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Aging and cholinergic responses in bovine trachealis muscle

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1 The relative potencies of muscarinic agonists on bovine tracheal smooth muscle were unchanged as a consequence of aging and were carbachol > oxotremorine > muscarine > pilocarpine > McNeil A-343.

2 During aging, the potencies of carbachol, oxotremorine, McNeil A-343 and pilocarpine, but not muscarine, were reduced.

3 Maximal induced tensions to all the agents studied were reduced as a consequence of age.

4 Irreversible antagonism with benzilylcholine mustard showed that agonist efficacy was significantly reduced during aging.

5 Estimated receptor occupancy at the EC_{50} was significantly greater in tracheal tissues from the mature versus immature cows for every agonist studied.

6 The dissociation constants for full agonists (carbachol, oxotremorine and methacholine) were decreased with maturation while the converse was observed with partial agonists (McNeil A-343, pilocarpine).

7 We conclude that there are significant changes in the properties and coupling of muscarinic receptors during aging. These changes may contribute to the reduced airway reactivity seen *in vivo*.

Introduction

A reduction in airway reactivity during aging is well documented both in man and animals (Derrick, 1971; Brink *et al.*, 1980; Weiss *et al.*, 1984) but there is a paucity of information regarding the mechanism(s) responsible for this change *in vivo*. This is, in part, because many neural and humoral inputs contribute to the regulation of airway reactivity *in vivo*. *In vitro* models, devoid of many neural and humoral factors, allow a determination of the changes which occur during aging directly at the level of smooth muscle. Further, techniques which are available allow a characterization of receptor properties from functional responses of isolated tissues to agonists.

Airway smooth muscle, especially that of the trachea, bronchi and central airways (Barnes *et al.*, 1983; Nadel & Barnes, 1984) is predominantly innervated by efferent vagal nerve fibres which terminate at muscarinic cholinoceptors on the smooth muscle. This neuronal supply is important in maintaining airway tone and in determining airway reactivity (Nadel & Barnes, 1984). Thus, changes in muscarinic receptor sensitivity might, in part, account for the decreased airway reactivity seen *in vivo*. Since the neurotransmitter at these parasympathetic nerve cell bodies is acetylcholine, this study has focussed attention on the effects of age on the sensitivity of airway smooth muscle to cholinomimetics. In addition, receptor affinity, estimated receptor occupancy at the EC_{50} and agonist coupling have been determined using the irreversible antagonist, benzilylcholine mustard.

Methods

Tissue preparations

Bovine tracheae from milk-fed Holstein cows were obtained from J.G. Fortes, North Branford, CT. Tracheae from mature Holstein cows (>5 years old) were obtained from Frank DeMartino and Son & Sons, Seymour, CT. The fresh tissues were transported in chilled Tyrode solution (4°C) to the laboratory.

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Tracheae were stripped free of all connective tissues. An incision was made down the dorsal medial line in order to expose the trachealis muscle. The cartilage rings were cut and everted to reveal the smooth muscle covered with an epithelial layer. This layer was stripped off the muscle with forceps. Small bundles of trachealis muscle were cut and tied at both ends with silk sutures. The average wet weight of a muscle strip was 10 mg–20 mg. Tissues were mounted in tissue baths (10 ml) and allowed to equilibrate for 2 h in Tyrode solution at 37°C, gassed with 5% CO_2 in O_2. The composition of the Tyrode solution (mM) was: NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.5, pH 7.4. Tissues were equilibrated under an initial load of 2 g. At the end of the equilibration period, the length of each preparation was recorded. Contractions were determined isometrically using Statham strain gauges (Model UC3) and the outputs were displayed on strip chart recorders (Kipp & Zonen, Holland; Model BD41). Upon completion of the experiment, tissues were removed from the organ baths, blotted and weighed to allow a determination of cross sectional area (Brink *et al.*, 1980).

Contractile responses to full agonists

Preliminary experiments were carried out to determine the reproducibility of sequential cumulative concentration-effect curves to the same agonist. Three sequential cumulative concentration-effect curves were generated for either carbachol, oxotremorine or muscarine. Tissues were washed until they had returned to baseline tension once a maximal response was achieved. Results of these experiments suggested that an alternate protocol was necessary to ensure reproducible results. Therefore, concentration-effect curves were generated in the presence of and after tissues had been pretreated for 20 min with indomethacin (17 μM). Based on results from these experiments, all subsequent experiments were carried out using the indomethacin pretreatment described above.

Contractile responses were expressed as a percentage of the maximal response to the agonist. EC_{50} values of agonists were determined from individual concentration-effect curves which were fitted by eye. These results are presented as the negative of the log molar EC_{50} value. Maximal tension (g mm^{-2}) was determined using the formula:

$$\text{Tension} = \text{Force/Cross sectional area}$$

i.e. Tension

$$= [\text{Force (g)} \times \text{Tissue length (mm)}]/\text{Wet weight (mg)}.$$

Irreversible antagonism

The dissociation constants of muscarinic cholinoceptor agonists (carbachol, oxotremorine, muscarine) at the muscarinic receptor in bovine trachealis muscle were determined according to the modification of the method described by Furchgott & Bursztyn (1967). A control cumulative concentration-effect curve was generated in the presence of indomethacin (see above). Tissues were washed to restore the basal tone after which preparations were incubated with freshly prepared benzilylcholine mustard (1 μM, final bath concentration) for 10 min. At the end of the incubation period, tissues were washed several times with fresh Tyrode solution. A second cumulative concentration-effect curve was now generated for the agonist under study.

Contractile responses to partial agonists

Cumulative concentration-effect curves were initially generated to carbachol. After washout to baseline, a cumulative concentration-effect curve to either pilocarpine or McNeil A-343 was generated. Responses to partial agonists were normalized as a % of the maximal carbachol response. $-\log EC_{50}$ values and maximal tensions were calculated as described above.

Data analysis

Irreversible antagonism Several equieffective concentrations were interpolated from concentration-effect curves to each agonist before (A) and after (A′) treatment with benzilylcholine mustard and were determined graphically. 1/[A] was plotted versus 1/[A′] and a straight line was fitted to the data by linear regression analysis. From the slope and the intercept on the ordinate scale, the dissociation constant (K_D) of the agonist receptor complex and the fraction of receptors still active (q) were calculated (Furchgott & Bursztyn, 1967). The efficacy of each agonist was determined from the EC_{50} value of the agonist and its dissociation constant (K_D) using data from individual experiments. The EC_{50} value was that concentration of each agonist which induced a response which was 50% of maximum. In addition, % fractional receptor occupancy was estimated for each agonist at its EC_{50} value using the following relationship:

$$100 \times [RC]/[RT] = 100 \times [EC_{50}]/(K_D + EC_{50})$$

where [RC] is the concentration of receptor-agonist complex and [RT] is the total receptor concentration (Besse & Furchgott, 1976). In some experiments, concentration-effect curves were fitted iteratively using ALLFIT (DeLean *et al.*, 1978). The equation

used to fit the line was the four parameter logistic equation:

$$Y = (a - d)/(1 + (X/c)^b) + d$$

where X and Y are the concentration of agonist and the induced response, respectively. 'a', 'b', 'c' and 'd' are the four fitted parameters, namely, response at zero concentration, slope factor, EC_{50} and response at infinite dose. After curve fitting, the parameters were substituted into the algorithm to yield concentrations giving known responses. These concentrations derived for both control ([A]) and benzilylcholine mustard concentration-effect curves (1[A']) were then analysed by linear regression analysis to determine the dissociation constant as described above. There were no significant differences in the data whether analysed using iterative curve fitting or using concentration-effect curves drawn and analysed by eye.

Determination of dissociation constant using partial agonists

The technique for the determination of dissociation constants of partial agonists is based on methods described by Barlow *et al.* (1967) and Waud (1969). The relationship between the concentration-effect curves to a partial agonist (P) and the full agonist (C) may be described as follows:

$$1/[C] = [ec/(K_D \times e_p)] + (e_c \times K_p)]/e_p \times K_D \times [P]$$

where [C] and [P] are the respective concentrations of full and partial agonist inducing equal responses; e_c and e_p are the respective efficacies of the full and partial agonist and K_D and K_p are the respective dissociation constants of the two agonists at the receptor. The dissociation constant (K_p) and efficacy (e_p) of the partial agonist at the muscarinic receptor were determined from double reciprocal plots of equiactive concentrations of carbachol and partial agonist. K_p was calculated from the slope and intercept on the ordinate scale of the straight line fitted to the data points by linear regression analysis (Waud, 1969). A small number of experiments were analysed by computer as described above. These data were not different from those analysed by eye.

Materials

The drugs used and their sources were: carbamylcholine chloride, pilocarpine hydrochloride, oxotremorine sesquifumarate, (±)-muscarine chloride, indomethacin (Sigma Chem. Co., St. Louis, MO); benzilylcholine mustard (gift from Dr D. Triggle School of Pharmacy, Buffalo); 4-(*m*-chlorophenylcarbamoyloxy)-2-butynyl-trimethylammonium chloride (McNeil A-343; McNeil Pharmaceutical, Spring House, PA). Indomethacin was dissolved in a minimal quantity of absolute ethanol and the solution was diluted with Tyrode solution.

Results

Reproducibility of cumulative concentration-effect curves

Both agonist potency and tissue contractility were significantly greater in second and third concentration-effect curves than the values obtained during the generation of the initial concentration-effect curve (Figure 1). Following treatment with indomethacin, no significant differences were observed between sequential concentration-effect curves (Figure 2). Based on these observations subsequent experiments involving the generation of sequential concentration-effect curves were conducted in the presence of indomethacin.

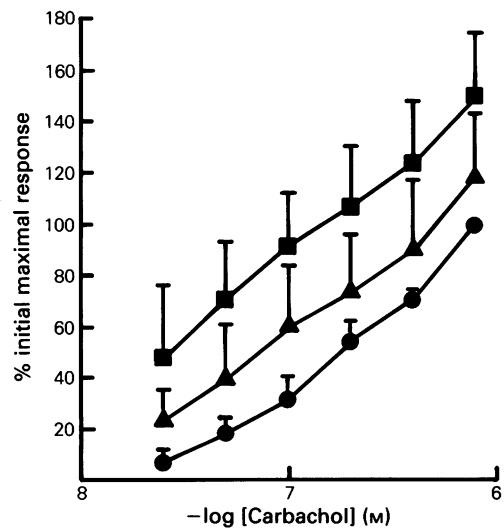

Figure 1 Reproducibility of sequential cumulative concentration-effect curves to carbachol. The figure shows the first (●), second (▲) and third (■) cumulative concentration-effect curves to carbachol in bovine tracheal tissues. Tissue response is expressed as a % of the maximal tension induced by carbachol during generation of the first concentration-effect curve. There was an increased potency of agonist and increased tissue contractility. Values are the mean of 4 determinations; vertical lines indicate s.e. mean.

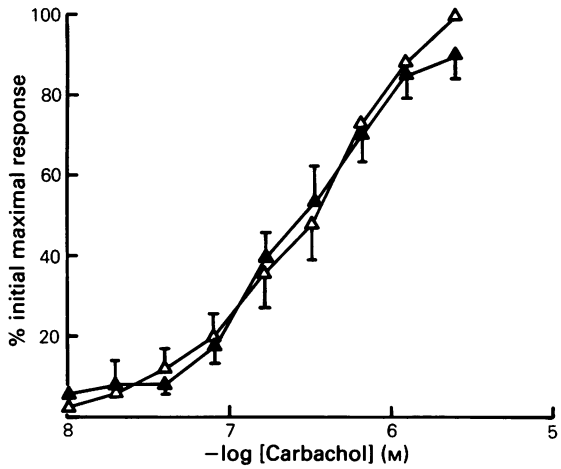

Figure 2 Effect of indomethacin treatment on concentration-effect curves to carbachol. Concentration-effect curves to carbachol before (△) and after (▲) treatment of tissues with indomethacin (17 μM; see Methods) are shown. Note the reproducibility of these experiments. Values are the mean of 4 determinations; vertical lines indicate s.e. mean. Where no s.e. mean is shown the value falls within the size of the symbol.

Contractile responses to full agonists

Concentration-effect curves to the muscarinic cholinoceptor full agonists, carbachol, oxotremorine and muscarine were generated in preparations from both immature (<2 weeks old) and mature (>5 years old) cows. There were significant decreases in tissue sensitivity to carbachol and oxotremorine during aging (Figure 3; Table 1). In contrast, there was no significant difference in tissue sensitivity to muscarine during aging (Figure 3; Table 1). The maximal tensions induced with carbachol, oxotremorine and muscarine were significantly decreased as a consequence of aging (Table 1).

Contractile responses to partial agonists

Concentration-effect curves to pilocarpine and McNeil A-343 were generated in both immature and mature tissues and responses were expressed as a % of the maximal response to carbachol. Significant decreases in the $-\log EC_{50}$ values were seen with both agonists during aging (Figure 4; Table 1). The maximum tensions developed were also significantly decreased with aging (Table 1).

Receptor affinity and drug efficacy

Treatment of tissues with benzilylcholine mustard caused a depression of maximum contractility and a large shift of the concentration-effect curve to the right (Figure 5). Analysis of these data showed that during aging, affinity of the receptor (K_D) for full agonists was increased (Table 1). In contrast, receptor affinity for partial agonists was reduced in tissues from mature animals. For all agonists except muscarine, there were increases in estimated receptor occupancy at the EC_{50}. Efficacy was decreased for all agonists (Table 1). Although there were considerable differences in the efficacies of full agonists in tissues from immature animals, these differences were not apparent in tissues from mature cows.

The order of potency ($-\log EC_{50}$) of the muscarinic agonists used was not changed with age

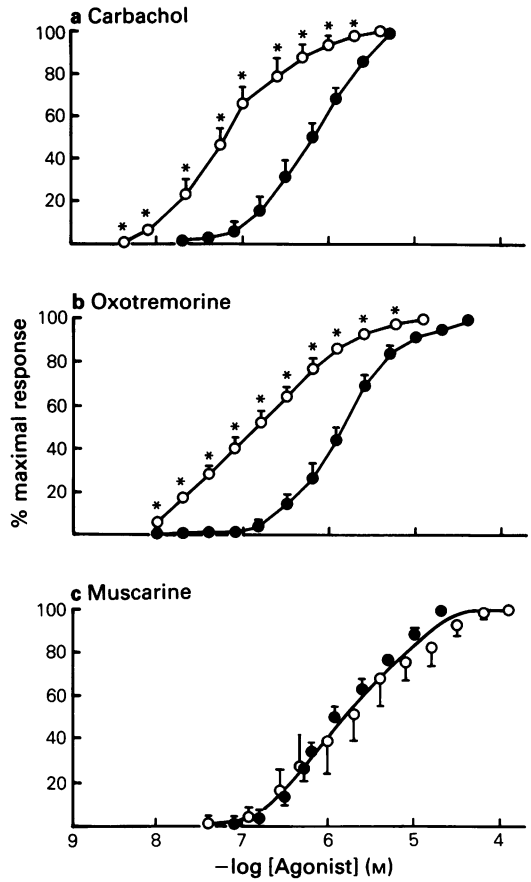

Figure 3 Concentration-effect curves to full muscarinic agonists. Each panel shows a concentration-effect curve to an agonist ((a) carbachol, (b) oxotremorine or (c) muscarine) in tissues from immature (○) and mature (●) cows. Data are the means from 4–8 experiments; vertical lines indicate s.e. mean. * Denotes values significantly different from data of mature tissues ($P < 0.001$).

Table 1 Potency, receptor affinity and efficacy of muscarinic agonists during aging in bovine tracheal tissues

Agonist	*Age*	T_{max}	$-log\ EC_{50}$	pK_d	*% occupancy*	*Efficacy*
Carbachol	Immature	5.57 ± 0.57*	7.14 ± 0.18*	4.12 ± 0.11*	0.31 ± 0.22*	2920 ± 270
	Mature	3.93 ± 0.91	6.28 ± 0.08	4.94 ± 0.22	6.9 ± 2.2	42 ± 19
Oxotremorine	Immature	7.19 ± 1.79*	6.89 ± 0.14*	4.23 ± 0.38	0.42 ± 0.14*	407 ± 115
	Mature	4.08 ± 1.09	5.90 ± 0.16	4.99 ± 0.09	2.31 ± 0.56	57 ± 14
Muscarine	Immature	6.15 ± 1.83*	5.89 ± 0.18	4.44 ± 0.21	2.26 ± 0.97	68 ± 19
	Mature	4.35 ± 1.0	5.90 ± 0.05	4.57 ± 0.26	1.91 ± 0.36	44 ± 13
Pilocarpine	Immature	6.30 ± 1.93*	5.77 ± 0.18*	5.42 ± 0.35	34.0 ± 12.5	4.9 ± 1
	Mature	1.74 ± 0.68	4.33 ± 0.25	4.40 ± 0.41	50.7 ± 15.8	2.7 ± 1
McNeil A-343	Immature	3.25 ± 0.14*	4.88 ± 0.05*	4.19 ± 0.10*	18.0 ± 3.5	6.6 ± 2
	Mature	2.44 ± 0.55	3.85 ± 0.10	3.34 ± 0.16	23.7 ± 2.3	4.3 ± 1

Values are the mean ± s.e. mean of 8 experiments using tissues from immature and mature animals except for data derived from irreversible antagonism experiments (pK_d, % occupancy and efficacy) which uses tissues from mature animals where data from four experiments are presented.
* Denotes values significantly different from values in tissues from mature animals.
$-log\ EC_{50}$: negative logarithm of the agonist concentration inducing a response which was 50% of maximal.
pK_d: negative logarithm of the dissociation constant.
T_{max}: maximal induced tension (g mm^{-2}).

and was carbachol > oxotremorine > muscarine > pilocarpine > McNeil A-343. The partial agonists were 200 times less efficacious and exhibited higher receptor occupancies when compared to carbachol.

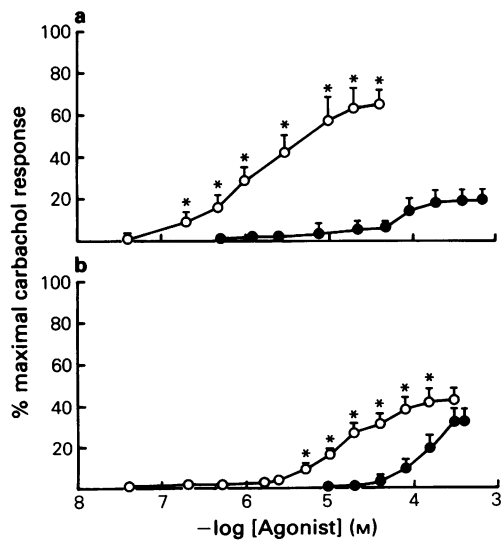

Figure 4 Concentration-effect curves to partial agonists (a) pilocarpine and (b) McNeil A-343. Each panel shows concentration-effect curves in tissues from immature (○) and mature (●) cows. Contraction (ordinate scale), expressed as a % of maximal carbachol response, is plotted against the negative logarithm of the agonist concentration (abscissa scale). Values are the mean of not less than 4 determinations; vertical lines indicate s.e. mean. *Denotes values significantly different from data of mature tissues ($P < 0.001$).

Discussion

Alterations in many responses to neurotransmitters and hormones as a consequence of aging have been well documented (Cohen & Berkowitz, 1967; Fleisch *et al.*, 1970; Toda & Hayashi, 1979; Roth, 1979; Scott & Reid, 1982). Generally, there is a reduction in tissue response to neurotransmitters which may be the result of either decreased agonist potency and/or reduced functional response. Similarly, airway smooth muscle tone is known to be affected by age and characteristically there is a reduction in response to stimulatory agents. For example, responses to bronchoconstrictor agents in man are greater in young individuals (Schlenker & Jaeger, 1980; Weiss *et al.*, 1984). Changes in tissue sensitivity and/or

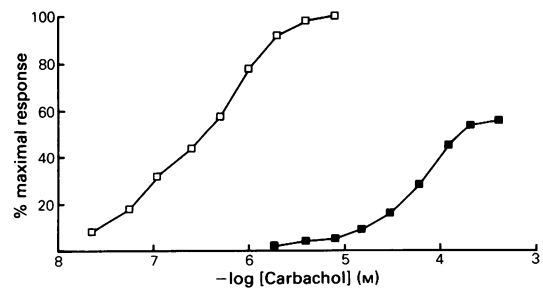

Figure 5 Effect of benzilylcholine mustard on carbachol-induced contraction. The figure shows a typical concentration-effect curve to carbachol in a tissue from an immature animal, before (□) and after (■) benzilylcholine mustard treatment (1 μM for 10 min, see Methods). Data are analysed as described in Methods and are presented in Table 1.

responsiveness may be particularly important in disease states such as bronchial asthma where airway hyperreactivity is a major contributing factor (Sears *et al.*, 1987). Indeed, the remission of childhood asthma (Morrison-Smith, 1961; Williams & McNicol, 1969) is characterized by a loss of bronchial hyperreactivity (Kelly *et al.*, 1987). Experiments in guinea-pigs have also shown a decreased airway reactivity to histamine and salbutamol during aging (Brink *et al.*, 1980; Duncan *et al.*, 1983). These *in vivo* changes in airway reactivity are also demonstrable in *in vitro* airway preparations suggesting that the changes occur, in part, at the smooth muscle. For example, there are reduced functional responses to adrenoceptor, cholinoceptor and histamine receptor agonists and to leukotrienes during aging (Brink *et al.*, 1980; Douglas *et al.*, 1984; Duncan & Douglas, 1985). In a limited number of experiments, it appears that these functional changes are correlated with altered receptor/post-receptor processes e.g. reduced receptor densities (Rothberg *et al.*, 1987), reduced receptor coupling (Scarpace & Abrass, 1983) and/or reduced second messenger generation (Nakagawa *et al.*, 1986; Andersson *et al.*, 1978). Regardless of aging, there are clear differences in respiratory tissues from different species. It is therefore important to examine all of the potential factors which affect tissue sensitivity during aging in tissue from one species. Further, since biochemical studies are necessary, the choice of tissue should be one that allows a plentiful supply of pure airway smooth muscle, hence the use of bovine tissues in this study.

The objective of the experiments described was to determine whether there were age related changes in bovine airway smooth muscle and if so what mechanisms might account for these alterations. These experiments showed (1) decreased agonist potency (Table 1; Figures 3 and 4) and (2) decreased tissue contractility (Table 1). To determine the mechanism(s) responsible for these *in vitro* changes in tissue properties, it is necessary to examine the properties of the receptor and its ensuing cascade. Functionally, this can be achieved by determining the effects of irreversible antagonists on tissue response. Studies using benzilylcholine mustard, an irreversible muscarinic receptor antagonist, showed that, during aging, for all full agonists except muscarine, there was an increased affinity, decreased efficacy and increased estimated receptor occupancy at the EC_{50}. The reasons why muscarine did not show changes with age are unclear. For partial agonists, a decreased affinity, increased estimated receptor occupancy and decreased efficacy were noted.

Without additional measurements of the receptor cascade, a precise interpretation of these data is difficult. However, a number of salient points can be made from these results. The large difference between the potency of an agonist and its dissociation constant is indicative of a large receptor reserve. This is substantiated by the small estimated receptor occupancy at the EC_{50} for full agonists versus partial agonists (Furchgott & Bursztyn, 1967; Grandordy *et al.*, 1986). Aging increases the number of receptors which must be occupied to give an equivalent functional response and there is a concomitant decrease in agonist efficacy. It is significant that in tissues from mature animals the efficacy of full agonists is essentially identical. This result contrasts with data from tissues of immature animals where different agonists have large differences in efficacy. These results suggest a loss in signal generation and/or transduction subsequent to drug receptor interaction. It may be of significance that the rank order of the dissociation constants for agonists is reversed as a consequence of aging (Table 1). The significance of this finding can only be determined from ligand binding studies but may indicate quantitative and/or qualitative changes in cholinoceptors.

In summary, changes occur in bovine tracheal tissues during aging which render agonists less efficacious. These changes may include reductions in the density of high affinity binding sites, increased rigidity of membrane resulting in a loss of signal transduction and/or a reduced coupling of the receptor to the guanine nucleotide regulatory proteins. Further, studies which elucidate age related changes in these biochemical processes are clearly necessary to allow an elucidation of the mechanism(s) of greatest importance to altered functional responses during aging. An understanding of these changes may provide insights into the mechanisms of airway hyperreactivity and the remission of bronchial asthma in children as a consequence of aging.

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