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INHIBITION OF NEUTROPHIL RESPONSE BY MEPACRINE*

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Abstract—Clinical and experimental evidence supports neutrophil involvement in the pulmonary complications of adult respiratory distress syndrome. Preliminary evidence indicates that mepacrine salvages pulmonary function in experimental models of adult respiratory distress syndrome, possibly by inhibiting neutrophil activation [E. M. Canham *et al.*, *Am. Rev. resp. Dis.* **127**, 594 (1983)]. This study examines the effect of mepacrine on neutrophil responses involved in pulmonary dysfunction associated with adult respiratory distress syndrome. A comparison is made between the ability of mepacrine to inhibit a specific neutrophil response utilizing different stimuli and the ability to inhibit different neutrophil responses to a single stimulus. Neutrophils were activated by a soluble stimulus, phorbol myristic acetate, and a particulate stimulus, heat-inactivated opsonized group B streptococcus. Mepacrine inhibited superoxide production in response to both phorbol myristic acetate ($IC_{50} = 5.3 \pm 1.2\ \mu M$) or opsonized group B streptococcus ($16.1 \pm 1.7\ \mu M$). Chemotaxis in response to *n*-formylmethionylleucylphenylalanine was also inhibited ($41.1 \pm 2.2\ \mu M$). Finally, aggregation stimulated by either streptococcus or phorbol myristic acetate was inhibited by mepacrine ($73.0 \pm 9.8\ \mu M$ and $77.0 \pm 19.2\ \mu M$ respectively). A comparison of the IC_{50} values demonstrates that the inhibitory effect of mepacrine is response dependent and stimulus independent. The results of this study are consistent with the proposal that mepacrine protects against the pulmonary complications associated with adult respiratory distress syndrome by its action as an inhibitor of neutrophil function.

Clinical studies have demonstrated enhanced neutrophil chemotaxis [1], superoxide production [1], and aggregation [2] accompanying patients with adult respiratory distress syndrome. Plasma isolated from these patients also aggregates normal neutrophils, *in vitro* [3]. In animal models of adult respiratory distress syndrome [4–8], protein-rich pulmonary edema probably results from neutrophil-mediated superoxide production [5, 7], whereas changes in hemodynamics are due to the release of mediators during neutrophil aggregation [4] as well as to superoxide production [5, 7]. Mepacrine preserves pulmonary function in these animal models, possibly by inhibiting neutrophil-mediated damage [6].

Previous investigations have demonstrated inhibition of phorbol myristic acetate (PMA‡)-induced superoxide production [6, 9] and *n*-formylmethionylleucylphenylalanine (FMLP)-induced chemotaxis [10] by preincubation of the neutrophils with mepacrine. However, no examination to determine whether the inhibitory effect of mepacrine on neutrophil activation was stimulus dependent or response dependent has been published. The inhibitory effect of mepacrine on a specific neutrophil response utilizing different stimuli was examined and compared to the effect of mepacrine on several different neutrophil responses utilizing a single stimulus. Aggregation and superoxide production in response to a soluble stimulus, PMA, and a particulate stimulus, heat-inactivated opsonized group B streptococcus, was examined. Chemotaxis was examined using FMLP as a stimulus. Upon infusion into animals, each of these stimuli produces similar pathophysiologic responses as seen in patients with adult respiratory distress syndrome [6, 11, 12].

METHODS

Materials. Hanks' balanced salt solution (HBSS) with 0.1% albumin (Sigma) was prepared as previously described [13]. Solutions of mepacrine, catalase, superoxide dismutase (SOD) and cytochrome *c* (all from Sigma) were prepared in HBSS. A stock solution of phorbol myristic acetate (25 μg/ml) was prepared in dimethyl sulfoxide and then diluted with HBSS. The final concentration of phorbol myristic acetate in all assays was 5 ng/ml.

Culturing, heat inactivation, and opsonization of group B streptococcus. Group B streptococci were grown overnight in Mueller–Hinton broth, heat-inactivated (100°, 30 min), and opsonized as previously described [11]. Following heat inactivation, the bacteria were centrifuged (3440 *g*, 5°, 30 min) and resuspended in sterile saline to a concentration of 10^{11} organisms/ml. After centrifugation (12,000 *g*, 2 min), the group B streptococci were opsonized by incubating with an equal volume of human plasma for 30 min at 37°. The opsonized streptococci were centrifuged (12,000 *g*, 2 min), resuspended in an equal volume of saline, and chilled (0°) until use.

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‡ Abbreviations: FMLP, *n*-formylmethionylleucylphenylalanine; GBS, group B streptococcus; HBSS, Hanks' balanced salt solution; PMA, phorbol myristic acetate; and SOD, superoxide dismutase.

Isolation of neutrophils and plasma. Neutrophils were prepared according to the method of Grisham *et al.* [14] using a Ficoll–Hypaque procedure. Neutrophils were diluted to a concentration of 20,000/μl for measurement of aggregation and superoxide production and to 400/μl for chemotaxis. Plasma was isolated from whole blood by centrifugation (2750 *g*, 5°, 15 min) and then chilled (0°) until use.

Aggregation of neutrophils. Aggregation of neutrophils was determined by nephelometry [13] assigning a 100% transmission value to a 50% HBSS dilution of the stock neutrophil suspension. Neutrophils were preincubated with mepacrine for 3 min at 37° prior to stimulation with phorbol myristic acetate (5 ng/ml) or group B streptococcus (5×10^9 organisms/ml). Aggregation followed for 5 min post-stimulation. Aggregation was defined as the maximum change in light observed during the 5-min post-stimulation period [13].

Neutrophil superoxide production. The production of superoxide was measured as SOD-inhibitable cytochrome *c* reduction as previously described [15]. Prior to assays neutrophils were preincubated with mepacrine for 3 min at 37°. Reactions were then initiated by addition of the stimuli at concentrations of 5 ng/ml and 1×10^9 organisms/ml for phorbol myristic acetate and group B streptococcus respectively.

Neutrophil chemotaxis. Neutrophils were preincubated with mepacrine at various concentrations for 3 min at 37° prior to chemotaxis. Chemotaxis was then assayed in response to *n*-formylmethionylleucylphenylalanine (10^{-7} M) as previously described [14].

Statistics. All data are expressed as mean ± standard deviation of the mean. Analysis of variance, with consideration for treatment, daily response, or repeated measurements, was conducted to determine significance within a system. Protected one-tailed *t*-tests were utilized to determine the significance of inhibition (of each group) from the control within the system. The IC_{50} values for mepacrine inhibition were determined by logit-log analysis. To determine the significance of the fit of the data to the model, a *T*-statistic for the correlation coefficient of each curve was performed. The IC_{50} values were compared utilizing a protected *T*-test. To enhance data interpretation, individual experiments were normalized to percent control diluent (100%) response.

RESULTS

Inhibition of superoxide production. Both phorbol myristic acetate and opsonized group B streptococci stimulated superoxide production in neutrophils (Fig. 1). Preincubation with mepacrine (1–200 μM) was effective at inhibiting superoxide production stimulated by either agent. Mepacrine at concentrations of 100 μM or greater did not inhibit superoxide-mediated reduction of cytochrome *c* stimulated by either KO_2 or xanthine-xanthine oxidase, whereas neutrophil-mediated superoxide production was inhibited by 80% at concentrations of

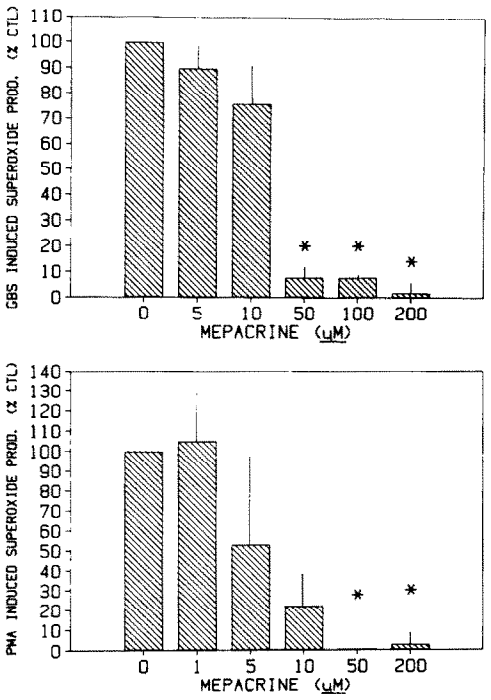

Fig. 1. Inhibition of superoxide production by mepacrine. Neutrophils preincubated with mepacrine were measured for superoxide production when stimulated with phorbol myristic acetate (PMA) or heat-inactivated opsonized streptococci (GBS). An asterisk (*) indicates that the difference between the experimental and control groups is statistically significant ($P < 0.0001$). Values are means ± SD; the N for experiments involving streptococci was 4 and that for phorbol myristic acetate was 5. Control values for superoxide production stimulated by streptococci and phorbol myristic acetate were 0.84 ± 0.21 and 2.14 ± 1.37 μmoles/min respectively.

100 μM mepacrine. These observations indicate that mepacrine is acting as a direct inhibitor of neutrophil-mediated superoxide production rather than a radical scavenger.

Inhibition of aggregation and chemotaxis. Opsonized bacteria and phorbol myristic acetate stimulate neutrophil aggregation by two different cellular mechanisms. While opsonized particles stimulate the production of aggregatory eicosanoid metabolites [16, 17], phorbol esters are ineffective at stimulating endogenous arachidonic acid release in the neutrophil [10, 17]. However, both agents stimulated neutrophil aggregation (Fig. 2). Mepacrine (10–200 μM) was equally effective at inhibiting aggregation induced by either stimuli. Chemotaxis in response to FMLP was also inhibited by mepacrine (10–100 μM) (Fig. 3).

Comparison of IC_{50} values for superoxide production, aggregation and chemotaxis. Logit-log modeling of the various PMN responses induced by the different stimuli resulted in significant correlations ($P < 0.01$) for all responses (data not shown). Mepacrine was most effective as an inhibitor of superoxide production regardless of the stimulus (Table 1). The IC_{50} for inhibition of chemotaxis was significantly greater than that required for superoxide production,

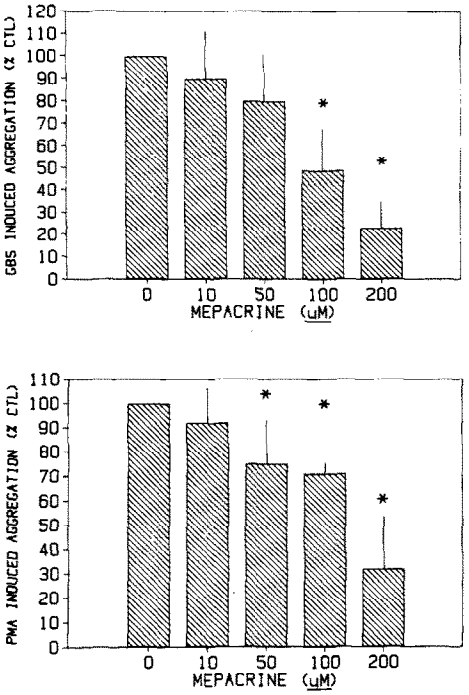

Fig. 2. Inhibition of aggregation by mepacrine. Neutrophils preincubated with mepacrine were aggregated with either phorbol myristic acetate (PMA) or heat-inactivated opsonized streptococcus (GBS). An asterisk (*) indicates that the difference between the experimental and control groups is statistically significant ($P < 0.0001$). Values are mean ± SD; N = 4. Control values for aggregation stimulated by streptococci and phorbol myristic acetate were 9.8 ± 3.5 and 9.5 ± 0.94 units/min respectively.

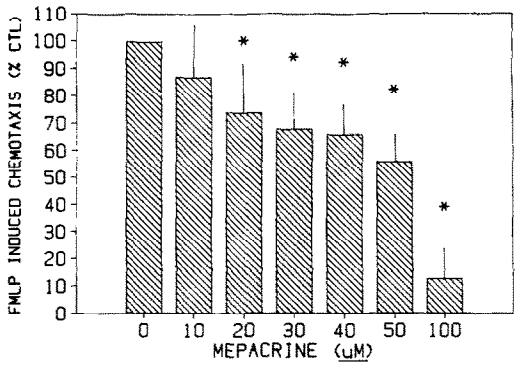

Fig. 3. Inhibition of chemotaxis by mepacrine. Neutrophils preincubated with mepacrine were assayed for chemotatic response to *n*-formylmethionylleucylphenylalanine (FMLP). An asterisk (*) indicates that the difference between the experimental and control groups is statistically significant ($P < 0.0001$). Figure 3 represents three separate experiments replicated five times; values are means ± SD. The control value for chemotaxis stimulated by *n*-formylmethionylleucylphenylalanine was 117 ± 53 (cells/field).

but less than that required for aggregation. Mepacrine was least effective at inhibiting aggregation.

DISCUSSION

Neutrophil activation appears to play an important role in the pulmonary dysfunction associated with adult respiratory distress syndrome [1–3]. Mepacrine preserves pulmonary function in *in vivo* models of the syndrome possibly by inhibiting neutrophil activation [6].

Table 1. Inhibition of neutrophil responses by mepacrine

Group	IC_{50} (μM)	vs 1	2	3	4	5	N
1. AGG (PMA)	77.0 ± 19.2			*	*	*	16
2. AGG (GBS)	73.0 ± 9.8			*	*	*	16
3. CHEM (FMLP)	41.1 ± 2.2	*	*		*	*	90
4. O_2^+ (GBS)	16.1 ± 1.7	*	*	*		*	20
5. O_2^+ (PMA)	5.3 ± 1.2	*	*	*	*		24

The IC_{50} values (mean ± SD) for mepacrine-dependent inhibition of aggregation (AGG), chemotaxis (CHEM), and superoxide production (O_2^+) were determined. Significance is shown by an asterisk (*) when the group in the row was different from the group in the column as determined by a two-tailed protected *t*-test ($P < 0.001$).

In the present report mepacrine was found to inhibit aggregation and superoxide production stimulated by PMA and group B streptococci. The concentrations of mepacrine required to inhibit aggregation and superoxide production varied significantly regardless of stimulus. Chemotaxis induced by FMLP was also inhibited by mepacrine. Neutrophil superoxide production was most sensitive and aggregation least sensitive to mepacrine inhibition. The effectiveness of mepacrine inhibition on chemotaxis was between that of aggregation and superoxide production.

Previous investigations have also demonstrated inhibition of PMA-induced superoxide production ($IC_{50} \sim 73$ μM) [6, 9] and FMLP-induced chemotaxis ($IC_{50} \sim 80$ μM) [10]. The effects of mepacrine on stimulated neutrophil aggregation and streptococci-induced neutrophil superoxide production have not been reported previously. Mepacrine inhibits other stimulated neutrophil responses *in vitro*. At similar concentrations mepacrine inhibits both phagocytosis ($IC_{50} \sim 100$ μM) [18, 19] and endogenous arachidonic acid release ($IC_{50} \sim 80$ μM) [10, 19]. High concentrations of mepacrine (>100 μM) have little effect on adherence [6, 18], phospholipid remodeling [9, 20], and direct inhibition of NADPH oxidase [9].

The mechanism by which mepacrine inhibits aggregation, chemotaxis, and superoxide production remains undefined. Inhibition of membrane phospholipid remodeling is doubtful due to the mepacrine concentration required. Previous work (data not shown) has demonstrated that opsonized streptococci aggregated neutrophils by stimulating eicosanoid metabolism. However, interference with arachidonic acid metabolism probably does not account for the inhibitory effect of mepacrine on neutrophil responses since phorbol myristic acetate does not stimulate significant arachidonic acid release [10, 17], nor is phorbol-induced aggregation inhibited by interference with arachidonic acid metabolism [21]. It is possible that inhibition of membrane depolarization [9] is the mechanism by which mepacrine decreases aggregation, chemotaxis, and superoxide production. In this study different

[mepacrine] IC_{50} values were obtained for aggregation, chemotaxis, and superoxide production regardless of the stimuli. These findings suggest that either mepacrine is not inhibiting all three responses through a common mechanism, or that it is not acting through a classical drug–receptor interaction.

In conclusion, mepacrine inhibited stimulated neutrophil responses which result in pulmonary dysfunction *in vivo*. The exact mechanism(s) by which mepacrine attenuates these responses is presently undetermined. Evidence presented suggests the need for wider examination of mepacrine in models investigating *in vivo* pulmonary dysfunction.

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