTION. Luminol (5-amino-2, 3-dihydrol,4 phthalazinedione) was obtained from Aldrich Chemical Co., Milwaukee, WI, dissolved in 1 ml of dimethyl sulfoxide (DMSO) and then diluted in 99 ml of HEPES buffer without calcium so that the final concentration of the luminol was $5x10^{-4}M$. This preparation served as stock concentrated luminol. A further dilution to $5x10^{-7}M$ of luminol was made for use in the assays.

INSTRUMENTATION. Chemiluminescence assays were performed with a Berthold Multi-Biolumat LB 9505-C (EG & G Berthold Analytical Instruments Inc., Nashua, NH) six channel luminometer linked to an Osicom Executive 286/12 computer programmed with an appropriate software to analyze the data obtained. The LB 9505-C was standardized with a light emitting diode (LED) specifically fitted for the Berthold instruments. Standard 3 ml disposable cuvettes made for the Berthold instrument were used.

REAGENTS

Dimethyl sulfoxide (DMSO). It was obtained from Fisher Scientific Co., Fair Lawn, NJ, and was heat-sterilized and kept as stock solution that was later used to differentiate the HL-60 cells into neutrophils.

A63162. The drug was a gift from Abbott Labs., North Chicago, IL. It was diluted in 1 ml of DMSO and then brought to a final concentration of $1x10^{-4}M$ in HEPES without calcium and stored to 2°C as a stock solution. The drug was prepared each week.

NDGA. Nordihydroguaiaretic acid (4,4'(2,3-dimethyl tetramethylene) dipyrocatechol), (NDGA) was obtained from Sigma Chemical Co., St. Louis, MO. It was diluted up to a final concentration of $1x10^{-4}M$ in HEPES buffer without calcium and stored to -2°C as a stock solution.

Tetrandrine. Tetrandrine was a gift from the National Cancer Institute (NCI) Chemical Carcinogen Reference Standard Repository, Chicago, IL. Tetrandrine was converted into the salt form by the addition of a few drops of 1 N HCl. The solution was titrated with diluted 0.1 N NaOH up to pH 6.8. Next, tetrandrine was diluted in HEPES without calcium up to a final concentration of $1x10^{-4}M$ and pH 7.2 calcium (which served as a stock solution) and stored to -2°C.

Silica. Silica particles with a diameter of 5 um or less was a gift from Dr. Vincent Castranova (National Institute of Occupational Safety and Health, Morgantown, WV). Silica particles were diluted with HEPES without calcium up to a final concentration of 0.5 mg/100 ul every time that one experiment had to be run.

AA861. This was a gift from Takeda Chemical Industries Ltd., Osaka, Japan.

METHODS

PREPARATION OF CELLS HL-60 cells were grown in Corning 100x20 mm style polystyrene culture dishes (Corning Glass Works, Corning, NY). DMSO of medium contained in the dishes) in a final concentration of 1.3% was used to differentiate the HL-60 cells into neutrophil-like cells. The culture cells were allowed to differentiate for

four days under the exposure of DMSO and then were removed from the dishes, centrifuged at 500Xg for 5 minutes and suspended in 1 ml HEPES buffer without calcium. The cells were counted by using a hemacytometer (Neubauer counting chamber) and then brought to a final concentration of $1x10^7$ cells/ml and kept on ice. Morphological assessment of the differentiated cells was performed by Wright-Giemsa staining, and the vitality of the cells was evaluated with the trypan blue exclusion test.

Human neutrophils were isolated from fresh heparenized human blood cells using Mono-Poly resolving medium. Three ml of blood were layered onto 3 ml of resolving media in a 13x100 mm tubes and centrifuged for 30 minutes at 300Xg. The upper cell layer containing monocytes, platelets and lymphocytes was discarded. The lower second cell layer contained neutrophils. Each layer was aspirated with a 3 ml plastic syringe fitted with an 18-gauge needle. Aspirated cells were placed in separate 50 ml conical centrifuge tubes with 50 ml non-calcium HEPES buffer. Then, the tubes were centrifuged to a 500Xg for 5 minutes. The supernatant solution was discarded. The cells were resuspended in 1 ml of buffer, counted using the Neubauer hemacytometer and brought to a final concentration of $1x10^7$ cells/ml. The vitality of the cells was tested by using trypan blue.

LIGHT MEASUREMENT - Chemiluminescence was measured using a Berthold LB 9505-LC luminometer. Before each assay the luminometer was adjusted to 37°C and all determinations were made after the background counts were stabilized. All reactions were carried out in 3 ml round-bottom plastic cuvettes, which can be inserted into the counting chamber of the luminometer. Each sample was counted for 20 minutes, and the integrated counts were read from the computer monitor linked to the luminometer. A printed copy of the data was obtained.

The specific experiments using HL-60 cells and silica were done as follows: each plastic cuvette contained 100 ul cells (final concentration $1x10^6$ cells/ml); 100 ul HEPES with calcium; 10 ul luminol (final concentration $5x10^{-7}$M). In all the cuvettes, except one (that served as blank) 100 ul silica (final concentration 0.5 mg) was added as stimulant of the reaction. Finally, A63162, tetrandrine or NDGA were added in amount of 100 ul each to all the cuvettes except to the blank cuvette and to a second one which served as control to compare the percentage of inhibition of the drugs. HEPES without calcium was added to each cuvette to complete a final volume of 500 ul.

The drugs were used in final concentrations of $1x10^{-5}$M except where noted and $1x10^{-7}$M and were evaluated in independent assays three times on different days under similar conditions.

In the assays where neutrophils (100 ul in a final concentration of $1x10^6$ cells) replaced the HL-60 cells, the experimental conditions were the same as described above for silica assays with HL-60 cells.

OPSONIZATION OF PARTICLES - 5 milligrams of silica is added to 5 ml heparinized serum (which was originally separated from the whole blood by centrifugation 1200Xg and aspiration of the clear yellow top layer of the blood). The mixture is incubated at 37°C for 30 minutes with intermittent agitation. The contents are centrifuged at 1200Xg for 5 minutes in a narrow tube and the supernatant liquid discarded. The coated silica particles are resuspended in HEPES buffer (50 ml) and recentrifuged at 1200Xg for 5 min. The supernatant solution is aspirated and discarded and the particles are resuspended in fresh HEPES buffer at the desired final concentration 0.5 mg/100 ul.

DATA ANALYSIS

Chemiluminescence results were expressed as integrated counts for 20 minutes. Integration of light curves was accomplished using a Osicom computer, monitor, printer and software from the Berthold Instrument Company. As light is produced in the tube it is recorded by the luminometer and displayed on the monitor. Once the proper commands are entered into the computer the display is integrated and can be printed on computer paper. The final integrated count response for each experiment was obtained after the counts from the blank cuvette were subtracted from the experimental counts. The values of inhibition were expressed as the percentage of decrease in the integrated counts that the drugs produced when compared with the counts from the control (without silica) cuvette. The values, thus obtained were statistically analyzed and the means, standard errors and confidence intervals were calculated.

RESULTS The morphological assessment of the differentiated HL-60 cells after 5 days of incubation with DMSO by using Wright-Giemsa staining, showed that 50 to 60% of the cells had neutrophil-like nuclei (banded nuclei). The trypan blue dye exclusion test showed that less than 10% of the cells were dead. These cells were brought to a final concentration of $1x10^7$ cells/ml and used for the assays.

The neutrophils isolated from fresh blood displayed 95% viable cells. These cells were brought to the final concentration of $1x10^7$ kept on ice and used for the assays.

MEASUREMENT OF CELL VIABILITY Cell viability in the presence of drugs was measured using the exclusion of trypan blue. $1x10^6$ cells were preincubated with 0.2 ml HEPES buffered medium at 37°C for 10 minutes. Then drug at appropriate concentration 10^{-5}M was added to the cell suspension. After 10 minutes incubation 0.04% trypan blue was added at room temperature for 4 minutes with stiring. Then cells were fixed with formalin (1% final concentration) and observed using light microscopy. Cell viability was determined as: % viability = number of cells excluding dye x 100%/total number of cells.

LIPOXYGENASE ENZYME PREPARATION

Buffer The assay for lipoxygenase is done in 0.2M sodium borate buffer at pH = 9.0.

Substrates The borate buffer (above) is degassed under vacuum for 45 minutes. A solution of 19 mMolar sodium arachidonate is prepared in the

degassed borate buffer.

Luminol To prepare a solution, a 9 mg of luminol (5 amino-2,3-dihydro 1,4 phthalazinedrone) was dissolved in 50 ml of borate buffer (pH 9.0).

Enzyme solution Lipoxygenase-1 (Sigma Chem Co St. Louis MO) from soybean was used. To prepare the solution 5.0 mg of the powdered enzyme (170,000 units/mg) solid (Sigma) was dissolved in 10.0 ml of 0.2 M sodium borate buffer pH = 9.0.

Lipoxygenase assay The lipoxygenase assay is conducted at 37°C using a Berthold Luminometer model 9505C-6 channel instrument; standard 3 ml round bottom plastic curvettes made to fit Berthold instruments are utilized. The assay is established in a total volume of 500 microliters as follows:

1. 350 microliters of 0.2M borate buffer, pH = 9.0
2. 100 microliters drug in borate buffer of buffer (borate) - no drug control
3. 20 microliter 10mM sodium arachidonate in deoxygenated borate buffer
4. 20 microliter of 10^{-4} M luminol
5. 10 microliter of soybean lipoxygenase in borate buffer, 5.0 mg enzyme dissolved in 10 ml of borate buffer, enzyme powder is 170,000 units/mg

Drug solution Drugs are dissolved in 1 ml of dimethyl sulfoxide and then HEPES buffer is added to 10^{-4}M drug to produce 100 ml total volume.

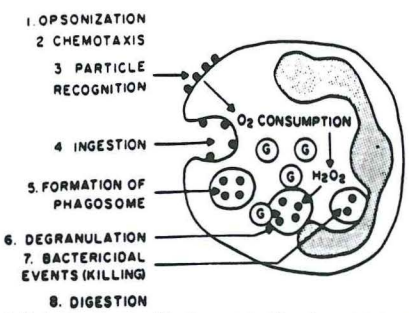

FIG. 1. The individual steps of neutrophilic phagocytosis. First, the particle is coated by plasma components, e.g., antibodies and/or complement (this step is not necessary with polystyrene beads). Second, the cell moves toward the particles (chemotaxis). Third, the particle is recognized by the cell. Fourth, the particles are ingested by the cell invaginating around them. Fifth, the cell closes around to form a structure known as the phagosome. Then granules within the cell (containing enzymes, proteins and oxidants) fuse with the phagosome. This is known as a phagolysosome. Sixth, the contents of the granules are poured into phagolysosome by a process known as degranulation. Seventh, post degranulation killing of bacteria occurs. In the case of polystyrene beads the same activity occurs but is of no relevance. Finally, bacterial digestion would occur. In the final step plastics may be degraded to a small extent but digestion generally does not occur.

Figure 1. The steps of phagocytosis (the engulfment of particles by macrophages, neutrophils, and human leukemia (HL-60) cells. In the case of neutrophils the particles must be coated with plasma or serum (opsonized) which contains the necessary antibodies and complement proteins.

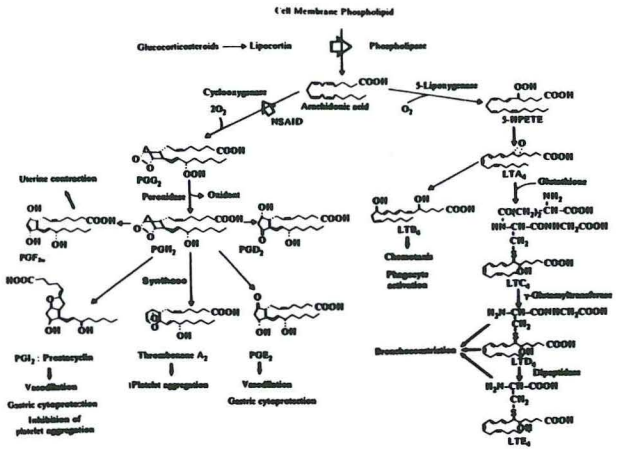

Figure 2. The arachidonate cascade depicting the cyclooxygenase and lipoxygenase metabolic schemes.

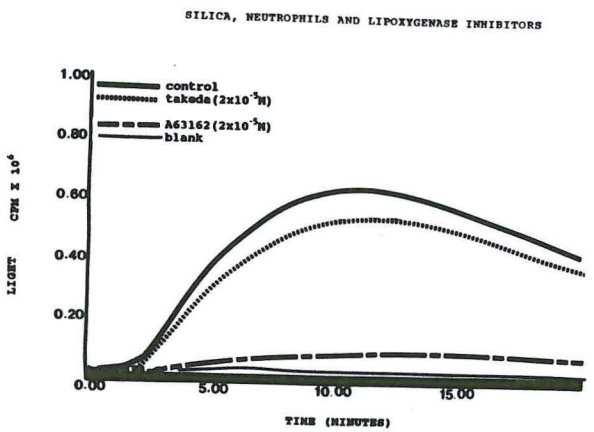

Figure 3. Human neutrophils (10^6) are reacted with 0.5 mg opsonized silica in the presence of luminol with and without various inhibitors of lipoxygenase at 37°C. This produces varying inhibition in the resulting cellular chemiluminescence over the 20 minute time course. Drugs included in this assay were the direct lipoxygenase inhibitors Takeda drug AA861, and Abbott drug A63162 all at $2x10^{-5}$M. The control contained no drug and the blank contained no silica and no drug. See methods for details.

RESULTS

In Figure 3, 0.5 mg silica was used to activate 10^6 human neutrophils in the presence of luminol to produce cellular luminescence. Then various lipoxygenase inhibitors at $2x10^{-5}$M were used to inhibit the production of light. The Takeda inhibitor (AA861) displayed minor inhibition. The Abbott inhibitor A63162 produced almost complete inhibition of luminescence. The actual pattern of inhibition appears to vary somewhat depending on the origin of the cells.

In Figure 4, human promyelocytic leukemia 60 (HL 60) cells were differentiated using DMSO (dimethyl sulfoxide). 0.5 mg silica was added to 10^6 HL 60 cells 5 days post differentiation with DMSO. Differentiation causes a loss of the myeloperoxidase system and an activation of the lipoxygenase system. Drugs were screened at a concentration of $2x10^{-5}$ M or with tetrandrine (T) at $2x10^{-6}$M (1/10 the dose of the others). T produced the least inhibition - approximately 1/3 - while A63162 inhibited about 50%. Nordihydroguairetic acid and AA861 were about equally inhibitory but AA861 was more active in the first half of the reaction while NDGA was more inhibitory in the second half.

In Figure 5, is depicted the kinetic reaction of silica with rat alveolar macrophages in the presence of lipoxygenase inhibitors. With these cells AA861 was the least active while A63162 was slightly less than 50% inhibitory to luminol luminescence. However NDGA was totally inhibitory to CL.

In Figure 6, using a soybean lipoxygenase assay, it is shown that tetrandrine has no direct effect on the lipoxygenase CL assay.

In Figure 7, both tetrandrine and A63162 appear to be equally inhibitory (42%) to silica activated DMSO differentiated HL-60 cells. NDGA almost totally inhibited cellular CL.

In Figure 8, neither tetrandrine nor A63162 were very inhibitory to CL from silica activated neutrophils but as before, NDGA was the most inhibitory to cellular CL.

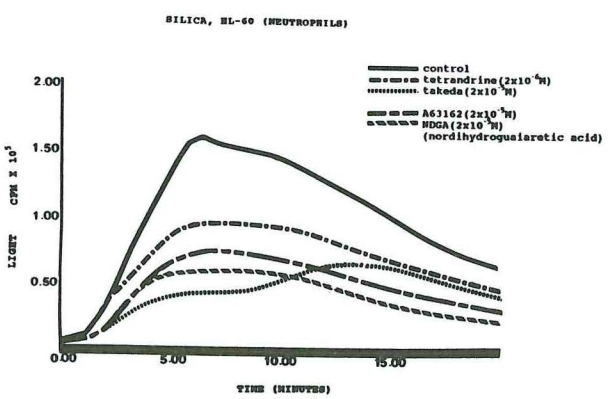

Figure 4. Five day-post DMSO differentiated HL-60 cells (10^6) are reacted with luminol and 0.5 mg silica in the presence and absence of various lipoxygenase inhibitors at 37°C. This produces varying inhibition in the resulting cellular chemiluminescence over the 20 minute time course. Drugs included in the assay were the direct lipoxygenase inhibitors Takeda - AA861, Abbott A63162, and the radical scavenge nordihydro guaiaretic acid (NDGA). The indirect inhibitor is tetrandrine. All drugs were at $2x10^{-5}$M concentration except tetrandrine which was $2x10^{-6}$M. See methods for details.

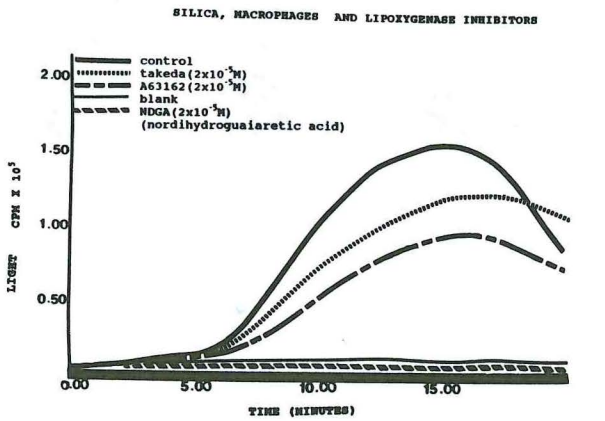

Figure 5. Rat alveolar macrophages (10^6) are reacted with 0.5 mg silica and luminol in the presence or absence of various lipoxygenase inhibitors at 37°C. This produces varying inhibition in the resulting cellular chemiluminescence over the 20 minute time course. Drugs included in the assay at $2x10^{-5}$M are the direct lipoxygenase inhibitors Takeda AA861, and Abbott A63162 and the free radical scavenges nordihydro guaiaretic acid. See methods for details.

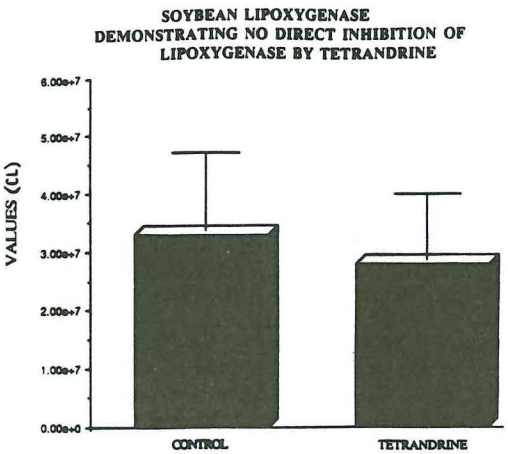

Figure 6. Soybean lipoxygenase is reacted with sodium arachidonate in borate buffer producing chemiluminescence in presence and absence of tetrandrine ($2x10^{-5}$M). The standard error of three experiments are shown. The assay indicates there is no direct inhibition of lipoxygenase by tetrandrine. See methods for details.

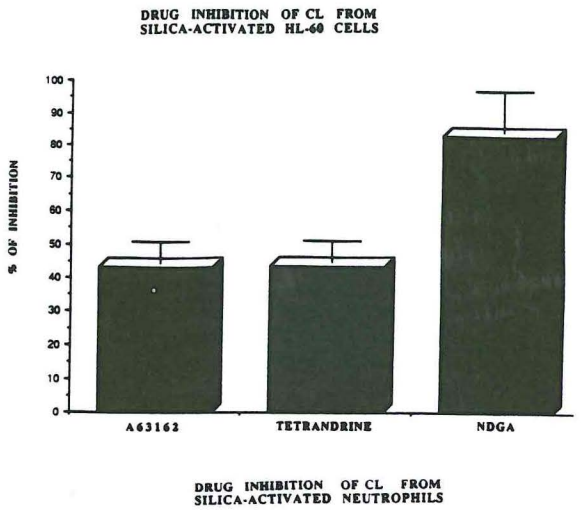

Figure 7. Five-day-post DMSO differentiated HL-60 cells (10^6) are reacted with 0.5 mg silica in the presence of luminol with various lipoxygenase inhibitors at 10^{-5}M producing inhibition of cellular chemiluminescence relative control without drug. The experiment was done 3 times with varying batches of cells and the standard errors of the mean are depicted. See methods for details.

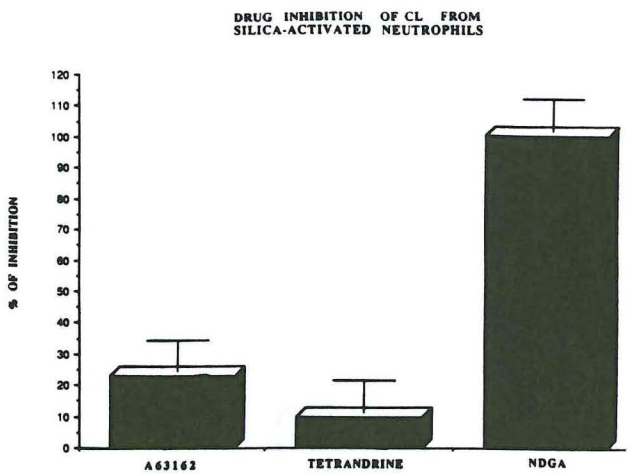

Figure 8. Inhibition of cellular chemiluminescence from silica activated human neutrophils (10^6) in the presence of luminol and various lipoxygenase inhibitors. The experiment was done 3 times with varying batches of cells and the standard errors of the mean are depicted. See methods for details.

DISCUSSION

Inhibition of 5' lipoxygenase (5L) can occur by direct or indirect attack of the complex necessary to produce the products 5 HPETE and leukotriene A. Inside the cell the activation of the cytoplasmic lipoxygenase causes the enzyme to move to the membrane where it attaches to a protein known as five lipoxygenase activating protein (FLAP) (12). The binding requires ATP and calcium ion and possibly other unknown factors (13). The enzyme apparently reacts with free arachidonate (15) or other long-chain unsaturated fatty acids or those same fatty acids conjugated (esterified) into phospholipids in the cell membrane (16). The 5L reaction requires oxygen to react with the iron (active site) which produces the peroxide free radicals and hence chemiluminescence. The system is susceptible to a variety of different drugs which can inhibit the system. Some drugs can bind to the iron (A63162) or AA861 or the free radical from the iron catalytic site can be scavenged by antioxidants such as NDGA. Since the cellular reaction requires 5L binding to FLAP, a drug can interfere with FLAP, ATP binding or Ca^{++} required in the active complex. These drugs would be indirect inhibitors.

In Figure 3, the direct antilipoxygenase drugs display widely different inhibitory properties. The AA861 does not appear to be particularly inhibitory while A63162 is almost completely inhibitory toward luminol dependent CL from human neutrophils. In whole cells, inhibition related to a combination of drug penetration and its particular mechanism and ability to act. A lack of activity could indicate a problem of penetration or limited direct inhibitory action against the enzyme. In addition in human neutrophils there are multiple light-producing mechanisms. The main CL activity is myeloperoxidase and the lesser, secondary CL activity is lipoxygenase. The exact interpretation of inhibition of CL is difficult to dissect except by selective inhibition.

However in Figure 4, lipoxygenase is the main activity in 5 day post DMSO differentiated cells (18) because myeloperoxidase is lost after 3-4 days (16) while the lipoxygenase gene is active at 5 days post differentiation (18). Therefore HL-60 (neutrophil-like) cells should produce CL linked primarily to 5' lipoxygenase activity. Under these conditions the AA861 and NDGA appeared to be most active inhibitors while the indirect inhibitor tetrandrine seemed less active; A63162 seemed intermediate between the other direct and indirect inhibitors.

In macrophages (Figure 5) AA861 seemed least inhibitory while A63162 inhibited less than half the total CL. NDGA produced inhibition below the blank and thus complete inhibition. Trypan blue was excluded from 98% of NDGA-treated cells indicating essentially complete viability and lack of major drug toxicity.

Since most of the inhibitors of 5' lipoxygenase are understood in terms of mechanism of inhibition, we tested tetrandrine. We found that it did not inhibit soybean lipoxygenase directly (Figure 5). Tetrandrine may inhibit via an

action on calcium channels (19). Rather than displaying the CL kinetic plots from the various cell types we did repetitive assays (Figure 6) with silica activated DMSO differentiated HL-60 cells and the drugs (A63162, tetrandrine and NDGA). Tetrandrine and A63162 appeared to be equivalent inhibitors at equipotent doses (10^{-5}M), while NDGA is about 85% inhibitory. Interestingly, a direct and an indirect lipoxygenase inhibitor are similar in activity while the NDGA free radical antioxidant (scavenger) is the most effective.

In multiple experiments with silica-activated-human neutrophils neither tetrandrine nor A63162 were major inhibitors while NDGA was totally inhibitory to the luminol dependent CL.

Although the human neutrophil is not a selective 5' lipoxygenase system in terms of production of CL, the drugs studied are still somewhat effective but scavenging radicals appears to be the most efficient mechanism to inhibit cellular CL.

However in the HL-60 DMSO treated 5' lipoxygenase activated system, drugs like A63162 or tetrandrine become more active and a similar molar dose of NDGA appeared to be less effective in HL-60 than in other cell types tested.

Since NDGA probably would be too toxic in vivo (20), but maybe a combination of direct and indirect inhibitors might be most effective against cellular oxidative mechanisms which produce free radicals and consequently CL (see Figure 8). Further maybe these newly developed inhibitors would be effective in the inflammatory conditions caused by silica. Hopefully early pharmacological interdic tion with lipoxygenase inhibitors might prevent the fibrogenic consequences of fibrosis due to deposition of silica in the lung. Only in vivo experimentation will demonstrate this and we plan to do such experiments in the future.

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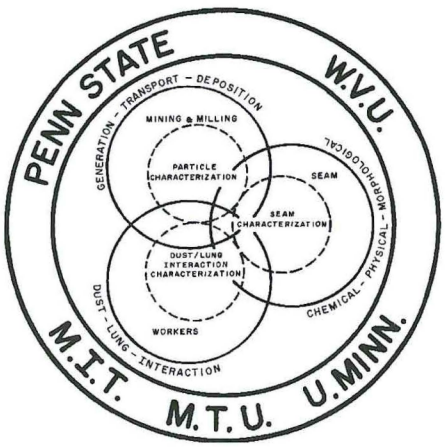

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