TANNIN INDUCED DOWNREGULATION AND DESENSITIZATION OF THE AIRWAY ß-ADRENERGIC RECEPTOR

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Abstract

Tannin, isolated from cotton bracts and implicated in the pathogenesis of byssinosis, inhibits Cl- secretion in isolated bovine tracheal epithelial cells, desensitizes airway cells to isoproterenol stimulation and decreases β-adrenergic receptor surface density. We have hypothesized that tannin exposure in cotton bracts results in a cycle of desensitization of the airway epithelial β-adrenergic receptor with partial recovery upon removal from the environment. We have also hypothesized that this cycle of desensitization/partial recovery results in the downregulation of the β-adrenergic receptor and this downregulation contributes to the pathogenesis of byssinosis. In these studies, increasing duration of exposure to tannin inhibited isoproterenol-stimulated cAMP release and the inhibition was reversible after 24 h. Repetitive (3 days), cyclical (8 h) exposures to tannin were associated with increasing inhibition of isoproterenol-stimulated cAMP release with partial recovery and incremental decreases in β-adrenergic receptor surface number. Tannin exposure (25 µg/ml for 24 h) also decreased β-adrenergic receptor mRNA. We conclude that tannin exposure downregulates the β-adrenergic receptor and results in increasing desensitization with repetitive exposure.

Introduction

Byssinosis is an occupational lung disease of cotton mill workers associated with acute symptoms of chest tightness, wheezing and shortness of breath which begin several hours after exposure to cotton dust in susceptible workers and are accompanied by across-shift changes in lung function and the development of an acute pulmonary inflammatory reaction in the airways (Kawamoto, et. al., 1987; Kennedy, et. al., 1987; Mundie, et. al., 1985; Rylander, et. al., 1983). Unlike other types of occupational lung disease, symptoms are worse on Mondays and after prolonged absences from the mill; eventually, however, these transient changes result in irreversible pulmonary function abnormalities.

Endotoxin and tannin, isolated from cotton bracts, have been implicated in the etiology of byssinosis (Rohrbach, 1994; Rylander, 1982). Nothing, however, is known about what causes the diminishing across-shift changes in pulmonary function and in respiratory symptoms during the work week.

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The purpose of this study was to determine whether repetitive exposure to tannin, isolated from cotton bracts, could produce changes in airway function compatible with a role for tannin in the change in symptom severity which occurs during the work week. Tannin, isolated from cotton bracts, inhibits chloride secretion and adenylyl cyclase, decreases surface β-adrenergic receptor number without affecting the dissociation constant, promotes phosphorylation of specific membrane proteins and uncouples the receptor from its stimulatory protein in short-term exposure studies (Cloutier and Rohrbach, 1986; Cloutier and Guernsey, 1995; Cloutier, et. al., 1997; Cloutier, et. al. 1994). We have hypothesized that repetitive tannin exposure progessively desensitizes the β-adrenergic receptor, decreases β-adrenergic receptor surface number and downregulates the β-adrenergic receptor resulting in a state of hyporesponsiveness or tachyphylaxis to further tannin exposure.

Materials and Methods

Bovine tracheas were obtained from a local slaughterhouse and placed in cold Hanks buffered saline solution (HBSS). Cell suspensions were prepared by scoring, stripping and cutting the bovine tracheal epithelium into small pieces using sharp dissection as previously described (Cloutier and Guernsey, 1995; Cloutier, et. al., 1994). Cells were isolated by gently stirring the strips at room temperature for two hours in 50 ml 50% Dulbecco's modified Eagle's medium and 50% Ham's F-12 medium (DMEM-F12) with 5% fetal calf serum containing dithiothreitol (DTT, 5 mM, Sigma Chemical Co., St. Louis, MO), deoxyribonuclease I (100 mg/ml, Sigma) and 0.1% protease, type XIV (Sigma). Cells were centrifuged, resuspended in media and allowed to rest for 1 hr at 37°C to remove any contaminating fibroblasts. Cells were then plated onto collagen-coated plastic culture dishes at 250,000/cm^2 and grown in culture medium consisting of DMEM-F12 supplemented with 5% fetal calf serum and (per ml) 80 µg gentamicin, 2.5 µg fungizone, 100 U penicillin and 100 µg streptomycin. After 3-4 days in culture, the culture medium was replaced with HBSS containing 20 mM HEPES (pH 7.4). Cells were then exposed to tannin (25 µg/ml) for up to 8 h and the cAMP response to isoproterenol was measured. The tannin was then removed, the cells were extensively washed and the cAMP response to isoproterenol was monitored for the next 24 h. In other experiments, cells were exposed to tannin (25 µg/ml) for 8 h and the cAMP response to isoproterenol and the cell surface receptor number were measured. The tannin was removed, the cells were extensively washed and allowed to recover for 16 hr at which time the cAMP response to isoproterenol and the cell surface receptor density were again measured. This cycle of 8 h of exposure to tannin followed by a 16 h tannin-free recovery period was repeated three times.

cAMP was measured using a radioimmunoassay kit (Amersham, Arlington Hts., IL). The cells were then

treated with 1N NaOH to dissolve cellular protein which was measured according to the method of Lowry (Lowry, et. al., 1951) using bovine serum albumin as the standard. cAMP activity was calculated as pmol cAMP per mg protein.

The effect of tannin on cell surface number was determined using ^{3}H-CGP 12177 (44 Ci/mmol; New England Nuclear). BTE cells in culture (~4 x 10^{5} cells) were exposed to tannin as above and then incubated in 1 ml DMEM containing 25 mM HEPES and 30 µg/ml BSA (Sigma) (pH 7.4) at 4°C for 3 h in the presence of a saturating concentration (1 nM) of ^{3}H-CGP 12177. Cell surface receptor density was calculated from one-point analysis in which 10^{-6} M propranolol was used to assess nonspecific binding. Results from tannin experiments were compared to similar experiments using isoproterenol (10^{-5} M for 3 h), which is known to cause a rapid decrease in cell surface receptor number (Penn, et. al., 1994).

The effect of prolonged tannin exposure on β-adrenergic receptor mRNA was examined using reverse transcriptase-polymerase chain reaction (RT-PCR). Confluent BTE cells were incubated in the presence or absence of tannin (25 µg/ml) for 24 h and total RNA was isolated using guanidium thiocyanate and a modification of the method of Chomcyzynski and Sacchi (Chomczynski and Sacchi, 1987).

β-adrenergic cDNA was synthesized from BTE cells using a reaction mixture consisting of 2.5 µg oligo(dT), 5 µg total RNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 10 mM DTT which was incubated for 3 min at 65°C. After being chilled on ice, 0.5 mM dNTPs and 500 units of maloney murine leukemia virus reverse transcriptase (Gibco/BRL) were added to the mix and the incubation was continued at 37°C for 90 min. The resulting cDNA was extracted with phenol/chloroform and ethanol precipitated in the presence of ammonium acetate. The cDNA was collected by centrifugation, washed with 70% ethanol, resuspended in 15 µl of H_2O and stored at -20°C.

PCR amplification of the bovine β-adrenergic receptor cDNA was performed using a DNA sequence of a 376 bp region lying within the putative transmembrane segments VI and VII of the bovine β-adrenergic receptor and was obtained from GenBank. Using this information, the appropriate primers (Primer 1: 5'-ACAACGCAGGACCTCCAAGTTC-3'; Primer 2: 5'-TCCACTCTGTTCCCCTGTGTAGTC-3') which flank a 340 bp segment of the bovine receptor were commercially synthesized (Gibco/BRL). A reaction mixture containing 50 pmol of each primer, 1 µl cDNA, 30 mM tricine (pH 8.4), 5 mM β-mercaptoethanol, 0.01% gelatin, 0.1% Thesit (Sigma), 20 mM $MgCl_2$ and 1.25 units of Taq DNA polymerase were incubated at 94°C for 5 min before the addition of 200 µM dNTPs. Samples were amplified by 30 repeated cycles at 94°C for 15 sec for denaturing, 60°C for 15 sec to allow annealing, and 72°C for 40 sec for elongation. A final incubation at 72°C for 5 min insured extension of all PCR products to their full length. An aliquot from each sample was electrophoresed through a 1.6% agarose gel and visualized by ethidium bromide staining.

Condensed tannins were isolated from the 1985 crop of bracts from Acala SJ-5 cotton grown in Texas utilizing sequential Amicon ultrafiltration and a modification of the procedure of Taylor and associates as previously described (Cloutier and Rohrbach, 1986; Taylor, et. al., 1971). Stock solutions of the tannin (molecular weight >10,000 dalton) were prepared daily, immediately before use, by dissolving the tannin at a concentration of 19.2 mg/ml in water. This represented the tannin concentration in the cotton bracts extract used in our original study (Cloutier and Rohrbach, 1986). Tannin concentrations are reported as µg/ml.

Data were analyzed using analysis of variance and Student's *t* test or as described (Bruning and Kintz, 1977).

Results and Discussion

Using BTE cells in culture, the intracellular cAMP response to a 10 minute exposure to 10^{-5} M isoproterenol was measured in cells prechallenged with tannin (25 µg/ml) for 5 min - 8 h (Figure 1). Isoproterenol-stimulated cAMP release was blunted in cells exposed to tannin compatible with desensitization. Inhibition began within 5 min and reached 52 " 5% (mean " SEM, n=6) at 60 min. After an 8 h exposure to 25 µg/ml, there was further inhibition (82 " 9%, $p<0.001$) in isoproterenol-stimulated cAMP accumulation. Inhibition was dose-dependent. Five µg/ml tannin inhibited isoproterenol-stimulated cAMP by 17 " 4% after 10 min and by 33 " 9% (n=6, $p<0.001$) after an 8 h exposure. Tannin was removed after 8 h, the cells were extensively washed and recovery was monitored. Reversibility began within 4 h and approached baseline by 24 h.

In BTE cells grown to 90% confluence, the effects of repetitive 8 h exposures to tannin with a 16 h recovery period on isoproterenol-stimulated cAMP release were measured (Figure 2). In these cells, the cAMP response to isoproterenol decreased to 57" 6% (mean " SEM, % of control) after the first 8 h tannin exposure and recovered to 72 " 10% after a 16 h washout period. During day 2, the cAMP response to isoproterenol further decreased to 36 " 6% after the second 8 h tannin exposure, recovered to 54" 12% after a 16 h washout and then further decreased to 15 " 4% after the third 8 hr exposure to tannin. Thus, repeated tannin exposure progressively inhibited the response to isoprotererenol and with each exposure, recovery was more incomplete. The cAMP response to isoproterenol incrementally decreased in general 40% with each 8 h tannin exposure and recovered approximately 15% in the 16 h during which tannin had been removed. If this pattern had

continued, the cAMP response to isoproterenol would have been negligible on days 4 and 5 of exposure. Cell viability was monitored by light microscopy (normal), trypan blue exclusion (<1%), protein recovery (unchanged) and LDH release (unchanged) in the cytosol. Longer exposures were not attempted because of concerns about cell viability for this length of time.

The effect of repetitive tannin exposure on surface β-adrenergic receptor cell number was also investigated (Figure 3). In preliminary experiments, cell surface receptor number decreased after tannin exposure and recovery after the initial tannin exposure was incomplete. After the second tannin exposure, cell surface receptor number further decreased without recovery. This decrease in cell surface receptor number could be due to an increase in receptor turn-over, a decrease in receptor synthesis or sequestration.

RT-PCR revealed a single band migrating at approximately 340 bp in untreated, control cells (Figure 4). This is the expected size for the region of the β-adrenergic receptor that was amplified. Interestingly, this fragment was absent in cells exposed to tannin (25 µg/ml) for 24 h. This fragment will be sequenced to confirm its identify. These data suggest that at least part of the decrease in cell surface receptor number is due to downregulation of the β-adrenergic receptor.

These changes in receptor density and cAMP levels after acute and "chronic" exposure to tannin, could contribute to the clinical syndrome of byssinosis. The Monday across-shift symptoms which occur with acute exposure to cotton dust and the diminishing across-shift symptoms that occur during the remaining weekdays are compatible with rapid desensitization of β-adrenergic receptors with β-adrenergic receptor-Gs uncoupling and sequestration. Recovery occurs overnight but is accompanied by relative hyporeactivity and a diminished responsiveness the rest of the week. Epithelial cells release both relaxing (eg. cAMP, epithelium-derived relaxing factor, PGE) and contracting factors (eg arachidonic acid, PGF 2") which modulate airway tone (Boucher, 1994). Increased airway tone can be caused by an increase in contracting factors or a decrease in relaxing factors or both. We have hypothesized that the Monday symptoms observed in some mill workers are, in part, caused by tannin in the cotton dust. Tannin deposits on the large airways and causes a change in epithelial mediator production. Some mediators such as non-metabolized arachidonic acid are released from airway cells and have direct effects upon airway smooth muscle and secondary effects upon other resident airway cells (Cloutier and Guernsey, 1996). In the airway epithelial cell, initial exposure to cotton tannin uncouples the β-adrenergic receptor from Gs, inhibits cAMP and results in a decrease in relaxing factors. This shifts the balance in the airway to an excess of contracting factors resulting in across-shift changes in large airway function (ie decrease in FEV1). Tannin exposure during the shift also decreases β-adrenergic receptor number which partially recovers overnight but which produces a state of relative hyporeactivity to subsequent tannin exposure on the following day since the number of receptors available to interact with tannin is decreased. As a result, on the second day tannin produces a smaller absolute change in receptor number and a smaller absolute decrease in cAMP which when coupled with the continued release of contracting factors shifts the balance toward moderation of contraction. The decrease in receptor number is similar to the mechanism whereby repeated isoproterenol exposure results in a state of relative hyporeactivity (tachyphylaxis) to continued isoproterenol exposure through a decrease in β-adrenergic receptor number. In this case, the decrease in receptor number for interaction with tannin limits the airway response to subsequent exposure. By the end of the work week, there are so few receptors available for interaction that the only observed effect is due to the contraction factors in cotton dust. Whether these factors also change with repeated tannin is not known but is under investigation. Recovery after tannin exposure is partial during the week but is complete upon absence from the mill over the weekend. This hypothesis is supported by clinical studies which demonstrate changes in large airway function after dust exposure, transient hyporesponsiveness in individuals after repeated short-term exposure to cotton dust, and a small decrease in baseline pre-shift FEV1 at the end of the work week compared to the beginning (Edwards, 1981; Glindmeyer, et. al., 1994; Warburton, et. al.,1992). In support of this hypothesis we have demonstrated that after repeated exposure to tannin, the cAMP response to isoproterenol is further inhibited and basal levels of cAMP are decreased. We have also demonstrated partial recovery upon short-term and complete recovery upon long-term removal (24 h) from tannin. Such a mechanism of rapid desensitization with the development of tolerance followed by downregulation has been proposed for patients with asthma on β-adrenergic drugs except in asthma, the stimulus is rarely continuous (Collins et. al., 1992). Tannin exposure, however is one of many occupational lung diseases where exposures are repetitive, frequent and occur over long periods of time.

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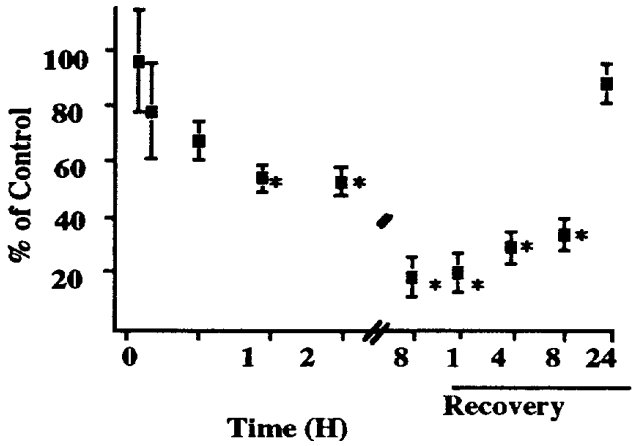

Figure 1. Effect of time of exposure to 25 µg/ml tannin on cAMP response to isoproterenol and reversibility.

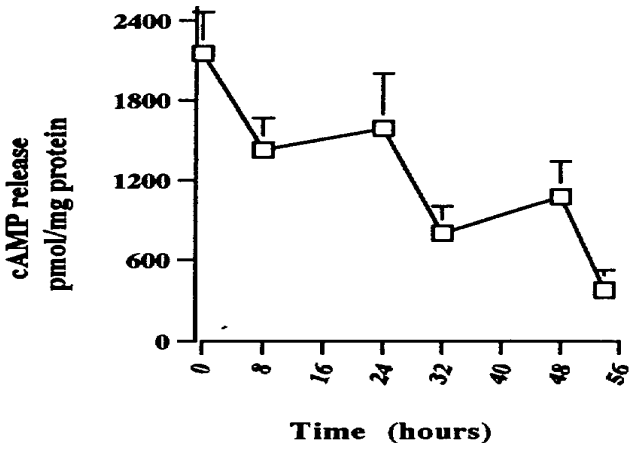

Figure 2. Effect of repeated tanning (25 µg/ml) exposure on isoproterenol-stimulated cAMP release. Cells in culture were exposured to tannin for 8 hours followed by a 16 h recovery period on 3 successive days. Data are expressed as the mean ± SEM of 2 observations.

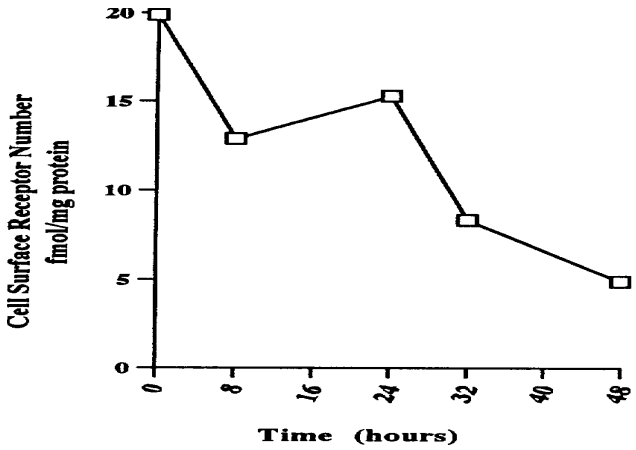

Figure 3. Effect of repeated tanning (25 µg/ml) exposure on cell surface β-adrenergic receptor number. Cells in culture were exposured to tannin for 8 h followed by a 16 h recovery period on 2 successive days.