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The Therapeutic Effect of Progesterone in Papain-Induced Emphysema (38372)

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Numerous studies have used anatomic and physiologic criteria to define papain emphysema in animals as a valid experimental model of the human disease (1–6). The report that a deficiency of the antiproteolytic factor, α_1-antitrypsin, is associated with human panlobular emphysema (7) provided another reason for the interest in an emphysema model caused by a proteolytic enzyme. Progesterone has been reported to prevent emphysema-like conditions produced by papain (8, 9) or phytohemagglutinin (10). However, in these studies, progesterone was administered prophylactically during the period of papain administration; the therapeutic potential of progesterone is unknown. Also, the direct effect of progesterone on papain activity has not been evaluated. The following studies were initiated to study (a) the therapeutic potential of progesterone against an established papain-induced emphysema lesion and (b) the effect of progesterone on papain cleavage of casein *in vitro*.

Methods. Protocol. Male Sprague–Dawley rats (5–6 weeks old) were treated with papain[1] as previously described (4). A 3% solution of papain in water was delivered by four nebulizers into a closed plexiglass chamber (34 × 21 × 19 in.) containing the rats. The nebulizers were operated at a pressure of 10 lb/in^2. Four cages of rats (five per cage) were placed in the chamber and given papain aerosol treatments three times a week for a 1 hr duration during a 4 week period. A group of "control" rats received no papain treatment. It had previously been shown that saline challenge had no adverse effect on the measured pulmonary parameters. At the end of the 4 week papain-treatment period eight treated and eight untreated "control" rats were sacrificed and the lung sections prepared for measurement of air spaces and mean linear intercept (Lm).

Functional residual capacity (FRC) was measured in all of the remaining rats, which were then divided into three groups. Progesterone (5 mg/kg sc daily) was administered to seven papain-treated rats, while seven other papain-treated rats received vehicle. Eight untreated "control" rats were allowed food and water *ad libitum*, but received no other treatment. FRC measurements were repeated on each rat 2 and 4 weeks after initiation of progesterone therapy. Following the final (four week) FRC determinations, rats were decapitated and the lungs were prepared for histologic examination.

Physiologic evaluation of lung damage. FRC determinations were performed by a technique based on those of King (11), Palecek *et al.* (2) and Giles *et al.* (4), but modified for chronic measurements. Rats were treated with atropine sulphate (1 mg/kg ip) and anesthetized 15 min later with sodium pentobarbital (30 mg/kg ip). A miniature laryngoscope, similar to that described by Gross (1), was utilized to insert a length of PE 200 tubing through the oral cavity into the trachea. The open end of the polyethylene tube was then connected to a 10 ml syringe containing pure oxygen; the connection to the syringe was 2 mm from the exit point of the cannula from the oral cavity. Such attachment was made at the end of expiration and the rats respired in this closed system for 7 min. Carbon dioxide was removed by an 11% solution of KOH with sufficient oxygen added to maintain constant volume. The tracheal tube was disconnected from the syringe at the end of expiration and the syringe was closed to the atmosphere. The percentage of nitrogen in the syringe was determined by use of an oxygen analyzer (In-

[1] Supplied by Warner-Chilcott Laboratories.

strumentation Laboratory, Inc), *i.e.* 100% − % oxygen = % nitrogen. Nitrogen concentration was converted to FRC (ml) by means of a calibration curve previously obtained by equilibrating known volumes of air with the apparatus.

In a separate series of experiments, FRC determinations were performed in six rats as previously described. The trachea was then exposed and a tie was placed around the trachea at the level of the cannula; the FRC was again measured. There was no indication of leakage in the technique used for chronic measurements.

The significance of the differences in mean values was determined using Student's *t* test for paired or unpaired observations.

Histologic evaluation of lung damage. Following decapitation, the thorax of the rat was opened and the lungs were distended with buffered neutral formalin fixation fluid at a pressure of 10 cm H_2O and removed intact. Lungs were left in the fixative until sectioned. After fixation, 5 μm sections were cut from paraffin blocks and stained with hematoxylin and eosin (HE).

Air spaces were determined as previously reported (4). A grid with 30 cross marks 12 mm apart (6 × 5 rows) was placed over the viewing screen of a Unitron microscope. Slides were examined at a magnification of 150 ×. Eight fields were randomly chosen from a single lung slide of the right lower lobe of the lungs. Lung spaces were counted in the following manner: if the cross marks fell on alveolar septa, blood vessels, connective tissue or any large air vessel wall, no score was given; if the cross mark fell on an empty space, a rating of one was given. Thus, a maximum score of 240 points was possible. An increase in air spaces would indicate either a loss of tissue, enlarged air spaces or both. This counting system was adapted from the work of Dunnill (12) and Palecek *et al.* (2).

The average intraalveolar distance (mean linear intercept; Lm) was determined by a modification of the method of Thurlbeck (13). Slides stained with HE were examined at a magnification of 200 times on a Visopan Projection Microscope. Measurements were made on five random fields. On each field, the number of alveolar intercepts was counted on right angle crossed lines of known length (0.95 mm), at that particular magnification. Alveolar walls transecting the lines, alveolar walls touching but not crossing the upper side of the horizontal line and touching but not crossing the left side of the vertical line were included as intercepts. Alveolar walls touching but not crossing the lower border of the horizontal or the right border of the vertical arm were not counted as intercepts. The total distance (the number of fields × the length of the cross lines) divided by the number of alveolar intercepts gave the Lm.

Papain assay. Papain was assayed by the method of Arnon and Shapira (14) using casein as substrate. A 1 ml solution (various concentrations of casein in 0.15 *M* NaCl–0.05 *M* Tris buffer, pH 8.0, containing 0.005 *M* cysteine and 0.002 *M* EDTA) was added to 1 ml of solution containing 300 μg of papain. Following incubation for 10 min at 37°, the reaction was stopped by the addition of 5 ml of 5% TCA. After 1 hr the supernatants were filtered through Whatman #1 filter paper and the absorbancy of the solution at 280 mμ was determined. All readings were corrected for the reagent blank, in which TCA was added prior to enzyme and substrate. The reaction was linear for at least 20 min.

Progesterone was solubilized in a 25% absolute ethanol/distilled water mixture and added to the papain solution 30 min before addition of the casein. The alcohol mixture had no effect on the reaction.

For analysis of enzyme kinetic data, reciprocal velocities were plotted against the reciprocal of substrate concentration as described by Lineweaver and Burke (15). The data were then analyzed according to the method of Wilkinson (16). All calculations were performed by a digital computer using COBOL programs based on Fortran programs written by Cleland (17). The programs provided values of the Michaelis constant (Km), maximal velocity (V_{max}), slope, 1/v intercept and the standard error of these estimates.

Results. Papain aerosol produced an emphysema-like lesion, characterized by elevated values for FRC, Lm and air space measurements. The effect of progesterone on FRC measurements is shown in Table I. After 4 weeks of progesterone administration to papain treated rats, FRC values, both absolute and based on body weight, were significantly lower in the group receiving the hormone than in the group treated with the vehicle.

Progesterone produced a significant decrease in both absolute and corrected (for body weight) FRC values during the first two weeks of treatment. Absolute FRC values for papain treated

TABLE I. The Effect of Progesterone, Administered After the Completion of Papain Treatments, on FRC Values of Male Rats.

	Weeks 1–4		Weeks 5–8		
		Time of measurement of FRC		Time of measurement of FRC	
No. of rats	Treatment	end of fourth week ml//ml/kg	Treatment	end of sixth week ml//ml/kg	end of eigth week ml//ml/kg
8	None	1.64 ± 0.07[a]//5.24± 0.27	None	2.59 ± 0.13//6.39 ± 0.38	2.81 ± 0.13//6.40 ± 0.24
7	Papain	3.34 ± 0.27[b]//9.80 ± 0.83[b]	Vehicle	3.52 ± 0.17[b]//8.51 ± 0.42[b]	4.13 ± 0.30[b]//9.05 ± 0.52[b]
7	Papain	3.64 ± 0.45[b]//10.30 ± 1.08[b]	Progesterone (5 mg/kg sc daily)	2.89 ± 0.23[c]//7.25 ± 0.52	2.98 ± 0.26[c] ± //6.51 ± 0.48[c]

[a] Mean ± SE.
[b] Significantly different from untreated controls of same measurement period, $P < 0.05$.
[c] Significantly different from papain group treated with vehicle, $P < 0.05$.

rats not receiving progesterone increased during the seventh and eighth weeks, $P<0.05$; FRC values, corrected for body weight did not change significantly. FRC (corrected for body wt) values of control rats increased during the first two weeks of the therapy period. Significant differences in body weight were not observed among the various treatment groups.

The curative effect of progesterone was also demonstrated by histologic evaluations (Tables II and III). Values of the Lm and air space measurements of "emphysematous rats" treated with progesterone were significantly lower than the values for "emphysematous rats" treated with vehicle. However, the decrease in Lm was not to the level of the untreated "control" rats indicating that the cure was not complete.

The effects of progesterone on the kinetics of the hydrolysis of casein by papain are shown in Table IV. Progesterone was tested in concentrations ranging from 10^{-6} to $10^{-3}M$; the effects of the two highest concentrations are shown. The slope, 1/v intercept and apparent Km for casein hydrolysis were not changed, indicating no enzyme inhibition.

Discussion. The present work, using both physiologic and anatomic measurements, demonstrates that progesterone has a therapeutically beneficial effect in papain-induced emphysema. The beneficial response to progesterone is probably not due to inhibition of papain, since progesterone treatments were not initiated until papain treatment had been withdrawn. This hypothesis is substantiated by the finding that concentrations of progesterone as high as $10^{-3}M$ had no effect on the enzymatic activity of papain *in vitro*.

The difference in FRC values between papain-treated rats receiving progesterone and papain-treated rats receiving vehicle cannot be attributed to weight differences since (a) both absolute and corrected (for body wt) FRC values were significantly different and (b) the weights of the experimental groups were similar. Occlusion of alveoli in many parts of the lung due to several diseases (*i.e.*, cell infiltrates in pneumonia) could also decrease FRC: no histologic evidence of any physical impairment of the FRC measurement was observed in the test groups.

The mechanism of the therapeutic effect of progesterone is unknown. A deficiency of serum α_1-antitrypsin has been related to human pan-

TABLE II. The Effect of Progesterone, Administered After the Completion of Papain Treatments, on Mean Linear Intercept Measurements in Male Rats.

	Weeks 1–4		Weeks 5–8	
No. of rats	Treatment	Lm values for rats sacrificed at the end of 4 weeks (μ)	Treatment	Lm values for rats sacrificed at the end of 8 weeks (μ)
8	None	51.2 ± 1.2[a]	—	—
8	None	—	None	52.0 ± 0.8
8	Papain	72.1 ± 4.1[b]	—	—
7	Papain	—	Vehicle	65.5 ± 2.1[b]
7	Papain	—	Progesterone (5 mg/kg sc daily)	57.3 ± 2.0[b,c]

[a] Mean ± SE.
[b] Significantly different from untreated control of same time period, $P < 0.05$.
[c] Significantly different from papain group treated with vehicle, $P < 0.05$.

lobular emphysema (7). Serum antitrypsin activity is elevated in pregnancy (18–20) and in women taking oral contraceptive medication containing progesterone and mestranol (20). Studies in our laboratory (21), however, have indicated that neither progesterone nor aerosolized papain altered serum antitrypsin in rats, indicating no relationship between serum antiprotease and the beneficial effect of progesterone. The therapeutic activity of progesterone may be due to stimulation of healing processes in pulmonary tissue. Aerosolized papain has been reported to alter elastic tissue in the lung (22) and a loss of elastic tissue would be expected to elevate FRC values. It is conceivable that progesterone could protect and restore the integrity of elastic or other tissue elements.

Apart from the general hypothesis that was tested, the present experiments also show that the rate of increase in FRC may be greater than that of the total body weight. Thus, during the first two weeks of FRC measurements, values of FRC/kg of control rats increased significantly; during the latter two weeks of the measurement period, FRC and weight appeared to increase at a similar rate. This finding suggests that comparative drug studies in rats be conducted in animals with as similar weight and age characteristics as possible.

Summary. An emphysema-like lesion was produced in rats by the periodic administration of papain aerosol. Functional residual capacity (FRC) was measured at the completion of the papain treatments and at two week intervals during the next month. Progesterone (5 mg/kg sc) daily for one month after development of the

TABLE III. The Effect of Progesterone, Administered After the Completion of Papain Treatments, on Air Space Values of Male Rats.

	Weeks 1–4		Weeks 5–8	
No. of rats	Treatment	Air space values for rats sacrificed at the end of 4 weeks	Treatment	Air space values for rats sacrificed at the end of 8 weeks
8	None	47.6 ± 3.4[a]	—	—
8	None	—	None	45.0 ± 3.7
8	Papain	67.5 ± 4.1[b]	—	—
7	Papain	—	Vehicle	60.3 ± 3.2[b]
7	Papain	—	Progesterone (5 mg/kg sc daily)	51.4 ± 2.4[c]

[a] Mean ± SE.
[b] Significantly different from untreated control, $P < 0.05$.
[c] Significantly different from papain group treated with vehicle, $P < 0.05$.

TABLE IV. The Lack of Effect of Progesterone on the Kinetic Constants of the Hydrolysis of Casein by Papain.

Concentration of progesterone (M)	Slope	Intercept l/v	Km $M \times 10^5$
0	1.10 ± 0.10[a]	0.93 ± 0.12	1.18 ± 0.26
10^{-4}	0.95 ± 0.14	0.92 ± 0.15	0.87 ± 0.27
10^{-3}	1.26 ± 0.20	0.92 ± 0.12	1.38 ± 0.31

[a] Values ± SE; triplicate measurements were made for all points on a line.

papain lesion, caused a significant decrease in FRC values; untreated control emphysematous rats maintained elevated values. Measurements of (a) average intraalveolar distance (mean linear intercept) and (b) air spaces in histological sections prepared from the lungs of rats sacrificed at the end of the papain treatment period and rats sacrificed 4 weeks later, after progesterone therapy, also suggested a therapeutically beneficial effect by the hormone. *In vitro*, progesterone had no effect on casein hydrolysis by papain.

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