Biochemical Pharmacology, Vol. 34, No. 24, pp. 4269–4273, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

ATTENUATION OF PULMONARY FIBROSIS IN MICE BY AMINOPHYLLINE*

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(*Received* 30 *November* 1984; *accepted* 20 *May* 1985)

Abstract—Cyclic nucleotides have been shown *in vitro* to regulate fibroblast proliferation and/or collagen production. We have reported previously that propranolol, which decreases the cAMP/cGMP ratio, potentiates the amount of fibrosis produced in a damaged lung. The purpose of this study was to determine if elevations in the cAMP/cGMP ratio may attenuate collagen production by fibroblasts following lung damage. Lung injury was induced in mice by either butylated hydroxytoluene (BHT) (350 or 400 mg/kg intraperitoneally) or bleomycin (4 units/kg intratracheally). The mice were treated with a phosphodiesterase inhibitor, aminophylline (20 mg/kg twice daily), prior to induction of lung injury and for the duration of the study. Cyclic nucleotide changes in the lung were also determined during lung injury, with and without aminophylline. The administration of aminophylline, which increased the cAMP/cGMP ratio, resulted in attenuation of the increase in total lung collagen normally seen after injury, while having no effect on collagen levels in the undamaged lung. The results are compatible with the hypothesis that elevation of whole lung cAMP/cGMP ratio early in the damage and repair process correlates with decreased hydroxyproline deposition.

Pulmonary fibrosis is a chronic disease characterized by a deranged cellular composition of the alveolar zone with an abnormal accumulation of collagen [1]. Under normal conditions, the involvement of the connective tissue following injury is self-limited and fibroblast proliferation and collagen production cease when adequate repair has been achieved [2]. However, fibrosis often appears to develop when normal tissue repair fails to take place and fibroblastic proliferation with excessive collagen production results. This indicates that there may exist endogenous mechanisms capable of controlling the extent and duration of fibroblast activity.

Regulation of fibroblastic activity has been studied extensively *in vitro* [3–7]. These studies indicate that the intracellular concentrations of cAMP and cGMP are regulatory signals. By elevating intracellular cAMP levels, fibroblast collagen production is suppressed. Because *in vitro* studies have shown that a variety of pharmacological agents will increase cAMP in fibroblasts [3] and that even small changes in cAMP were effective in suppressing collagen production [5], it is reasonable to suggest that such agents may be helpful in suppressing collagen production in fibrotic disorders *in vivo*. Aminophylline, a soluble theophylline salt, elevates cyclic AMP via competitive inhibition of cyclic nucleotide phosphodiesterase, the enzyme responsible for the conversion of cAMP to 5′-AMP [8]. The purpose of this study was to determine if elevations in cAMP (increased cAMP/cGMP ratio) by aminophylline increase the regulation of fibroblastic activity and decrease the accumulation of collagen in normal or damaged lungs *in vivo*.

MATERIALS AND METHODS

Animals. Male BALB/c mice, 8- to 9-weeks-old, weighing 20–25 g, bred in the barrier facility at the Biology Division, Oak Ridge National Laboratory, were used. All animals were kept on a conventional laboratory diet (Purina Chow) with food and water *ad lib.* throughout.

Chemicals. Aminophylline (theophylline ethylenediamine) and butylated hydroxytoluene (3–5-di-*tert*-butyl-4-hydroxytoluene, BHT) were purchased from the Sigma Chemical Co., St. Louis, MO. Bleomycin sulfate (Blenoxane) was a gift from Bristol Laboratories, Syracuse, NY. Aminophylline was dissolved in sterile saline and administered subcutaneously at a volume of 0.1 ml/10 g body weight. BHT was dissolved in corn oil and injected intraperitoneally at a volume of 0.1 ml/10 g body weight. Bleomycin was dissolved in sterile saline and instilled intratracheally at a volume of 0.1 ml/20 g body weight.

Aminophylline pretreatment. The first study was to determine the effect aminophylline treatment had on collagen accumulation in the lung following chemical-induced damage. Aminophylline or normal saline was injected subcutaneously at a dose of 20 mg/kg body weight twice daily for 2 days prior to induction of lung injury (see below), on the day of lung injury, and for the duration of the study.

* Research was sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc.

† Research was performed under an appointment to the Laboratory Cooperative post-graduate research training program administered by Oak Ridge Associated Universities for the U.S. Department of Energy.

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Induction of lung injury. Injury was induced on day 0 by two methods: (1) BHT was administered intraperitoneally at a dose of either 350 or 400 mg/kg, while controls received corn oil, and (2) bleomycin was instilled intratracheally at a dose of 4 units/kg with controls receiving saline.

Aminophylline time study. In a separate study, three different dosing schedules of aminophylline were used with BHT (350 mg/kg) as the damaging agent. Aminophylline (20 mg/kg) was administered for 3 days on either days −2 to 0, +1 to +3, or +4 to +6.

Mice were killed, utilizing methoxyflurane anesthesia and exsanguination, 2 weeks following BHT injection and 3 weeks following bleomycin instillation. Lungs were perfused *in situ* through the right ventricle with normal saline, excised, frozen, and lyophilized. Hydroxyproline, an amino acid present virtually only in collagen [9], was measured as an estimate of total lung collagen. The hydroxyproline assay used has been described previously [10].

Cyclic nucleotide determination. The effect of aminophylline on cyclic nucleotide levels in the damaged lung was determined. Aminophylline (20 mg/kg) or saline was administered twice daily on days −2 to +7. On day 0, BHT (350 mg/kg) was administered to all animals. Animals were killed (N = 3/group) on days −2 to +7, and lungs were removed for cyclic nucleotide determination. Cyclic AMP and cGMP values were determined from each lung by radioimmunoassay (New England Nuclear) and corrected for protein content [11]. Data were expressed as the cAMP/cGMP ratio which appears to be a more sensitive and reliable indicator of the growth state of the cells than the individual nucleotide levels [12].

Statistical analysis. One-way analysis of variance was performed on the hydroxyproline data with comparison of means performed using Tukey's w-procedure [13]. Cyclic nucleotide data were analyzed by a paired *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Aminophylline treatment. The first series of experiments were designed to evaluate the effect of continuous aminophylline administration on lung damage induced by either BHT or bleomycin. Aminophylline treatment did not affect body weight significantly when compared with the appropriate controls (data not shown). This lack of effect on body weight by aminophylline was also seen in the other experiments in this study. The hydroxyproline values for 350 mg/kg and 400 mg/kg BHT-treated groups are shown in Fig. 1. BHT (350 mg/kg) elevated hydroxyproline values compared with corn oil controls. The continuous treatment of aminophylline, starting 2 days prior to BHT, completely blocked this elevation. BHT at 400 mg/kg raised hydroxyproline values even higher than BHT at 350 mg/kg. Continuous aminophylline treatment again significantly lowered hydroxyproline values, although they did not completely return to control levels.

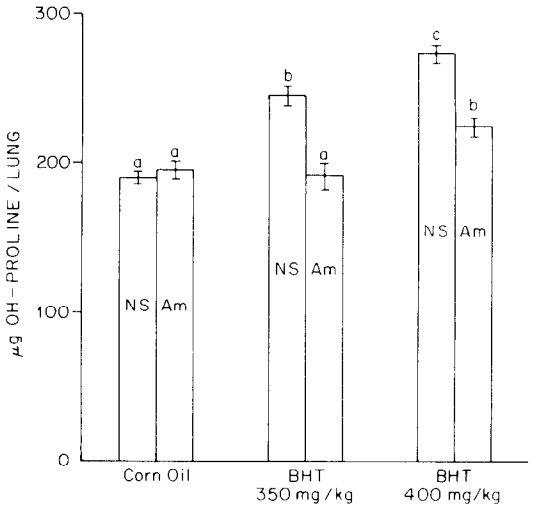

Fig. 1. Effect of continuous aminophylline administration on lung damage induced by BHT. Aminophylline (Am) (20 mg/kg) or normal saline (NS) was administered subcutaneously twice daily on days −2 to +14. Lung injury was induced on day 0 with butylated hydroxytoluene (BHT) (350 or 400 mg/kg) intraperitoneally. Animals were killed (N = 9–10/group) on day 14. Results are expressed as μg hydroxyproline per total lung (mean ± S.E.). Bars with different superscripts differ significantly ($P < 0.05$).

Figure 2 illustrates the effect of aminophylline on bleomycin-treated mice. Bleomycin alone significantly elevated hydroxyproline values above control. The continuous treatment of aminophylline significantly reduced the accumulation of hydroxyproline in the bleomycin-treated animals, although

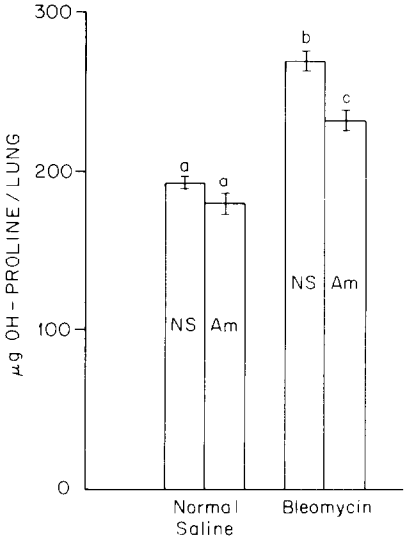

Fig. 2. Effect of aminophylline on bleomycin-treated mice. Aminophylline (Am) (20 mg/kg) or normal saline (NS) was administered subcutaneously twice daily on days −2 to +21. Lung injury was induced on day 0 with bleomycin (4 units/kg) instilled intratracheally. Animals were killed (N = 8–10/group) on day 21. Results are expressed as μg hydroxyproline per total lung (mean ± S.E.). Bars with different superscripts differ significantly ($P < 0.05$).

Table 1. Aminophylline time study

Treatment* (day 0)	Aminophylline† or normal saline	Hydroxyproline‡ (μg/lung)
Corn oil	Normal saline	186.7 ± 3.7§
BHT	Normal saline	251.3 ± 5.9‖
BHT	Aminophylline (days −2 to 0)	242 ± 7.0
BHT	Aminophylline (days +1 to +3)	256.1 ± 7.2
BHT	Aminophylline (days +4 to +6)	244.6 ± 9.1

* Male mice received BHT (350 mg/kg) i.p. or corn oil (0.1 ml/10 g) i.p. on day 0 and were killed 2 weeks later.

† Mice received aminophylline (20 mg/kg twice daily) or normal saline subcutaneously on the days indicated.

‡ Values are means ± S.E. from nine to ten animals/group.

§ Hydroxyproline values for corn oil treated animals administered aminophylline during the various time intervals were not different from saline control animals

‖ All BHT-treated groups were elevated significantly over control (corn oil) groups. However, there was no significant difference between the four BHT-treated groups.

these values were still above untreated control values.

Aminophylline time study. To determine if there was a critical time period that aminophylline produced its effect, animals were administered aminophylline at three different intervals relative to BHT administration. These time periods included: prior to BHT (days −2 to 0), during initial BHT damage (days +1 to +3), and a few days after BHT administration (days +4 to +6). As shown in Table 1, there was no significant effect on the BHT-induced elevation of hydroxyproline values with any of the treatment regimens.

Cyclic nucleotide determination. Since the previous results have shown that aminophylline influenced hydroxyproline levels in damaged lungs while having no effect in control lungs, the effect that aminophylline had on cyclic nucleotide levels in the damaged lung was determined. Alterations in the cyclic nucleotide ratio induced by administration of aminophylline or saline in a BHT-damaged lung are shown in Fig. 3. Aminophylline significantly elevated the

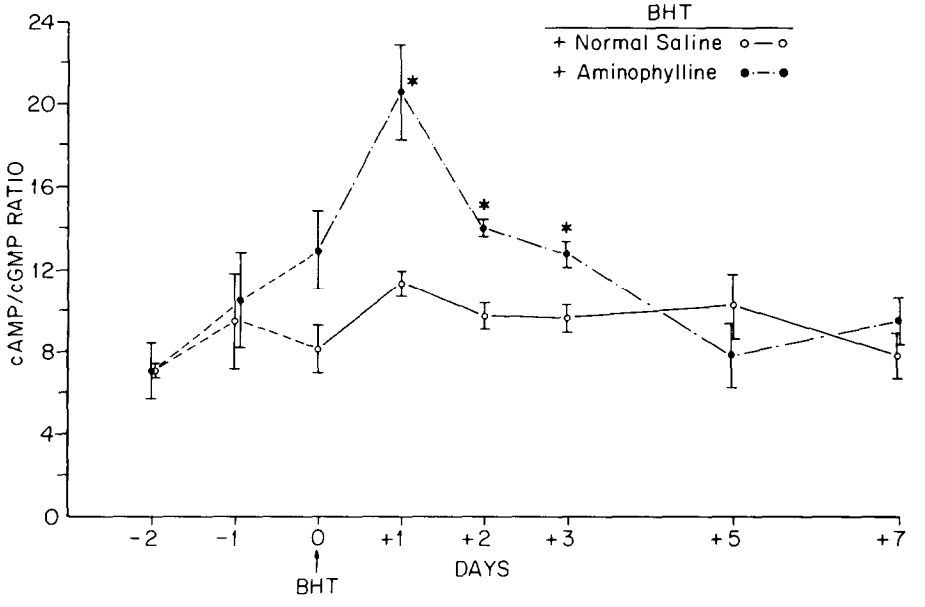

Fig. 3. Effect of aminophylline on cyclic nucleotide levels in BHT-damaged lungs. Aminophylline (20 mg/kg) or normal saline was administered twice daily on days −2 to +7. Butylated hydroxytoluene (BHT) was administered to all animals at a dose of 350 mg/kg on day 0. Animals were killed (N = 3/group) on the days indicated, and cAMP and cGMP values were determined for each lung by radioimmunoassay and corrected for protein content. Data are expressed as the ratio of cAMP/cGMP (mean + S.E.). An asterisk (*) = $P < 0.05$. Absolute values (mean ± S.E.) for control cAMP and cGMP at the start of the study were 36.9 ± 5.7 and 5.2 ± 0.4 pmoles/mg protein respectively.

cAMP/cGMP ratio on days +1, +2, and +3 in BHT-treated animals compared with saline-treated BHT-animals.

DISCUSSION

In the present study we have shown that aminophylline administration to mice prior to, and simultaneously with, acute lung damage prevented the increase in total lung hydroxyproline content compared with control animals with lung damage. This decrease in lung hydroxyproline back to untreated control values was complete when the lung damage was moderate (BHT 350 mg/kg), while the attenuation following more severe damage (BHT 400 mg/kg or bleomycin 4 units/kg) was only partial. Aminophylline treatment in animals without lung injury did not affect hydroxyproline content. Aminophylline also resulted in an elevated total lung cAMP/cGMP ratio during the initial days of lung injury.

Collagen is the most abundant protein synthesized by mammalian fibroblasts and plays an important role in the structure and function of most tissues. Production of collagen by fibroblasts appears to be rigidly controlled [14, 15]. One of the stimuli that can modify collagen production by fibroblasts is the alteration of cAMP levels within the cell [3]. Baum *et al.* [3] demonstrated that several agents, all of which elevate intracellular cAMP, cause an average reduction of 47% in the percentage of total protein synthesis represented by collagen. This occurred with little change in the production of other major extracellular proteins and was produced by a variety of agents, all with different mechanisms of elevating cAMP. These results suggest a central role for cAMP as a regulatory signal. Looking at this role in more detail, Baum *et al.* [4] demonstrated that the effect of cAMP in altering the amount of collagen produced is by modulation of the level of intracellular degradation. Berg *et al.* [5] have shown that even small changes in cAMP are effective in suppressing collagen production in cultured fibroblasts.

Several *in vivo* studies have supported the concept that cyclic nucleotides may play an important role in the processes that result in pulmonary fibrosis. We recently demonstrated in mice that administration of the beta-adrenergic antagonist, propranolol, at a dose demonstrated to decrease the ratio of cAMP/cGMP, results in an elevation in total lung collagen *in vivo* [10]. This increase in collagen is evident only when propranolol is administered prior to and during acute lung damage induced by either BHT, bleomycin, or high concentrations of oxygen. No effect is seen when propranolol administration is delayed following injury or when given to an undamaged lung. Kuo *et al.* [16] showed that BHT administration results in increased levels of cGMP within the lung. Since it appears that the ratio of cAMP/cGMP is important in the regulation of cell proliferation [17], an increase in cGMP would diminish the effects of cAMP. Giri and Krishna [18] made a similar finding using paraquat, an agent known to induce fibrosis [19]. They showed that, following paraquat administration to guinea pigs, there are marked elevations in guanylate cyclase activity, an enzyme that converts guanosine triphosphate to cGMP. These studies support the hypothesis that alterations in cyclic nucleotides (decreased cAMP or increased cGMP) may play a role in the resulting fibrosis.

Our experiments suggest that aminophylline may suppress collagen production by fibroblasts in a damaged lung. Several *in vitro* studies support this possibility [3, 4, 20, 21]. However, since our study was *in vivo*, versus the closed system of tissue cultures, there is lack of "direct" evidence linking aminophylline to fibroblast activity. Other possibilities exist that may have contributed to the aminophylline-induced suppression of collagen production. The alteration of other endogenous mediators by aminophylline, possible alterations in blood supply, or changes in the metabolism of the toxic agents may have played a role. However, if aminophylline had influenced the metabolism of BHT, one would have expected to see a similar prevention of collagen accumulation when aminophylline was given prior to, or during the first several days following, administration of BHT (Table 1). The lack of effect of the short-term dosing of aminophylline does not support the possibility of altering metabolism.

Alterations in the whole lung cAMP/cGMP ratio early in the damage and repair process appear to have influenced the subsequent deposition of collagen. If the cAMP/cGMP ratio is decreased, as in the case of the beta-adrenergic antagonist propranolol, excessive collagen production can result [10]. Conversely, the present data suggest that a lung involved in the normal reparative process may be affected by the aminophylline-induced elevation in the intracellular cAMP/cGMP ratio. This increase in the cAMP/cGMP ratio may correlate with a decreased hydroxyproline deposition *in vivo*.

Acknowledgements—We would like to gratefully acknowledge the assistance provided us by Mrs. June Whitaker and Mr. Tom Stephens.

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