Toxic effects of human eosinophil secretion products on isolated rat heart cells *in vitro*

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(Received 9 October 1981/Accepted 11 January 1982)

Rat heart cells and mitochondria were incubated with supernatants from eosinophils or neutrophils that had been stimulated with zymosan-C3b. Supernatants from eosinophils, but not neutrophils, were toxic to rat heart cells in a dose-dependent manner. This was associated with an increased O_2 uptake, which was blocked by either 1 mM-cyanide or 100 μ M-ouabain. Supernatants from eosinophils, but not neutrophils, caused a decrease in O_2 uptake by rat heart mitochondria utilizing pyruvate (+ malate) but not other substrates. The activity of pyruvate dehydrogenase (EC 1.2.4.1) from rat heart was inhibited by Ca^{2+} -free eosinophil supernatants. The activity of oxoglutarate dehydrogenase (EC 1.2.4.2) was also inhibited but not that of lipoamide dehydrogenase (EC 1.6.4.3). Prior incubation with heparin prevented these effects of eosinophil supernatants on heart cells, suggesting that they were caused by eosinophil cationic proteins. Other cationic proteins, including poly-L-lysine and poly-L-arginine were also toxic to rat heart cells, but these reduced O_2 uptake. It was concluded that granulocyte secretion products containing eosinophil cationic proteins are toxic to isolated rat heart cells *in vitro*. This may be due to an initial increase in membrane permeability, which may lead to activation of $(Na^+ + K^+)$ -dependent ATPase and increased O_2 uptake. A second step may involve inhibition of pyruvate dehydrogenase by the same products, leading to a decreased O_2 uptake. It is suggested that these mechanisms could contribute to the development of cardiac injury and myocardial disease in clinical situations where many degranulated eosinophils are present.

Patients with persistently raised blood eosinophil counts have a high incidence of endomyocardial fibrosis [Löffler's or eosinophilic endomyocardial disease (Chusid *et al.*, 1975; Parrillo *et al.*, 1979)] and it has been suggested that eosinophils themselves could cause the cardiac injury (Brockington *et al.*, 1970). Initially experiments *in vitro* to see whether eosinophils could damage antibody-coated cells including heart cells showed little cytotoxicity (van Epps & Bankhurst, 1978) unless complement was also present (Parrillo & Fauci, 1978). However, the presence of degranulated eosinophils in the blood and cardiac tissues of patients with endomyocardial disease supported the suggestion that this disease was caused by eosinophil secretion products released from degranulated eosinophils (Spry & Tai, 1976). The presence of degranulated

blood eosinophils before the development of heart disease in a number of patients has supported this hypothesis (Olsen & Spry, 1979). Other studies on the cytotoxic capacity of eosinophils have shown that they are toxic to a number of antibody- (Butterworth, 1977) or complement-coated (Ramalho-Pinto *et al.*, 1978) parasites *in vitro*, and it has been suggested that eosinophils could be responsible for tissue injuries in a number of diseases (Spry, 1976). The cationic proteins in eosinophil granules have been found to be toxic to a number of cells (Gleich *et al.*, 1979; Frigas *et al.*, 1980) and parasites (Butterworth *et al.*, 1979; McLaren *et al.*, 1981) *in vitro*, and eosinophil peroxidase has also been implicated in eosinophil-dependent cytotoxic reactions (Jong & Klebanoff, 1980). A low-molecular-weight protein from eosinophils has also been

shown to induce progressive demyelination when injected into the cerebral ventricles (Durack *et al.*, 1981).

These studies were designed to see whether products released from stimulated eosinophils could injure heart cells *in vitro*. Zymosan-C3b has been found to be a potent stimulus for eosinophil secretion, giving rise to release of lysosomal enzymes including histaminase and arylsulphate B *in vitro* (Herman *et al.*, 1979). Supernatants from stimulated eosinophils were added to suspensions of rat heart cells to see whether viability or metabolism would be altered. Additional experiments were then planned to see whether the plasma membrane and mitochondria were affected. It was hoped that this would show how eosinophils could be responsible for the development of endomyocardial disease in patients who have hypereosinophilia and large numbers of degranulated eosinophils.

Materials

The following materials were used: preservative-free heparin (Paines & Byrne), protamine sulphate, poly-L-lysine, poly-L-arginine, cytochrome *c* (type IV), 2,4-dinitrophenol, ouabain, Strophanthine-G, hyaluronidase (type 1), taurine, zymosan A from *Saccharomyces cerevisiae*, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide ('FCCP'), *NNN'*-tetramethylbenzene-1,4-diamine dihydrochloride ('TMPD'), rotenone (Sigma), lysozyme, pyruvate (Boehringer), collagenase (type 1) (Worthington), dimethyl sulphoxide (analytical grade) BDH, methyl-cellulose (Koch-Light) and sodium diatrizoate (Winthrop).

Methods

Isolation of rat heart cells

Single-cell suspensions of rat heart cells were prepared by the method of Clark *et al.* (1978). Briefly, hearts were removed from 200–250 g adult male PVG (local inbred strain) rats and perfused with 0.05% collagenase (type 1) and 0.2% hyaluronidase (type 1). The cells were filtered and re-suspended at 2.5×10^5 cells/ml in Ca^{2+} -free bicarbonate-buffered balanced salt solution, pH 7.4, containing 0.5 mM-EDTA, 30 mM-aurine and 10% dimethyl sulphoxide. Cell viability was assessed by phase-contrast microscopy and uptake of fluorescein diacetate (Ramasamy, 1974).

O_2 uptake by isolated heart cells

Heart cell suspension (2 ml) containing 5 mM-glucose or 10 mM-pyruvate was incubated in a shaking water-bath at 37°C for 3 min with continuous gassing with O_2/CO_2 (19:1). O_2 uptake was measured using an oxygen electrode (SEA

Metabolic O_2 Meter) stirred at 600 rev./min. Equilibration occurred for 2 min after which the basal O_2 consumption was measured. O_2 uptake was calculated as $\mu\text{mol consumed/min per } 2 \times 10^8$ cells.

Granulocyte secretion products

Venous blood was obtained from patients with the hypereosinophilic syndrome.

Eosinophils and neutrophils were purified by first sedimenting red cells with 2% methyl-cellulose and 34% sodium diatrizoate. Eosinophils were then separated using discontinuous metrizamide gradients of 18%, 20%, 22%, 23%, 24% and 25% (w/v) (Vadas *et al.*, 1979). The purity of eosinophils was >90% and of neutrophils >95%. For preparation of supernatants from eosinophils and neutrophils zymosan-C3b was prepared by incubating 10 mg of zymosan A (which had been boiled for 15 min in phosphate-buffered saline) to 1 ml of fresh human serum at 37°C for 30 min. After washing once, particles were incubated with eosinophils or neutrophils in the ratio of 2.5 particles/cell at 37°C for 30 min (Herman *et al.*, 1979). The mixture was centrifuged at 1000 *g* for 5 min, and the supernatant was removed and stored at -70°C. Ca^{2+} -free supernatants were prepared by dialysis overnight at 4°C against 0.01 M-EGTA. Protein was assayed by the method of Lowry *et al.* (1951) and concentrations of eosinophil cationic proteins were assayed by radioimmunoassay (Venge *et al.*, 1977).

Isolation of rat heart mitochondria

Rat heart mitochondria were isolated by the method of Chance & Hagihara (1960). The proteinase step was omitted. Hearts were homogenized in isolation medium (0.225 M-mannitol, 75 mM-sucrose, 10 mM-Tris, 100 μM -EDTA, pH 7.2) by using an Ultra-Turrax homogenizer for 5 s at full speed, then for 30 s in a Potter-type homogenizer.

O_2 -uptake measurements

O_2 uptake in mitochondria was measured polarographically using a Clark-type electrode (Yellow Springs Instrument Co., Ohio, U.S.A.) at 25°C. Mitochondrial samples (0.5–1.5 mg of mitochondrial protein) were introduced to 100 mM- K^+ respiration medium [100 mM-KCl/75 mM-mannitol/25 mM-sucrose/10 mM-phosphate-Tris/10 mM-Tris/HCl/50 μM -EDTA (pH 7.4)]. State-3 respiration was induced by the addition of 250 μM -ADP. The respiratory control ratio [$(\text{O}_2$ uptake in the presence of ADP)/(O_2 uptake in the absence of ADP)] was calculated (Chance & Williams, 1956). Mitochondria with respiratory control ratios of 4 or more with pyruvate and malate as substrates were used in the present study. Uncoupled mitochondria were prepared by the addition of 0.1 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide. For inhibition

studies, mitochondria were pre-incubated for 3 min with eosinophil supernatants before the addition of substrates.

Enzyme assays

Pyruvate dehydrogenase (EC 1.2.4.1) activity was assayed by monitoring the formation of NADH at 340nm on an SP. 8-100 recording spectrophotometer as described by Reed & Willms (1966). Oxoglutarate dehydrogenase (EC 1.2.4.2) activity was assayed as above except that oxoglutarate was the substrate used. Lipoamide dehydrogenase (EC 1.6.4.3) activity was assayed by the method of Ngo & Barbeau (1978).

Statistics

Differences between means were calculated by using a two-tailed *t* test, and regression analyses were calculated by the method of least-square fit.

Results

Isolated rat heart cells

Supernatants from zymosan-C3b-stimulated eosinophils were toxic to isolated rat heart cells in a

dose-dependent manner. Toxicity was found to be associated with an increase in O₂ uptake by the dying cells (Table 1). Neutrophil supernatants prepared in the same way and containing equivalent protein concentrations did not injure heart cells or alter O₂ uptake.

Eosinophil supernatants alone did not alter the oxygen electrode's performance or response, and NaCN (1.5–10mM) inhibited O₂ uptake of heart cells both in the presence and absence of eosinophil supernatants.

When heart cells were pre-incubated with 100μM-ouabain eosinophil supernatants failed to produce an increase in O₂ uptake but cytotoxicity was unaffected (see Table 1).

Isolated rat heart mitochondria

Table 2 shows the effects of eosinophil supernatants on isolated rat heart mitochondrial respiration. When the oxidation of NADH-linked substrates was studied (see Fig. 1), it was found that low concentrations of eosinophil supernatants inhibited pyruvate (Fig. 2) and oxoglutarate oxidation specifically. Oxoglutarate oxidation [control 93 ± 8

Table 1. Effects of increasing concentrations of eosinophil supernatants on the viability and O₂ uptake of isolated rat heart cells

The cells were killed and O₂ uptake increased. Abbreviation: ECP, eosinophil cationic protein. Results for heart cells are means ± s.d. for the numbers of observations shown in parentheses. Glucose (5mM) was the substrate in these experiments.

Eosinophil supernatant added		Heart cell parameters	
Total protein (μg/ml)	ECP (ng/ml)	Viable (%)	O ₂ uptake (μmol/min per 2 × 10 ⁸ cells)
Nil	Nil	85 ± 4 (8)	9.0 ± 2.8 (8)
75	40	63 ± 5 (4)**	13.4 ± 2.1 (6)*
150	80	46 ± 4 (4)**	18.9 ± 0.4 (6)**
225	120	13 ± 2 (4)**	21.5 ± 0.9 (6)**
300	160	13 ± 2 (4)**	29.0 ± 1.0 (6)**
300 + ouabain	160	14 ± 2 (3)**	8.4 ± 0.8 (3)

**P* < 0.01.

***P* < 0.001 (test versus result for nil addition).

Table 2. Effect of eosinophil supernatants on the oxidation of some mitochondrial substrates

Pyruvate oxidation was inhibited by eosinophil supernatants at each concentration tested (27–134 ng of protein/ml). The oxidation of other substrates was unaffected. Abbreviations used: TMPD, *NNN'*-tetramethylbenzene-1,4-diamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Results are means ± s.d. for the numbers of observations shown in parentheses.

Substrate	O ₂ uptake (ng-atoms/min per mg of mitochondrial protein)	
	Without eosinophil supernatant	With eosinophil supernatant
Pyruvate (5mM) + malate (2.5mM) + FCCP (0.1 μM)	160 ± 20 (11)	42 to 134
β-Hydroxybutyrate (10mM) + malate (2.5mM) + FCCP (0.1 μM)	51 ± 1 (3)	50 ± 6 (3)
Glutamate (10mM) + malate (2.5mM) + FCCP (0.1 μM)	52, 52 (2)	52, 52 (2)
Succinate (10mM) + rotenone (0.1 μM) + FCCP (0.1 μM)	57 ± 12 (6)	57 ± 0 (6)
TMPD (24ng/ml) + ascorbate (20 μM) + FCCP (0.1 μM)	160 ± 7 (3)	158 ± 7 (3)

Fig. 1. Outline of the mitochondrial electron-transport system

Abbreviations: TMPD, *NNN'*-tetramethylbenzene-1,4-diamine; Q, ubiquinone.

Fig. 2. Effects of increasing concentrations of eosinophil supernatants on pyruvate-dependent O_2 uptake and pyruvate dehydrogenase activity

Dose-dependent inhibition was found in both experiments.

Residual activity (%)

$$= \frac{\text{control activity} - \text{test activity}}{\text{control activity}} \times 100.$$

Control activity was 107.3 ± 3.9 ($n = 5$) nmol/min mg of mitochondrial protein.

($n = 5$) in the presence of eosinophil supernatants was reduced to between 22 and 74 n-atom/min per mg of mitochondrial protein. 3-Hydroxybutyrate oxidation was inhibited by 10% at protein concentrations of eosinophil supernatants, which caused 80% inhibition of pyruvate oxidation. Succinate oxidation was unaffected. Likewise the rate of respiration in the presence of the artificial electron donor *NNN'*-tetramethylbenzene-1,4-diamine plus ascorbate was unchanged.

This inhibition of pyruvate oxidation was studied further by assaying pyruvate dehydrogenase activity in the presence of Ca^{2+} -free eosinophil supernatants. The activity of the pyruvate dehydrogenase com-

Fig. 3. Effects of increasing concentrations of eosinophil supernatants on oxoglutarate dehydrogenase activity. The control activity is 134.4 ± 1.4 ($n = 3$) nmol/min per mg of mitochondrial protein.

plex was reduced by increased concentrations of the eosinophil supernatant (see Fig. 2). The activity of oxoglutarate dehydrogenase was more sensitive to eosinophil supernatant inhibition (Fig. 3). The pattern of oxoglutarate dehydrogenase inhibition was very different from that of pyruvate dehydrogenase. The activity of lipoamide dehydrogenase was unaffected [control, 480.9 ± 4.5 ($n = 3$) nmol/min per mg of mitochondrial protein; in the presence of high levels of eosinophil supernatants, 502.3 nmol/min per mg of mitochondrial protein (mean of two estimations)].

Eosinophil cationic proteins

Measurements of eosinophil cationic-protein concentrations in eosinophil supernatants showed that 40 ng/ml (2 nM) could cause increased O_2 uptake and be toxic. It has been shown that the effects of eosinophil cationic proteins are inhibited by heparin (Venge *et al.*, 1980). Heart cells that were pre-incubated with 200 units of heparin/ml showed no increase in O_2 uptake in the presence of eosinophil supernatants containing 80 ng of eosinophil cationic proteins/ml. To see whether this was a non-specific effect of cationic proteins, five other cationic proteins were assessed for their effects on isolated heart cell respiration and viability. Cells were pretreated with cytochrome *c*, protamine sulphate, lysozyme, poly-L-lysine and poly-L-arginine at 1 mM. Only lysozyme, poly-L-lysine and poly-L-arginine were toxic, and in each case there was a decrease rather than an increase in O_2 uptake. This suggests that the effects of eosinophil supernatants on heart cells are not due to their strong cationic properties.

Discussion

The principal findings of the present study were that eosinophil secretion products injured rat heart

cells *in vitro* and inhibited O_2 uptake of isolated rat heart mitochondria utilizing pyruvate (+ malate). Eosinophil supernatants affected O_2 uptake in two opposing ways: they caused an increased uptake with intact heart cells but a decreased uptake with isolated heart mitochondria.

This apparent paradoxical effect on O_2 uptake might be explained by the following scheme. The eosinophil supernatants might cause membrane damage to heart cells, which would cause an influx of Na^+ , hence stimulating the $(Na^+ + K^+)$ -dependent ATPase activity, or they might stimulate the $(Na^+ + K^+)$ -dependent ATPase activity directly. The increased activity of $(Na^+ + K^+)$ -dependent ATPase would lower the cytosolic ATP/ADP ratio, thereby stimulating oxidative phosphorylation, thus causing the increase in O_2 uptake. After this rapid burst in O_2 uptake the inhibition of pyruvate dehydrogenase and oxoglutarate dehydrogenase would decrease the O_2 uptake if the inhibitory components of eosinophil supernatants could enter the intact cell, but this remains to be determined. With $100 \mu M$ -ouabain, this increased O_2 uptake was not seen, but cell death still occurred.

Inhibition of mitochondrial respiration

Eosinophil supernatants were found to have a specific effect on pyruvate oxidation (Fig. 2) and oxoglutarate oxidation. As the metabolism of other substrates was not inhibited it is probable that the respiratory chain itself was unaffected. Mitochondria in the uncoupled state are not dependent on trans-membrane potential (or oxidative phosphorylation) for the transport of substrates into the matrix (Denton & McCormack, 1980; Denton *et al.*, 1980). The rate of substrate oxidation under these conditions is dependent on the activity of the specific dehydrogenase for the substrate. The inhibition of both pyruvate and oxoglutarate oxidation could be due to a direct effect on either their respective dehydrogenases or translocases. Ca^{2+} -free eosinophil supernatants also inhibited pyruvate oxidation (results not shown).

Although both pyruvate dehydrogenase and oxoglutarate dehydrogenase have catalytic mechanisms with much in common (Randle & Tubbs, 1979), the pattern of inhibition by eosinophil supernatants was different in each case. The activity of lipoamide dehydrogenase, which is a component of both complexes, was not affected. The activity of oxoglutarate dehydrogenase fell rapidly to a steady level, which was maintained over an increasing concentration of eosinophil supernatants. The range of eosinophil-cationic-protein concentrations responsible for this steady level of activity is approx. 1.5–3.6 nM. This is six orders of magnitude lower than the reported K_m for oxoglutarate (2.3 mM; Denton *et al.*, 1980) in rat heart mitochondria.

It is unlikely that the inhibitory factor binds at the active centre. It is possible that this factor binds either close to the active centre, thus denying substrates access to the active centre, i.e. 'steric hinderance', or binds distally and produces a conformational change that results in a lowering of activity.

We should like to thank Dr. Leanna Reed for help in setting up the rat heart digestion procedure, Dr. Inge Olsson for assaying the eosinophil-cationic-protein levels, Dr. Marion Stubbs for advice and Professor John Humphrey and Professor John Goodwin for their enthusiastic support. We thank Miss Ruth Frearson for typing the manuscript. The work was carried out under the auspices of the International Society and Federation of Cardiology, and was supported with grants from the British Heart Foundation and the Brain Research Trust.

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