Thermochimica Acta, 217 (1993) 49–55
Elsevier Science Publishers B.V., Amsterdam

Calorimetric analysis of the oxidative influence of granulocytes on erythrocytes

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(Received 22 July 1992; accepted 31 August 1992)

Abstract

The heat production of human granulocytes activated with phorbol-12-myristate-13-acetate was determined in the absence and presence of erythrocytes. The heat produced by the erythrocytes agreed with the enthalpy change calculated, assuming that glucose oxidation had occurred. Reaction of the heme with CO almost completely prevented the metabolic response of the erythrocytes. The primary site of the metabolic activation for the erythrocytes in response to oxidant stress, exerted by the toxic granulocyte metabolites, is presumed to be the heme group. The data obtained demonstrate that significant metabolic interactions occur between activated granulocytes and erythrocytes. The power evolution observed is explained in terms of glucose combustion.

INTRODUCTION

The application of biophysical methods to the analysis of cellular processes has brought about significant advances in recent years. Microcalorimetry allows direct and continuous measurement of cellular metabolic activity and gives the physicochemical value of the enthalpy change associated with the processes studied [1]. Calorimetric studies of blood cells have been conducted in several laboratories [2, 3] and this technique has been found to be useful in the measurement of heat production by activated granulocytes [4–6]. As demonstrated earlier with both flow and batch calorimetry, the activated granulocytes represent a unique model system, because the thermal energy produced is proportional to the oxygen consumed and the enthalpy change, resulting from aerobic

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glucose catabolism, and is not coupled to anabolic pathways [4,7]. Following specific membrane perturbation, the granulocytes exhibit a respiratory burst associated with the generation of O_2^-, H_2O_2, $^{\cdot}OH$, $^1O_2$, HOCl, chloramines and possibly other oxidizing agents [8–12]. These O_2^- metabolites are bactericidal, mutagenic, cytotoxic and cytolytic to normal and tumour cells and may also inhibit granulocyte metabolism [8].

Heat measurements can be performed directly on samples of granulocytes maintained in chosen environmental conditions. It is therefore much easier to simulate in vivo situations during a calorimetric study than by using other techniques. This consideration incited us to study calorimetrically the physiology of granulocyte activation in their natural environment, the blood. Thus this paper deals with the study of the metabolic interactions occurring between phorbol-12-myristate-13-acetate (PMA) activated human granulocytes and surrounding erythrocytes.

MATERIALS AND METHODS

Venous blood was drawn from healthy donors. Experiments on whole blood were carried out following dilution of the blood (1:1) with Gey's buffer solution, pH 7.4, depleted of Ca and Mg salts. Granulocytes were obtained by dextran sedimentation followed by Isopaque–Ficoll separation described by Böyum [13]. They were then incubated at the concentration of 2.5×10^6 cells ml^{-1} in Gey's buffer in the presence or absence of 5×10^9 erythrocytes ml^{-1}. The autologous erythrocytes were washed and the buffy coat removed; thereafter they were suspended in Gey's buffer.

Platelets and mononuclear cells were separated from the blood on density gradients and were tested to determine if they could be directly activated by PMA 100 ng ml^{-1} (Sigma, USA).

After incubation periods of varying duration, lysis of the erythrocytes was accomplished with 0.01% digitoxin; thereafter the mixture was centrifuged at 500*g* to remove cell debris and the supernatant was used for spectrophotometric determination of hemoglobin. All measurements were carried out using a Cary 219 (Varian, CA, USA) spectrophotometer at room temperature. Oxyhemoglobin and methemoglobin were determined by the sequential addition of NaCN, CO, and dithionite [14]. Membrane lipid peroxidation was evaluated with the thiobarbituric acid assay [15].

Lactic acid, pyruvic acid and 2,3-diphosphoglyceric acid levels were determined enzymatically with commercially available kits from Boehringer Mannheim, Mannheim, Germany.

Blocking of the erythrocyte anion channels was performed by 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS) and 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene (SITS), obtained from Sigma USA. Erythrocyte suspensions were incubated with 100 μM of DIDS and

SITS respectively for 10 min at room temperature prior to addition of granulocytes [16].

Inhibition of methemoglobin formation was achieved by incubation and by running the cell suspensions in 10% CO atmosphere.

The kinetics of oxygen consumption were measured using a Clark electrode in a Yellow Spring system (Yellow Spring Instrument Co, USA). The cellular samples were saturated with 100% O_2 before measurement. In this way complete saturation of hemoglobin was maintained during the experiments, otherwise the heat of O_2 release from hemoglobin has to be considered. For calculation purposes it was assumed that the O_2 concentration in the air-saturated saline solution was 0.4 μg of atomic O per ml at 37°C [17]. Glucose catabolism through the hexose monophosphate shunt (HMPS) was determined by measuring the $^{14}CO_2$ produced during oxidation of (1-^{14}C)-glucose and (U–^{14}C)-glucose. The $^{14}CO_2$ evolved was collected in an ionization chamber and measured using a vibrating-reed electrometer (Batec, Johnston Laboratories, USA).

A 2277 TAM calorimeter from Thermometrics AB, Sweden was used [18], with glass ampoules containing 2 ml of cell suspension. A cannula, which was sealed at the end with silicone grease, was inserted through the rubber septum and connected to a plastic syringe containing 0.2 ml of PMA solution. At thermal equilibrium, compression of the syringe forced out the silicon plug from the end of the cannula and thereby allowed the PMA solution to mix with the cell suspension. Complete homogeneity was achieved through a double movement of the syringe piston. The heat evolved was calculated by integration of the areas of the power–time curves obtained. The heat of dilution of both solutions was measured in parallel and subtracted from the measured energy. Calibration of the instrument was performed electrically.

RESULTS

Addition of PMA to the whole blood sample and the erythrocyte/granulocyte mixtures generated the same type of power–time curve, as shown in Fig. 1(b) and 1(c). Slow activation was observed in the first minutes and heat production gradually increased for about 40–60 min before reaching the maximum. The decline in power production was very slow: the base line value was not reached until about 3 h later. The power recorded was about twice as high as that obtained when PMA stimulation was accomplished on a pure granulocyte suspension (Table 1). No significant increase in heat production was observed upon addition of PMA to purified erythrocytes, lymphocytes, platelets or monocytes at the cellular concentrations present in the blood.

Furthermore, addition of platelets and lymphocytes to granulocyte sus-

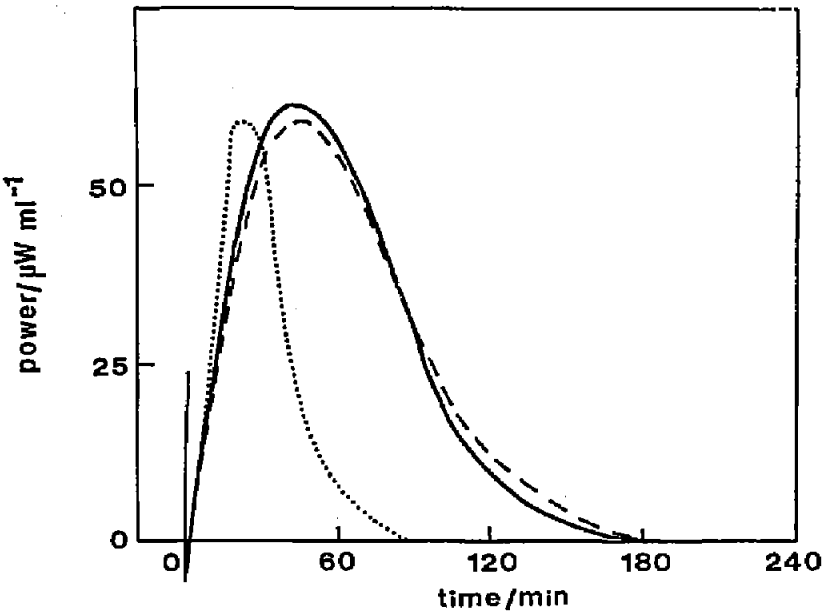

Fig. 1. Power–time curves of (a) human granulocytes (2.5×10^6 cells ml^{-1}) (·····) (b) whole blood (- - - -) and c) a cell suspension of granulocytes (2.5×10^6 cells ml^{-1}) and erythrocytes (2.5×10^9 cells ml^{-1}) (——), activated with 100 ng PMA ml^{-1} in 100% O_2 atmosphere. The curves from single experiments are representative of the eight different blood donors investigated.

TABLE 1

Thermochemical and biochemical results obtained from 2 ml cell suspensions of granulocytes (2.5×10^6 cells ml^{-1}), erythrocyte (2.5×10^9 cells ml^{-1})/granulocyte (2.5×10^6 cells ml^{-1}) mixtures, with and without treatment with CO, and whole blood after addition of PMA

	Granulocytes	Granulocytes+ erythrocytes	Granulocytes+ erythrocytes + 10% CO	Whole blood
Q exper. (J)	0.23 ± 0.05	0.56 ± 0.10	0.27 ± 0.05	0.53 ± 0.07
CO_2 produc. (μmol)	0.45 ± 0.06	0.82 ± 0.04	0.54 ± 0.06	
O_2 consump. (μmol)	0.46 ± 0.06	0.84 ± 0.06	–	
Q_1 calc. (J)	0.23 ± 0.03	0.41 ± 0.03	0.26 ± 0.03	
Pyruvic acid (μmol)	–	0.06 ± 0.02	–	
Lactic acid (μmol)	−0.03 ± 0.01	2.18 ± 0.70	0.32 ± 0.10	
Q_2 calc. (mJ)	−0.002	0.13 ± 0.04	0.20 ± 0.05	

Mean values (±SD) were calculated from eight different duplicate measurements referring to the period of activation: for granulocytes, 120 min; and for cell mixtures, 180 min. The ΔH values used in the calculations of the aerobic Q_1 and anaerobic Q_2 metabolism were −488 and −58.5 kJ mol^{-1} respectively [19, 20].

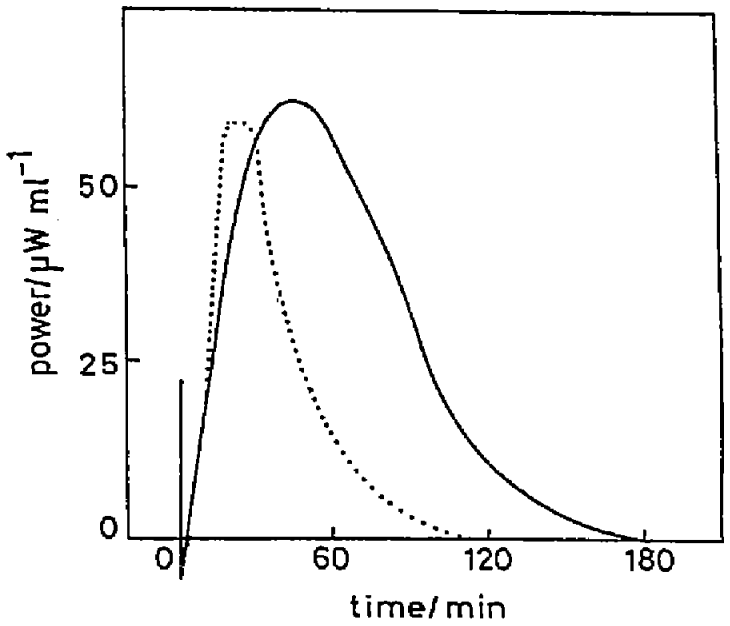

Fig. 2. Power–time curves of cell suspensions of granulocytes (2.5×10^6 cells ml^{-1})/erythrocytes (2.5×10^9 cells ml^{-1}) mixtures activated with 100 ng PMA ml^{-1} in (a) (——) 100% CO_2 atmosphere, and (b) (·····) 10% CO atmosphere.

pensions did not alter the total heat evolved or the kinetics of the heat production by activated granulocytes alone (see Fig. 1(a)). The role of oxidation of the heme group during activation of the red cell metabolism was investigated by using erythrocyte/granulocyte suspensions previously equilibrated with, and maintained in, a 10% CO atmosphere. The power–time curves obtained (Fig. 2) resembled those of the pure granulocyte suspensions. The CO treatment did not reveal any inhibitory effect on the metabolic burst of granulocytes alone [21] although most of the erythrocytic metabolic activation was prevented (Table 1).

The calorimetric signal for the erythrocyte/granulocyte mixture was unaffected upon pre-incubation of the erythrocytes with either SITS or DIDS. Thus the free radicals do not seem to have any influence on the power observed. In parallel experiments, oxygen consumption, CO_2 production during oxidation of ^{14}C-labeled glucose, and the production of pyruvic acid, 2,3-diphosphoglyceric acid and lactic acid were measured.

The results from the above determinations are presented in Table 1 except for the 2,3-diphosphoglyceric acid levels which did not show any significant difference between basal and PMA-activated samples of erythrocyte/granulocyte mixtures. The power $Q = 130$ mJ (Table 1) of anaerobic glycolysis was calculated from the lactic acid produced from 2 ml of cellular suspension, assuming no accumulation of intermediate metabolic products in activated cells. Possible sources of error in the thermochemical measurements were excluded. The absence of hemoglobin in the supernatant indicated no lysis of erythrocytes and no formation of methemoglobin.

No other hemoglobin degradative products were observed and no evidence of membrane lipid oxidation during the experiments was noted.

DISCUSSION

The results of the present study strongly indicate that the erythrocytes respond to the oxidant attack of the PMA-triggered granulocytes with a powerful metabolic activation [16, 21]. Previous studies have shown that lysis of erythrocytes caused by peroxides can be prevented when the hemoglobin is treated with CO [22]. In the present study, it was shown that after the saturation of erythrocyte hemoglobin with CO, formation of methemoglobin could be completely prevented. Other authors [23] have indicated the stroma as the principal target of the peroxide attack.

Blocking of the erythrocyte anion channels with the sulfonated stilbenes SITS and DIDS did not alter the calorimetric profile. This may indicate that the reactive oxygen metabolites produced by activated granulocytes interact with intracellular mechanisms in erythrocytes independent of membrane properties. The major production of thermal power observed in whole blood was correlated experimentally to the overall metabolic activity of the two cell populations, granulocytes and erythrocytes. As with granulocytes, the power produced by erythrocytes is related to glucose catabolism. In contrast to the behavior exhibited by granulocytes, a consistent fraction of the total power evolution (about 20–30%), can be attributed to anaerobic glycolysis.

In the cooperative erythrocyte–granulocyte system, power production can be explained in terms of glucose catabolism only as demonstrated by the stoichiometric correlation found between moles of O_2 consumed and CO_2 produced, and the relativley low production of pyruvic acid (Table 1). The predicted enthalpy change for glucose oxidation in aqueous solutions to CO_2 and O_2 (-488 kJ per mol O_2) gives a theoretical power effect of 410 mJ for 2 ml of cellular suspension. Adding to this the value calculated for anaerobic glycolysis (Table 1), a total power effect of 540 mJ is obtained. This value is 96% of the observed value of 560 mJ. In fact the Q per mole O_2 derived from the experiments, after subtracting the estimated contribution of anaerobic glycolysis, corresponds to -521 kJ per mol O_2, a value which is in agreement with that of glucose combustion (-488 kJ per mol O_2) reported by Monti and Wadsö [19]. This supports the hypothesis that all the oxygen consumed by both granulocytes and erythrocytes is finally reduced to water and the necessary reducing equivalent derives from the NAD(P)H produced by glucose catabolism.

From these experiments one can conclude that calorimetry is a suitable and simple technique for investigating the effects of cellular interactions on metabolic pathways.

ACKNOWLEDGMENTS

This work was supported by the Nordic Fund for Technology and Industrial Development, Denmark, the Påhlssons Foundation, Sweden, and the Consiglio Nazionale delle Ricerche, Italy. The authors thank Mrs B. Persson for her technical assistance.

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