COTTON BRACTS TANNIN PHOSPHORYLATES AIRWAY MEMBRANE PROTEINS

M.M. Cloutier and L. Guernsey
Pediatric Pulmonary Division
University of Connecticut Health Center
Farmington, CT

Abstract

In airway epithelial cells, tannin, isolated from cotton bracts, inhibits chloride secretion, decreases $-adrenergic receptor number, uncouples the $-adrenergic receptor from its stimulatory G protein and desensitizes the airway epithelium to $-agonists. We examined the effect of tannin on airway membrane protein phosphorylation, especially phosphorylation of the $-adrenergic receptor as a mechanism of rapid desensitization. Using bovine tracheal epithelial cells, gel electrophoresis and autoradiographic techniques, tannin promotes phosphorylation of 3 distinct membrane proteins, one of which is within the molecular weight range of the $-adrenergic receptor and is phosphorylated rapidly. The pattern and timing of the phosphorylation suggest a role for tannin stimulation of a $-adrenergic receptor kinase ($ARK) in tannin desensitization of airway epithelial cells.

Introduction

Inhalation of cotton mill dust by textile workers results in the development of the occupational lung disease, byssinosis (Bouhuys,1976; Shilling, 1956). While the etiology of byssinosis is not known, endotoxin and tannin isolated from cotton bracts, the thin, brittle leaves surrounding the cotton boll, have been implicated as important etiologic agents (Rohrbach, 1994; Rylander, 1982). Tannin has pronounced biologic effects on a diverse group of cell types including platelets, airway epithelial cells, pulmonary arterial endothelial cells, T lymphocytes and alveolar macrophages and alters many different signal transduction pathways (Hempel et.al., 1988; Ayars et.al.,1986; Johnson et. al., 1986; Vuk-Pavlovic et.al., 1988).

In airway epithelium, cotton dust or an aqueous extract derived from cotton bracts (CBE), inhibits active ion transport and increases the permeability of the paracellular pathway (Cloutier et.al., 1984). Tannin, isolated from CBE, accounts for approximately 75% of the decrease in short-circuit current by inhibiting net chloride secretion (Cloutier et. al.,1986). This inhibition demonstrates specificity for the apical membrane, is dose dependent and reversible. Tannin also has effects upon other signal transduction pathways including decreases in intracellular calcium in response to epinephrine, inhibition of protein kinase C activity and increases in nonmetabolized arachidonic acid release from bovine airway cells grown in culture (Cloutier et.al., 1994; Cloutier and Guernsey, 1995a; Cloutier and Guernsey, 1996).

The most striking of the effects of tannin on the airway epithelium, however, are effects on the b-adrenergic receptor/cAMP pathway. Tannin inhibits basal and epinephrine-stimulated intracellular cAMP levels in a dose-dependent manner in part by decreasing b-adrenergic receptor number without affecting the dissociation constant (Cloutier et.al., 1994). When the b-adrenergic receptor is bypassed by forskolin which acts directly upon the catalytic subunit of adenylyl cyclase, tannin noncompetitively and reversibly inhibits forskolin-stimulated adenylyl cyclase activity in a dose-dependent manner (Cloutier and Guernsey, 1995b). Thus, tannin decreases intracellular levels of cAMP stimulated by epinephrine by inhibiting b-agonist binding to its receptor, and by inhibiting adenylyl cyclase directly irrespective of agonist binding. The effects of tannin are both specific and selective. By inhibiting Cl^- secretion, tannin inhibits secondary water transport in the airways, mucus secretion and mucociliary transport and may also modulate airway smooth muscle reactivity to both contracting and relaxing agents. These effects could result in the pathologic pulmonary findings in patients with byssinosis. Decreases in mucociliary transport would also result in secretion retention and exposure of the epithelium to other substances in cotton dust, such as endotoxin, for longer than normal periods of time. Thus, tannin may directly and indirectly contribute to the pathogenesis of byssinosis.

Tannin could affect the b-adrenergic receptor and decrease b-adrenergic receptor number by uncoupling the receptor from Gs, a process associated with receptor phosphorylation, by sequestration of the receptor in intracellular vesicles and/or in the case of prolonged exposure, by receptor downregulation. We previously demonstrated that tannin uncouples the receptor from Gs (Cloutier et.al., 1996) In these experiments we expand our investigations into the effects of tannin on the b-adrenergic receptor/cAMP pathway by demonstrating that tannin rapidly desensitizes the b-adrenergic receptor to isoproterenol, phosphorylates specific airway epithelial cell membrane proteins including one protein at the molecular weight of the b-adrenergic receptor and with prolonged exposure, downregulates the receptor.

Materials and Methods

The materials and methods have been previously described by us (Cloutier et.al., 1994; Cloutier and Guernsey, 1995b). Bovine tracheas are obtained from a local abbatoir; the epithelial surface is stripped and cells are grown in culture for 2-3 days until 80-90% confluence is reached. Bovine tracheal epithelial cells (BTE) are composed of a

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 1:182-185 (1997)
National Cotton Council, Memphis TN

homogeneous population of b-adrenergic receptors of the b_2-subtype (Nogami et. al., 1993).

Tannin was isolated from Acala SJ-5 cotton as previously described and prepared fresh daily (Cloutier and Rohrbach 1986). cAMP was measured using a radioimmunoassay as previously described (Cloutier et. al., 1994; Cloutier and Guernsey, 1995b).

In phosphorylation experiments, BTE cells (~10^6) were incubated with $^{32}PO_4$, washed and challenged with either diluent control) or tannin (25 mg/ml) for various lengths of time (5 sec - 4 min). The reaction was terminated with perchloric acid and the material centrifuged. The pellet was washed twice, adjusted to pH 6.8 and heated before loading onto gels for electrophoresis. Equal amounts of b-adrenergic receptors (determined by ^{3}H-DHA) were electrophoresed on a 12% SDS-polyacrylamide gel with 4% stacking gel to separate lower mass proteins (<80 kDa), fixed in ethanol (40% v/v) and glycerol (5% v/v), dried and autoradiographed at -80° C with enhancing screens for 24-30 h. Appropriate molecular weight standards were used. Band density was determined by laser densitometry.

Results and Discussion

Using BTE cells in culture, the intracellular cAMP response to a 10 minute exposure to 10^{-5} Misoproterenol was measured in cells prechallenged with tannin (25 mg/ml) for 5 - 120 minutes. Isoproterenol-stimulated cAMP release was blunted in cells exposed to tannin compatible with desensitization. Inhibition began within 5 min and reached a maximum of 52 ± 5% (mean ± SEM, n = 5) at 60 min. (Figure 1)

Forskolin stimulates cAMP release in airway epithelial cells by bypassing the b-adrenergic receptor and acting directly upon adenylyl cyclase. Since tannin has effects upon adenylyl cyclase activity, we compared the effects of tannin (25 mg/ml) on isoproterenol (10^{-6} M)-stimulated cAMP release and forskolin (10^{-5} M)-stimulated cAMP release. Tannin pretreatment was more effective in inhibiting isoproterenol-stimulated cAMP release (64 ± 7% inhibition, n=4) than in inhibiting forskolin-stimulated cAMP release (30 ± 7% inhibition). This suggests that tannin inhibits both the b-adrenergic receptor and adenylyl cyclase and that the desensitization is not due to effects solely upon adenylyl cyclase.

In timed phosphorylation experiments, tannin phosphorylated 3 distinct protein bands in BTE cells. One band at ~61 kDa was phosphorylated within 5 seconds while 2 other bands at 41 kDa and 36 kDa were phosphorylated beginning at 2 min. The 61 kDa band had a maximum incorporation of phosphate of 184% at 5 sec and the 41 and 36 kDa bands had a maximum response of 156% and 128%, respectively, at 4 minutes. The 61 kDa band is within the molecular weight range for the b-adrenergic receptor (Connolly et. al., 1986). The 41 kDa band is similar to a protein kinase C-dependent phosphoprotein of 40-47 kDa, which has been identified by others as inositol triphosphate 5'-phosphomonoesterase while the 36 kDa band is unidentified at this time but has been observed in tannin-induced platelet protein phosphorylation studies (Hempel et. al., 1988) (Figure 2).

In another group of experiments, bovine tracheal epithelial cells were grown to 90% confluence and exposed to either 5 mg/ml or 25 mg/ml tannin for 10 min or 24 hrs. The isoproterenol-stimulated and forskolin-stimulated cAMP response were measured. Prolonged tannin exposure at both concentrations decreased basal levels of cAMP and blunted both isoproterenol-stimulated and forskolin-stimulated cAMP release in a dose-dependent manner. Compared to a 10 min exposure, a 24 h exposure to 25 mg/ml tannin further inhibited (63 ± 5%, n = 4, $p<0.05$) isoproterenol-stimulated cAMP release. The extent of inhibition was dose dependent. Five mg/ml tannin inhibited isoproterenol-stimulated cAMP release by 17 ± 4% after 10 min and by 33 ± 9% ($p<0.001$) after 24 h exposure. Inhibition of forskolin-stimulated cAMP release was tannin dose-dependent but was not time dependent. There was no difference in the extent of tannin inhibition of forskolin-stimulated cAMP between a 10 min exposure and a 24 h exposure for either 5 or 25 mg/ml. No morphologic changes were noted in cells exposed to either concentration of tannin for 24 h (Figure 3).

These experiments demonstrate that tannin desensitizes bovine tracheal epithelial cells to isoproterenol and promotes phosphorylation of specific membrane proteins. Phosphorylation results in a rapid short-term desensitization due to covalent modification of the b_2-adrenergic receptor and would result in relative hyporeactivity and a diminished responsiveness the rest of the work week. Hyporeactivity has been clinically observed in mill workers during the work week and is not associated with worsening baseline pulmonary function during the week (Edwards, 1981). With longer absences, receptor recovery is complete and the acute across shift changes recur. Longer exposure could result in downregulation which presumably could contribute to an overall reduction in receptor number and responsiveness and the blunting of clinical symptoms later in the week. This process would produce acute symptoms on Monday with diminishing symptoms during repeated exposure the remainder of the week and recovery of sensitization during the weekend. Incomplete recovery might occur after many years of exposure to cotton dust and result in secondary changes in the airways and progressive permanent changes in pulmonary function. Such a mechanism of rapid desensitization with the development of tolerance followed by downregulation has been proposed for patients with asthma on b-adrenergic drugs except in asthma the stimulus is rarely continuous (Collins et. al., 1992). Tannin exposure, however, is one of several occupational

lung diseases where exposures are repetitive, frequent and occur over long periods ot time.

In summary, these preliminary data suggest that tannin-induced b-adrenergic receptor desensitization is due to specific membrane phosphorylation with uncoupling of the receptor from Gs resulting in decreases in adenylyl cyclase activity and decreases in cAMP activity. Longer exposures result in receptor downregulation by mechanisms yet to be determined. Thus, tannin may play an important role in the Monday across-shift symptoms and in the chronic disease of byssinosis.

Acknowledgments

We are indebted to Ellen Berger for secretarial services. This work was supported by Grant HL-28669 from the National Heart, Blood and Lung Institute and by a grant from the University of Connecticut Health Center.

References

Ayars, G.H., L.C. Altman, C.D. O'Neill, B.T. Butcher and E.Y. Chi. 1986. Cotton dust-mediated lung epithelial injury. J. Clin. Invest. 78:1579-1588.

Bouhuys, A.1976. Scheduled asthma in the textile industry. Lung. 154:3-16.

Cloutier, M.M. and L. Guernsey 1995a. Tannin inhibition of protein kinase C in airway epithelium. Lung. 173:307-319.

Cloutier, M.M. and L. Guernsey 1995b. Tannin inhibits adenylate cyclase in airway epithelial cells. Am. J. Physiology 268 (Lung Cell. Mol Physiol.12) L851-L855.

Cloutier, M.M. and L. Guernsey 1996. Tannin stimulates arachidonic acid release from bovine tracheal epithelial cells. Am.J. Physiol. 270 (Lung Cell. Mol. Physiol 14): L613-L618.

Cloutier, M.M., L. Guernsey and R.I. Sha'afi 1994. Tannin inhibits cAMP pathways in bovine airway epithelium. Am. J. Respir. Cell. Mol. Biol. 10:106-111.

Cloutier, M.M., K. Lesniak, J.A. Russell and M. S. Rohrbach 1984. Effect of cotton bracts extract on canine tracheal epithelium and shunt pathway. Am. Rev. Respir. Dis. 130:1087-1090.

Cloutier, M.M. and M.S. Rohrbach 1986. Effects of endotoxin and tannin isolated from cotton bracts on the airway epithelium. Am. Rev. Respir. Dis. 134:1158-1162.

Cloutier, M.M., C.M. Schramm and L. Guernsey 1996. Tannin uncouples the $-adrenergic receptor from G Proteins. IN: Proc. 20th Cotton and Other Organic Dusts Res. Conf. P. Wakelyn, RR. Jacobs and R. Rylander, Eds. National Cotton Council, Memphis, TN. pp327-330.

Collins, S., M.G. Caron and R.J. Lefkowitz 1992. From ligand binding to gene expression: New insights into the regulation of G-protein-coupled receptors. Trends Biochem. Sci. 17:37-39.

Connolly, T.M., W.J. Lawing and P.W. Majerus 1986. Protein kinase C. phosphorylates human platelet inositol triphosphate 5'phosphomonoesterase, increasing the phosphatase activity. Cell. 46:951-958.

Edwards, J. 1981. Mechanism of disease induction. Chest 79:38S-43S.

Hempel, S.L., C. L. Wheatley and M.S. Rohrbach 1988. Protein phosphorylation during tannin-mediated activation of human platelets. J. Lab. Clin. Med. 111:299-306.

Johnson, C.M., M. Nyberg-Hanson and M S. Rohrbach 1986. Toxicity to endothelial cells mediated by cotton bract tannin. Am.J. Pathol. 122:399-409.

Nogami, M., D.J. Romberger, S.I. Rennard, and M.L. Toews 1993. Agonist-induced desensitization of $-adrenoceptors of bovine bronchial epithelial cells. Clin. Sci. 85:651-657.

Rohrbach, M.S.1994. Biological activity of condensed tannins. IN: Organic dusts: Exposure, Effects and Prevention. R. Rylander and R.R. Jacobs (Ed). Lewis Publishers, Inc. Chelsea. pp69-91.

Rylander, R.1982. Bacteria as etiological agents in byssinosis. Eur. J. Respir. Dis. 123:34-36.

Shilling, R.1956. Byssinosis in cotton and other textile workers. Lancet. 11:261-265.

Vuk-Pavlovic, Z., J.A. Russell, and M.S. Rohrbach 1988. Cotton bract tannin: A novel human T-lymphocyte mitogen and possible causative agent of byssinosis. Int. Arch. Allergy Appl. Immunol. 87:14-18.

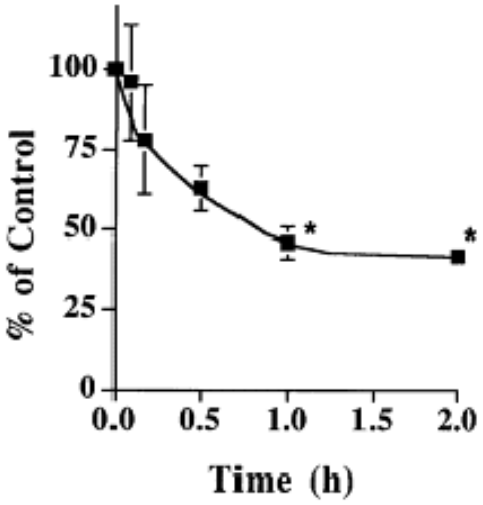

Figure 1. Effect of time of tannin (25 µ/ml) exposure on cAMP response to a 10 min exposure to isoproterenol. Data are expressed as a percent of control (cAMP response to isoproterenol in the absence of tannin)

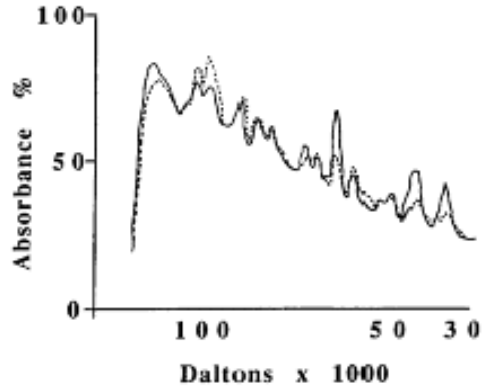

Figure 2. Densitometry tracings comparing the phosphorylation changes of discrete airway proteins before and after exposure to 25 µg/ml tannin. Control (dashed line). Tannin (solid line).

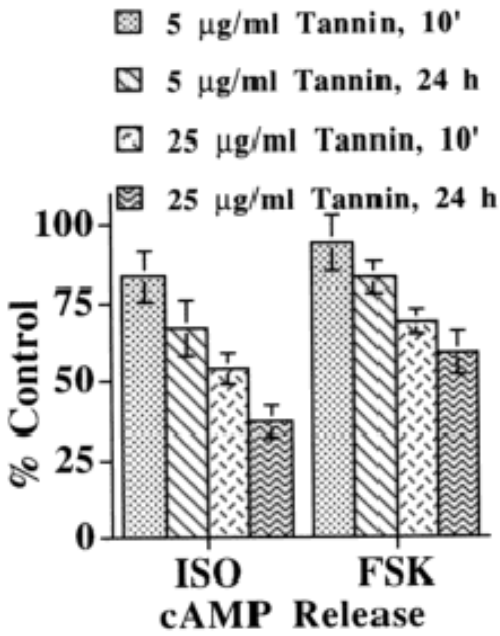

Figure 3. Efeects of tannin concentration and duration of exposure on isoproterenol and forskolin stimulated cAMP release.