Inhibition of human platelet lipoxygenase by cyanide[1]

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Summary. Potassium cyanide inhibited the lipoxygenase activity of a human platelet cytosolic fraction in a concentration-dependent manner ($ID_{50}=2$ mM). The inhibition was monitored by spectrophotometry (conjugation of diene bonds at 236 nm), by chromatography (inhibition of formation of 12-hydroperoxy eicosatetraenoic acid) as well as by measuring suppression of oxygen consumption. The lipoxygenase activity of intact platelets was also inhibited by KCN as evidenced by the reduction in 12-hydroxy-eicosatetraenoic acid formation in response to thrombin.

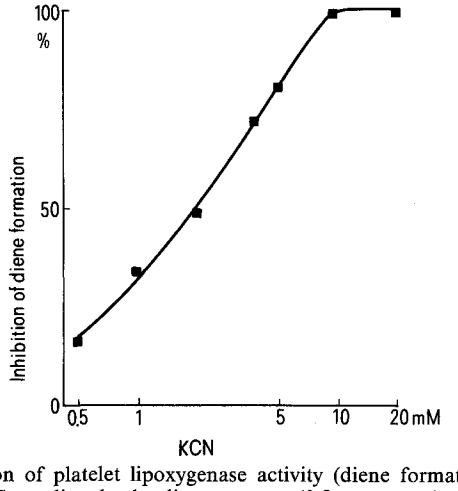
Soybean lipoxygenase[3] as well as bovine platelet lipoxygenase[4] were reported to be cyanide insensitive. Recently, however, it has been shown that soybean lipoxygenase[5] as well as lipoxygenases from rat testis[5], winged bean[6] and cowpea[7] are sensitive to cyanide inhibition. In this study we report that cyanide inhibits the activity of human platelet lipoxygenase in a cell-free system as well as in intact platelets.

Materials and methods. Human platelets were obtained from healthy donors who had not taken any drugs for 2 weeks before the experiments. Lipoxygenase was prepared by ammonium sulfate fractionation of platelet cytosol as described[8].

Spectrophotometric measurements of cytosolic lipoxygenase activity at 236 nm as well as monitoring for oxygen consumption using a Clark-type electrode were carried out using arachidonic acid as substrate as described elsewhere[9]. Production of radioactive metabolites from (1-^{14}C)-arachidonic acid (AA) was followed by extraction and separation on TLC plates[10].

To prelabel platelet phospholipids, intact platelets were incubated with 0.1 μCi (1-^{14}C)-arachidonic acid and washed according to procedures published elsewhere[11]. Stimulation of the release of AA from phospholipids and its subsequent oxygenation was achieved by incubation of 5×10^8 platelets/ml with 5 U/ml thrombin for 3 min at 37 °C.

Results. 1. Inhibition of lipoxygenase activity in the platelet cytosolic fraction. The figure shows that the lipoxygenase activity of platelet cytosol was inhibited by KCN in a concentration-dependent fashion with an ID_{50} of 2 mM.



Inhibition of platelet lipoxygenase activity (diene formation[9]) by KCN. Cytosolic platelet lipoxygenase (0.5 mg protein in 1 ml 25 mM tris-HCl pH 8.2) was preincubated for 10 min at 4 °C with increasing concentrations of KCN. The samples were warmed to 21 °C and reaction was initiated by adding 10 μM AA in 0.5 μl ethanol. The progress of the reaction was followed spectrophotometrically. The initial rate was calculated from the increased absorbance at 236 nm observed in the 1st 1-min of the reaction.

This was followed by spectrophotometric monitoring of conjugation of diene bonds at 236 nm. Inhibition of lipoxygenase activity was confirmed by including 0.05 μCi (1-^{14}C) in the assay and quantitatively determining the formation of radioactive products. The formation of radioactive 12-hydroperoxy-eicosatetraenoic acid (HPETE) was reduced by 42 and 80% at 2 and 4 mM, respectively. Finally, measurements of oxygen consumption were performed. The lipoxygenase fraction consumed 2.67 ± 0.43 nmoles O_2/min/0.5 mg protein when 10 μM AA was used as a substrate and this was reduced to 0.88 ± 0.32 nmoles O_2/min/0.5 mg protein when 2 mM KCN was preincubated for 10 min with the enzyme ($N=3$).

2. Inhibition of oxygenases in whole platelets. The table shows that thrombin but not vehicle (control) induced the release of radioactive metabolites of AA from prelabeled platelets including both prostaglandins (PGs) (30.3% of released cpm) and 12-hydroxy-eicosatetraenoic acid (HETE) (57%). Indomethacin, a cyclooxygenase inhibitor[12], inhibited the platelet cyclooxygenase (only 4.1% of released cpm was present as PGs compared to 30.3% in control) but not the total release of AA (693 ± 80 cpm for thrombin plus indomethacin compared to 779 ± 87 cpm for thrombin alone). Thus indomethacin 'shunted' more AA towards the lipoxygenase pathway (76.5% compared to 57% of released cpm). KCN, however, inhibited both lipoxygenase and cyclooxygenase. Most of the AA liberated in the presence of KCN was not metabolized (83% AA, 9.5% PG and 7.5% HETE). The release of AA from phospholipids induced by thrombin in cyanide-treated platelets was reduced compared to thrombin alone (606 ± 24 cpm compared to 779 ± 87 cpm). However there was still more than a 7-fold increase in the amount of metabolized and unmetabolized AA released from phospholipids in cyanide-treated, throm-

Effect of KCN on the release of AA from human platelet phospholipids and the formation of its metabolites in response to thrombin

	Thrombin 5 U/ml	Indomethacin (10 μM) + thrombin (5 U/ml)	KCN (5 mM) + thrombin (5 U/ml)
cpm released from phospholipids**	779 ± 87	693 ± 80	606 ± 24
% of amount present in phospholipids	9.9 ± 1.1	8.7 ± 0.1	7.7 ± 0.3
% of cpm released in PG's	30.3 ± 1.1	4.1 ± 0.1	9.5 ± 1.5
% of cpm released in HETE	57.0 ± 2.2	76.5 ± 0.5	7.5 ± 1.5
% of cpm released in AA	12.8 ± 2.4	19.5 ± 0.5	83.0 ± 3.0

* (Mean of 4 determinations ±SD). ** Total radioactivity in platelets was 7873 ± 75 cpm per 3.4×10^8 platelets/ml. When labeled platelets were incubated with the vehicle instead of thrombin solution only 87 ± 7 cpm were released from the phospholipids and of these 80 ± 3 cpm were present in AA.

bin-stimulated platelets (606 ± 24 cpm) compared to the unstimulated control (87 ± 7 cpm).

Discussion. We have shown by 3 different methods, that lipoxygenase present in the cytosol of human platelets is inhibited by KCN. Lipoxygenase activity (as well as cyclooxygenase activity) in intact platelets stimulated with thrombin was also inhibited. In intact platelets metabolism of AA by both the lipoxygenase and the cyclooxygenase enzymes was low although the total amount of AA released from phospholipids (and presumably available as substrate for these enzymes) was considerable. In conclusion, KCN behaves like 5,8,11,14-eicosatetraynoic acid (ETYA)[13] in that it inhibits both cyclooxygenase and lipoxygenase activities in platelets.

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Proteolytic activity on endogenous substrates in cell-free extracts of *Trypanosoma cruzi*

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Summary. Cell-free extracts of *Trypanosoma cruzi* contain proteolytic activity able to degrade endogenous substrates. The activity was maximal at pH 3 to 4, had an 'optimal' temperature of 65 °C, and was strongly inhibited by N-α-p-tosyl-L-lysine chloromethyl ketone.

The American trypanosomiasis, Chagas' disease, affects about 20 million people in Central and South America. The causative agent is a parasitic flagellate, *Trypanosoma cruzi*, which has a rather complex life cycle, involving bloodstream trypomastigotes and intracellular amastigotes in the mammalian host, and epimastigotes and metacyclic trypomastigotes in the insect vector[2]. Epimastigotes, apparently identical to the insect form of the parasite, are readily obtained in axenic culture, and have been employed in most biochemical studies on the parasite[3]. The epimastigotes have been shown to contain several proteolytic activities[4-8]. The study of the proteases in *T. cruzi* is interesting from several points of view. First, these enzymes initiate protein catabolism, which is probably intense in the parasite, and might explain, together with lipid oxidation, the high rate of endogenous respiration observed[3]. Second, proteases might be involved in the penetration of the nondividing trypomastigotes into mammalian cells[6], a process required for their transformation in intracellular amastigotes, able to multiply and thus essential for the perpetuation of chagasic infection. Finally, proteases are able to degrade proteins with enzymatic and/or antigenic properties during their isolation. It is very important from a practical standpoint to obtain knowledge about the response of these activities to inhibitors, in order to prevent their action.

Itow and Camargo[4] reported the presence in cell-free extracts of epimastigotes of *T. cruzi*, Y strain, of proteases acting on α-L-benzoyl-D-L-arginine-p-nitroanilide (BAPA), azocasein, and carbobenzoxy-L-tyrosine-p-nitrophenylester, and also of aminopeptidase activities. Torruella et al.[5] confirmed in 5 different strains of the parasite the presence of the proteases acting on azocasein and BAPA, and described an activity on casein, with a considerable difference in optimal pH with respect to that acting on azocasein. The protease acting on BAPA was purified and studied by Bongertz and Hungerer[6]. Avila et al.[7] detected in crude extracts of epimastigotes of the Y strain 3 activities which were classified as peptidases, and 2 classified as cathepsins. Recently Rangel et al.[8] purified a protease able to act optimally on casein at pH 6.0 and on hemoglobin at pH 3.0, and inhibited by thiol reagents. All these proteolytic activities have been detected using extraneous substrates such as casein, azocasein, hemoglobin and BAPA. From the practical standpoint of the prevention of proteolytic action on antigenic and enzymatic proteins of *T. cruzi* during isolation, it is perhaps more important to study the degradation of the proteins present in the cellfree extracts themselves. This paper presents the results of a study of the proteolytic activity on endogenous substrates in cell-free extracts of *T. cruzi*, including its response to pH and temperature, and the effects of different inhibitors.

Materials and methods. Culture epimastigotes of the Tulahuén strain, Tul 2 stock[5], were disrupted by compression-decompression in a Sorvall-Ribi cell-fractionator[9]. The homogenate was centrifuged at 1000 × g for 15 min; part of the supernatant (S 1000) was kept, and an aliquot was centrifuged at 105,000 × g for 1 h. Both supernatants (S 1000 and S 105,000) were used without further treatment for the experiments described. After incubation of the reaction mixtures (described in the legends to figures) the proteolysis was stopped by addition of 1 ml of 3% trichloroacetic acid (TCA), and the TCA-soluble peptides liberated were determined by reaction with Folin and Ciocalteu's phenol reagent[10]. Zero-time controls, to which TCA was added before the supernatant, were substracted in all cases. The reaction was linear with time for at least 15 min at 30 °C with both supernatants either at pH 5.0 or 7.2.

Results and discussion. The proteolytic activity on endogenous substrates, expressed per ml of supernatant, was