Biochemical Pharmacology, Vol. 34, No. 10, pp. 1757–1761, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00

MECHANISM OF PROTECTION AGAINST "REPERFUSION INJURY" BY APROTININ

ROLES OF POLYMORPHONUCLEAR LEUCOCYTES AND OXYGEN RADICALS

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(*Received* 3 *October* 1984; *accepted* 27 *November* 1984)

Abstract—We have proposed that reperfusion injury results from the overproduction of reactive oxygen metabolites by PMN as a result of increased sensitivity to oxygen during the low oxygen phase. Aprotinin was demonstrated to inhibit this overproduction and also radical production evoked by chemotactic peptide, 50% inhibitions occurring in the range 5–20 μM. Over this range, no effect on cell viability was observed. This inhibitory effect may provide a scientific basis for the protective effect of aprotinin in reperfusion induced injury.

The reperfusion of ischaemic tissues can cause major tissue damage. This has important implications to clinical situations, particularly in the reperfusion of mesenteric ischaemia. The protease inhibitor, aprotinin (Trasylol Bayer U.K.), has been used for the prophylaxis of post-operative complications [1, 2], although its mechanism of action in protecting against this reperfusion-induced injury is unknown. Recently it has become established that "reperfusion injury" is a consequence of the overproduction of highly reactive species of oxygen, particularly the oxygen radicals [3, 4]. It was proposed that the key step leading to this overproduction was the conversion of xanthine dehydrogenase to the superoxide generating enzyme, xanthine oxidase [5, 6]. Although this conversion can occur experimentally by limited proteolysis, the mechanism by which it can be triggered *in vivo* remains unknown. Furthermore, the xanthine dehydrogenase content and the conversion times do not correlate with the susceptibility of the tissues to reperfusion damage. We have therefore proposed an alternative mechanism for the overproduction of reactive oxygen species during reperfusion, involving polymorphonuclear leucocytes (PMN) either endogenous to the tissue or attracted there as a result of local trauma induced by disease or surgery. The involvement of PMNs has also previously been implicated in ischaemic myocardial injury [7, 8]. These cells have a unique oxidase system which has evolved specifically to produce oxygen metabolites. It has recently been demonstrated that the oxidase can adapt to anoxic conditions by increasing its affinity for oxygen and that re-oxygenation of "sensitized" cells results in overproduction of reactive oxygen metabolites [9]. It is proposed that this reaction provides the basis for oxygen-radical-mediated "reperfusion injury".

The aims of this paper are (i) to demonstrate and characterize the ability of aprotinin to inhibit oxygen radical production by stimulated and "reperfused" PMNs, and (ii) to determine whether this inhibition plays a significant role in the mechanism of protection by aprotinin.

MATERIALS AND METHODS

Human PMNs were isolated from blood taken from healthy volunteers, by a one-step centrifugation procedure through Ficoll-Hypaque ($d = 1.114\ g\,ml^{-1}$) adapted from Ferrante and Thong [10]. The following methods were performed as previously described; rat PMN preparation [11], luminol-dependent chemiluminescence monitoring [11], oxygen consumption measurement [12] and oxygen-controlled luminescence monitoring [9, 13]. The key reagents were obtained as follows; F-met-leu-phe and luminol (Sigma, Poole, UK), latex beads (Polysience, Cambridge, U.K.), neutrophil isolation medium (Ficoll-hypaque United Technologies Packard, U.K.), and aprotinin, purified and stored with the preservative, benzyl alcohol (0.9%) as Trasylol (Bayer, F.R.G.).

RESULTS AND DISCUSSION

Addition of aprotinin (5–80 μM) to human peripheral PMNs inhibited the luminol-dependent chemiluminescence response to the chemotactic peptide, f-met-leu-phe (Fig. 1a). This inhibition was rapid in onset being maximal within 30 sec and was not reversed by washing. These observations are consistent with the fast on rate and slow dissociation rate of aprotinin with protease (e.g. trypsin, $t_{\frac{1}{2}}$ on = 6.3 sec, $t_{\frac{1}{2}}$ off = 17 weeks [14]). The inhibition was not attributed to (i) an effect on cell viability, because maximally inhibited cells retained the ability to exclude trypan blue (99%), (ii) the effects of the preservative, benzyl alcohol (maximum concentrations = 0.09%) or (iii) interference with the ability of luminol to detect the response, because chemi-

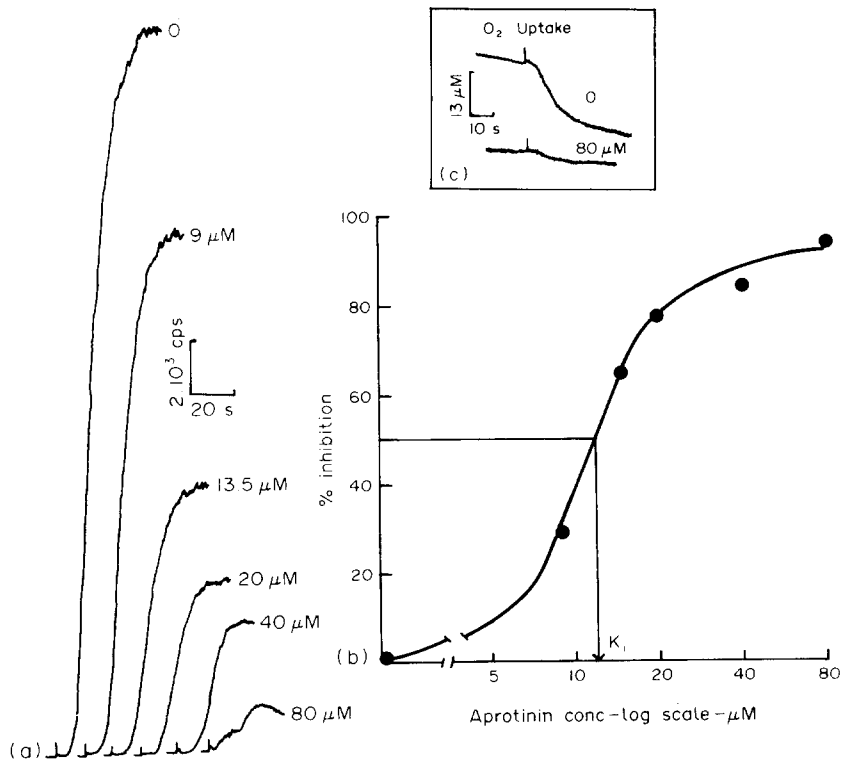

Fig. 1. Inhibition of PMN oxidase activation by aprotinin. (a) The traces shown are the luminol-dependent chemiluminescent response of human PMN (approximately 5×10^5 ml^{-1}). At the time indicated on each trace, the chemotactic peptide f-met-leu-phe (1 μM) was added to the cell suspension. The responses are calibrated in luminescence counts per sec (cps). The concentrations shown at the end of each trace indicate the aprotinin concentration present throughout the period of the recording. Results are from a typical experiment. (b) The relationship between the percentage inhibition of the peak luminescent rate against aprotinin concentration is shown, using the data taken from the experiment shown in (a). In the experiment K_i (50% inhibitory concentration) was approximately 12 μM. (c) The rate of oxygen consumption was measured using a Clark-type electrode, from approximately 10^7 ml^{-1} cells. The upper trace shows the increased oxygen uptake in response to chemotactic peptide (1 μM) and the lower trace the inhibited response observed in the presence of aprotinin.

luminescence could still be triggered with H_2O_2 and microperoxidase. The increased oxygen consumption that accompanies activation of the oxidase was also inhibited by aprotinin (Fig. 1c). This provides further evidence that the effect was the result of inhibition of the oxidase. However, the possibility exists that aprotinin may act as an oxygen radical scavenger, since such a mode of action may result in both an inhibition of chemiluminescence, due to a reduction in the concentration of reacting oxygen species, and an apparent inhibition of oxygen consumption by re-generation of oxygen from oxygen radicals.

This latter possibility would seem unlikely for the following reasons (i) aprotinin did not inhibit luminol-luminescence triggered by chemically generated reactive oxygen metabolites, (ii) unopsonized beads stimulated maximal chemiluminescence (albeit with a slower rising phase) in the presence of aprotinin (see below and Fig. 2) and (iii) other proteins scavenge oxygen radicals at far higher concentration, for example the K_i measurement for human serum albumin (HSA) scavenging in a similar PMN system was of an order of magnitude higher than that reported here for aprotinin, i.e. approximately 70–140 μM for HSA, compared with 2–20 μM for aprotinin [15]. The latter possibility would seem unlikely in view of the finding that a second stimulus, unopsonized latex beads (d = 1.01 μM) was capable of stimulating maximal chemiluminescence from cells treated with inhibiting concentrations of aprotinin, although aprotinin slowed the rising phase of this response (Fig. 2). It was concluded that aprotinin inhibited the production of reactive oxygen metabolites by PMNs stimulated by chemotactic peptide.

Figure 1a shows a typical experiment which demonstrates the dose-dependent inhibition of both the peak chemiluminescence and the rate of the linear rising phase. It was noted that the "lag time" between addition of stimulus and onset of response was unaffected. From the relationship between aprotinin concentration and percentage inhibition, a value for the 50% inhibitory dose, K_i, was found to be approximately 12 μM. Similar inhibitory effects were found with rat peritoneal PMN, (Fig. 2), K_i being approximately 20 μM. These concentrations are similar to those which inhibit macrophage-mediated cytotoxicity, 15 μM [16], platelet aggregation, 4–10 μM

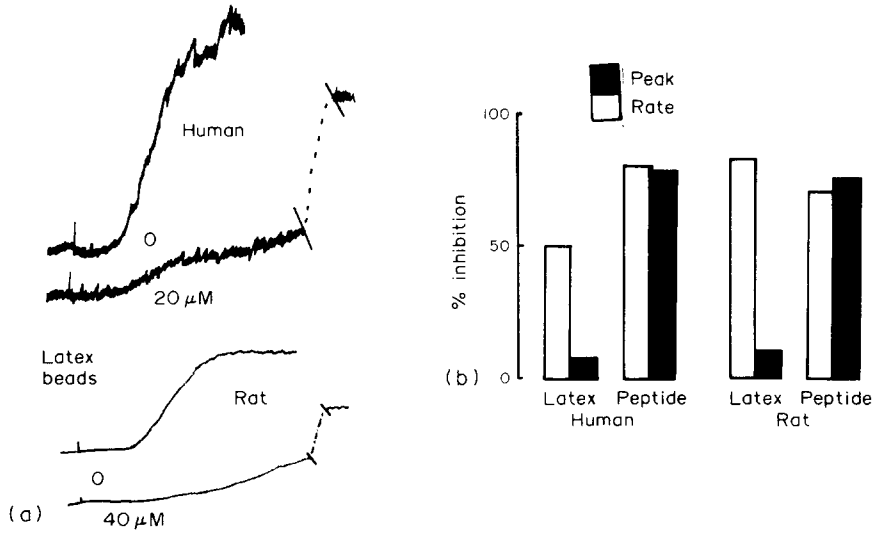

Fig. 2. Comparison of inhibition by aprotinin of responses evoked by latex beads and chemotactic peptide. (a) Typical responses are shown which demonstrate the effect of aprotinin on the latex bead-induced response from human PMN (upper set of traces) and rat PMN (lower traces). The upper trace of each set shows the unhibited response, peaking within 2 min, and the lower trace in each set show response in the presence of the aprotinin (concentration as indicated). The gaps in the lower traces indicate 15 min when the approximate plateau level was reached. (b) The histogram compares the effect of aprotinin on the latex bead and chemotactic peptide induced response. The filled columns show the inhibition of the peak luminescent response achieved and the open columns show the effect of the linear rising rate of the response. The data for the human PMN are taken from Figs. 1(a) and 2(a), for 20 μM aprotinin and the data for rat PMN are shown for latex beads in Fig. 1(a) for 40 μM aprotinin.

[17, 18], and protease activity [14]. Our studies indicate that aprotinin is one of the most potent of the serine esterase inhibitors of PMN function, previously reported K_i values being lima bean trypsin inhibitor 430 μM [19], soybean trypsin inhibitor 80 μM [19] or approximately 200 μM [20], phenylmethylsulphonyl fluoride (PMSF) approximately 500 μM [20], and benzamidine, approximately 60 mM [19]. However, the only previous report of the inhibition by aprotinin of PMN superoxide production failed to achieve 50% inhibition even with concentrations above 100 μM [20]. The reason for this discrepancy is not known, but it is noted that these authors also reported the value for soybean trypsin inhibitor more than two-fold higher than a previous report.

In order to evaluate whether aprotinin could inhibit "reperfusion induced" oxygen radical production, experiments of the type shown in Fig. 3 were performed. After monitoring the initial stable resting chemiluminescence rate, the oxygen concentration was reduced by equilibration with argon. The chemiluminescence rate declined, providing evidence that the resting chemiluminescence was monitoring an oxygen metabolite. On re-aeration there was a rapid initial rise followed by a slower phase of rising chemiluminescence. This confirmed our previous observations in which the effect was shown to result from a "K_m shift" of the oxidase as it adapted to low oxygen [9]. In the experiment shown in Fig. 3, the anaerobic to aerobic transition was repeated to induce a further increase in the chemiluminescent rate to more than twice the initial resting rate. Before repeating this transition in oxygen concentration for a second time, aprotinin 40 μM was added to the cells. The stimulating effect of reaeration was abolished, the new chemiluminescent rate was 25% of the previous re-aerated rate and approximately 50% of the initial resting rate. Furthermore the new chemiluminescence rate was stable (no significant rise over 100 sec) and it was not possible to induce further enhancement of the chemiluminescent rate by subsequent anaerobic to aerobic transitions. From the inhibition of the "re-aeration enhanced" response, the K_i for aprotinin was approximately 15 μM. However, assuming that each re-aeration in the absence of inhibitor would produce enhancement by the same increment (see Fig. 3) it can be calculated that an aprotinin concentration of approximately 5 μM would be required to prevent the "re-aeration enhancement" phenomenon.

The question now arises as to the significance of these results to clinical "reperfusion" situations in which aprotinin has beneficial effects. Since oxygen radicals must be produced extracellularly in order to have the potential to inflict damage to surrounding matrix and cells, it is significant that aprotinin inhibits two stimuli, chemotactic peptide and reaeration that produce radicals extracellularly [21]. Latex beads, which induce radical production directed within the phagosome [21], were not totally inhibited by aprotinin, although the rising rate was significantly reduced. Differential inhibition of this kind would be beneficial if occurring *in vivo* since it would enable intracellular killing of endocytosed bacteria without allowing the pathogenic extracellular production of radicals. In order to determine the significance of the inhibitions reported it is important to relate the

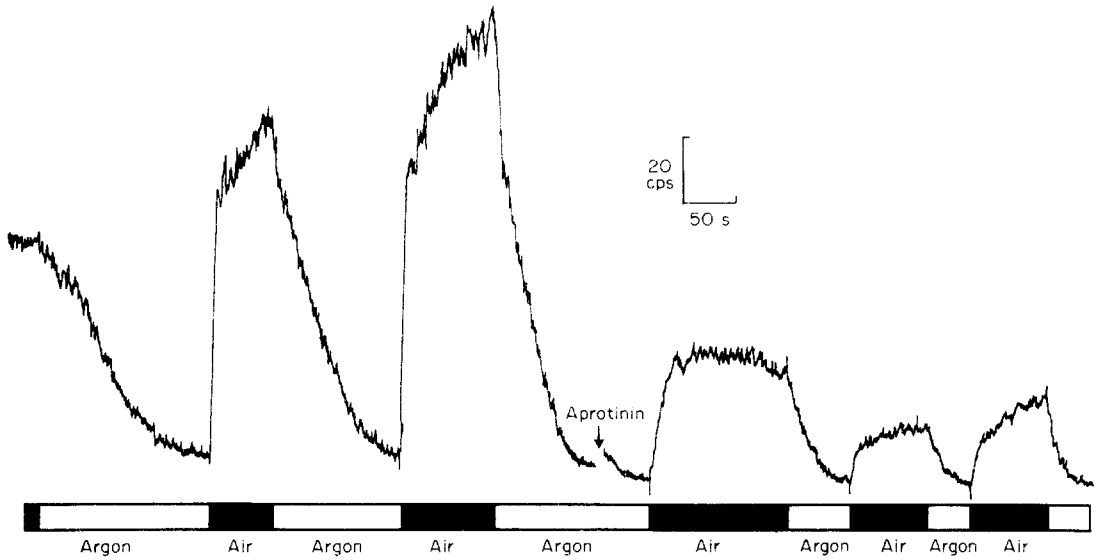

Fig. 3. Effect of aprotinin on the re-oxygenation induced chemiluminescent response. The trace shows the record of a typical experiment in which unstimulated cells were subjected to the oscillations of oxygen concentration by gassing alternately air and argon through the medium as indicated by the bars beneath the trace. The chemiluminescent rate was monitored continually during these manipulations. At the point indicated on the trace aprotinin, 40 μM, was added to the cell suspensions. The small rise in the luminescent rate at this point was due to the introduction of a small amount of oxygen. The increased sensitivity of the luminescence detecting system is seen from the calibration bar.

in vivo inhibitory concentrations to those occurring at the site of action *in vivo*. In rats, the injury resulting from reperfusion of ischaemic skeletal muscle was inhibited by aprotinin 1.25×10^5 units/kg i.v. [22], equivalent to approximately 17 μM if distributed uniformly throughout the extracellular body fluid, or approximately 60 μM if restricted to the plasma. This is comparable to the K_i value for aprotinin in rat PMN of 20 μM (Fig. 2). A typical dose for humans (10^6 units) distributed uniformly throughout the body extracellular fluid is equivalent to approximately 2 μM, or if restricted to the plasma, circulating PMNs would be exposed to 7 μM. These concentrations must be compared with the aprotinin concentrations required to prevent oxidase activation by chemotactic peptide, K_i, 12 μM, and by reaeration 5 μM. The possibility therefore exists that at least part of the clinical action of aprotinin may be due to the inhibition of oxygen radical production reported here. No attempt has been made to establish the molecular mechanism by which aprotinin inhibits PMN activation, but since aprotinin has been shown to bind to the surface of PMN [23] and is not expected to enter the cell cytoplasm (M.W. = 6.5×10^3), it is likely that the site of its action is at the PMN plasma membrane. It is intriguing to speculate that the recently discovered dual control of PMN activation by intracellular Ca^{2+} and membrane-derived diacylglycerol [24–26] may be disturbed by aprotinin, since the formation of diacylglycerol by phospholipase-C is prevented by serine esterase inhibitors [27] and phospholipase-C induced activation of PMN is the most susceptible to inhibition by this class of inhibitor [20].

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