Proton Secretion by Stimulated Neutrophils

Significance of Hexose Monophosphate Shunt Activity as Source of Electrons and Protons for the Respiratory Burst

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Abstract. Phagocytosis by neutrophils is accompanied by a burst in O_2 consumption and activation of the hexose monophosphate shunt (HMPS). Proton secretion equal to the amount of O_2 consumed is an additional feature of the respiratory burst, but its source has not been identified, nor has the source of all electrons donated to O_2 in the respiratory burst. We chemically quantitated total CO_2 generation in human neutrophils and found that proton secretion elicited by phagocytosis was accompanied by a stoichiometric increase in CO_2 generation. Addition of carbonic anhydrase and its inhibitors had no effect on either the quantities of CO_2 measured or the quantities of protons secreted. Therefore, the CO_2 generated in the respiratory burst of stimulated neutrophils is hydrated to form H_2CO_3, which then dissociates, accounting for the observed proton secretion. Furthermore, the CO_2 generated corresponds to the O_2 consumed with a respiratory quotient of nearly 1. We conclude on the basis of this and previous studies that the HMPS activity is the source of both the electrons for the NADPH oxidase and of protons secreted in association with the respiratory burst.

Introduction

Stimulation of neutrophils with a variety of soluble and particulate agents is known to elicit a burst of O_2 consumption (1–3). This nonmitochondrial (4) redox activity has been studied as an activatable electron transport chain functioning as an NADPH-oxidase (5–7) located in the plasma membrane or phagosomal membrane (8, 9). The hexose monophosphate shunt (HMPS)[1] is the likely source of the reduced pyridine nucleotides because activity of this pathway increases concomitantly with the O_2 consumption during stimulation (4, 10, 11). Also, neutrophils from patients with chronic granulomatous disease, which totally lack activatable NADPH-oxidase activity (12, 13), exhibit no enhanced HMPS activity during stimulation (14). Finally, neutrophils with almost complete deficiency of glucose-6-phosphate (Glc-6-P) dehydrogenase are unable to mount a respiratory burst (15, 16). Despite these strong indications of a functional association between the NADPH-oxidase and the HMPS, quantitation of HMPS activity by isotope transfer studies have not always shown a satisfactory stoichiometric relation between O_2 consumption and HMPS activity (7). In a recent report (17), HMPS activity was found to account for <10% of the reduced pyridine nucleotides used for O_2 consumption. In the same report, H^+ secretion was found intimately coupled to O_2 consumption with a stoichiometry of 1:1. The source of the protons could not be identified.

We speculated that, if as indicated above, the HMPS is the only significant source of electrons for the NADPH oxidase, then amounts of CO_2 equivalent to the amounts of O_2 consumed would be generated during the burst; and from the hydration product, H_2CO_3, proton generation equivalent to the O_2 consumption would be expected. To avoid the inherent problems of determining the specific activity of relevant intracellular substrates, which limits the use of the previously employed isotope transfer method in quantitative studies of CO_2 generation, we chemically quantitated the CO_2 generated by stimulated neutrophils using a conductometric method. By correlating CO_2 generation with H^+ secretion and O_2 consumption, we showed that the HMPS donates both the electrons and the protons consumed by the NADPH-oxidase, and via CO_2, generates the extra protons secreted by the activated cells.

Methods

Neutrophils were isolated from healthy volunteers as described (18) by dextran-induced erythrocytes sedimentation followed by density sepa-

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1. *Abbreviations used in this paper:* Glc-6-P, glucose-6-phosphate; HMPS, hexose monophosphate shunt.

ration of the leukocytes (19), and were stimulated with serum opsonized zymosan (Nutritional Biochemicals, Cleveland, OH) (20). For studies of proton secretion, the neutrophils were suspended in 145 mM NaCl, 5 mM KCl, 0.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose plus 1 mM Na_2HPO_4/NaH_2PO_4, pH 7.41. For studies of O_2 consumption, the neutrophils were resuspended in 135 mM NaCl, 5 mM KCl, 0.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, 1 mM Na_2HPO_4/NaH_2PO_4, plus 20 mM Hepes, pH 7.41.

Proton secretion. A Radiometer pH-titration unit (Radiometer, Copenhagen, Denmark) was used to titrate H^+ secretion at constant pH. Cells were diluted to varying concentrations from 5×10^6 to 2×10^7 cells/ml in 10 ml of buffer at 37°C with constant stirring in a YSI model 5301 bath stirrer assembly (Yellow Spring Instruments, Yellow Spring, OH) connected to a Haake water circulator (Haake Instruments, Inc., Saddle Brook, NJ). The pH was monitored with a model PMN 62 pH meter. The pH meter was connected to the titration unit set to hold pH at 7.41. This was accomplished by automated addition of 0.015 N NaOH from the autoburette unit (ABU 12), controlled by the titrator. The amounts of NaOH added were continuously recorded.

CO_2 generation. CO_2 generation was quantitated by using a modification of the conductometric method described by Maffly (21). 5 ml was withdrawn from samples in which H^+ secretion or O_2 consumption were measured until the time of sample removal. These 5-ml samples were then immediately injected into a test tube containing 2 ml of 1 M lactic acid (Sigma Chemical Company, St. Louis, MO) and 100 μl of Dow Corning Silicone Defoamer (Arthur H. Thomas Co., Philadelphia, PA). Ultrapure nitrogen (Med Tech, Bedford, MA) was bubbled through the sample at a rate of 120 ml/min to expel and carry the CO_2 for mixing with a 1 mM NaOH solution that was pumped up from a 4 liter reservoir through a conductivity cell (Radiometer). Mixing took place in a tube (diameter, 4 mm; length, 5 m) arranged as a double helix and submerged in the NaOH reservoir. After mixing, the gas was separated from the NaOH, which was returned to the reservoir through a second conductivity cell. The difference in conductivity of the NaOH before and after mixing with gas was measured by a differential conductivity meter (Wescan Instruments Inc., Santa Clara, CA) and recorded. Total CO_2 in the samples was measured as the magnitude of the recorded peaks, which were linearly proportional to the CO_2 content from 0 to 4 μmol for 5 ml of samples. For each experiment, a calibration curve was obtained with standards of $NaHCO_3$ in 5 ml buffer.

Oxygen consumption. Oxygen consumption was measured at 37°C by a Clark type oxygen electrode (Yellow Spring Instruments). Neutrophils were incubated in 10 ml of buffer in a concentration from 5×10^6 to 2×10^7 cells/ml. The oxygen concentration was calculated by using a solubility coefficient of O_2 at 37°C of 0.024 ml/ml (22).

Carbonic anhydrase. Carbonic anhydrase was assayed in a homogenate of neutrophils obtained after sonication as described by Kernohan (23). Carbonic anhydrase (bovine erythrocytes) was from Sigma Chemical Co. Ethoxzolamide, a generous gift from Dr. Thomas H. Maren, University of Florida College of Medicine, Gainesville, FL, was dissolved in dimethyl sulfoxide. Protein was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Results are expressed as means±SD. Statistical analysis was performed by using a statistical computer package (version 2.1, Northwest Analytical, Portland, OR).

Results

A constant rate of proton secretion (Fig. 1) was observed with unstimulated neutrophils of 1.34±0.41 fmol/cell per min (*n*

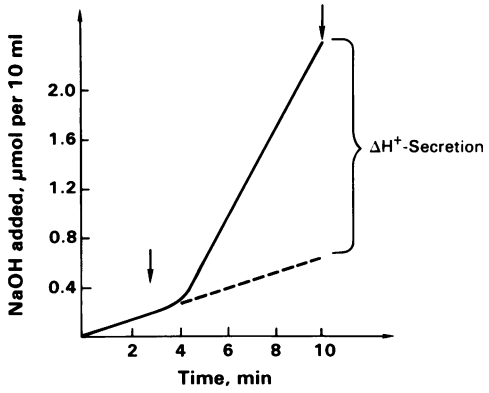

Figure 1. Proton secretion. 5×10^7 cells were incubated in 9.75 ml of buffer at 37°C. After equilibration for 3 min, recording of proton titration was started and as indicated by the first arrow, opsonized zymosan in buffer (250 μl) was added to a final concentration of 1.8 mg/ml. The experiment was stopped by withdrawing 5 ml of the cell suspension (second arrow) and injecting it into a test tube with lactic acid (Methods). The burst of H^+ secretion, ΔH^+, was calculated by subtracting the extrapolated H^+ generation by the cells before stimulation (- - -) from the total amount of H^+ titrated (——).

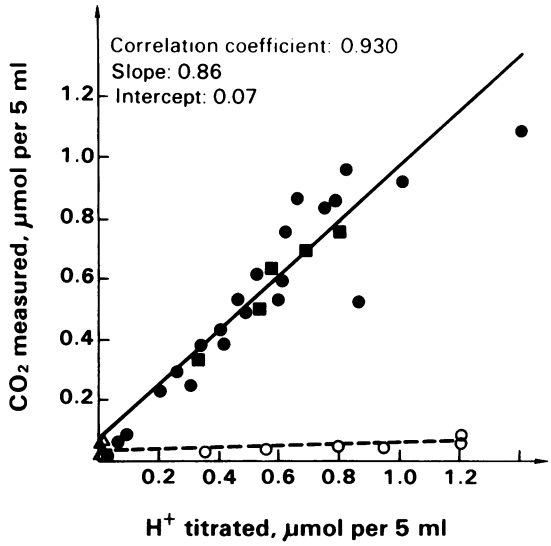

Figure 2. O_2 generation and H^+ secretion. The burst of protons secreted by neutrophils (5×10^6–2×10^7 cell/ml) during phagocytosis of opsonized zymosan, 1.8 mg/ml, was calculated as described in the legend to Fig. 1 and correlated with total CO_2 measured in the 5 ml samples withdrawn (●). The line defined by regression analysis is drawn and specified. It is not significantly different from a line with a slope of 1 and an intercept of O ($P > 0.5$). For some experiments, carbonic anhydrase, 300 units/ml, was present during proton titration (■). Open circles represent experiments where stimulation of the cells was omitted. Open triangles: 2-deoxyglucose, 5 mM, was substituted for glucose in the buffer, and the cells, 5×10^6/ml, were incubated with opsonized zymosan for 10 min.

= 11), which is in agreement with previous reports (17). As seen from Fig. 2, this H^+ secretion was not accompanied by significant CO_2 generation and can be accounted for by lactate production, which we have previously measured to be 1.3 fmol/cell per min under similar conditions and found not to have increased significantly during phagocytosis (24). As illustrated in Fig. 1 and Table I, activation by opsonized zymosan elicited a burst of H^+ secretion, which after a lag period of 90 s was maximal at 5.53 fmol/cell per min for 3–4 min and then gradually diminished. In contrast to van Zweiten et al. (17), we found that the burst of H^+ generation, which is the difference between H^+ generation during activated and resting states (Fig. 1), correlated stoichiometrically with total CO_2 generation during phagocytosis (Fig. 2). Addition of exogenous carbonic anhydrase did not affect the H^+ secretion or the CO_2 generation during phagocytosis (Table I and Fig. 2). Therefore, the CO_2 measured is present in the samples as HCO_3^-, and the demonstrated stoichiometry between CO_2 generation and H^+ secretion identifies H_2CO_3 as the source of protons generated in association with the burst.

A close stoichiometric association between O_2 consumption and CO_2 generation is seen in Fig. 3. Substitution of glucose with 2-deoxyglucose completely abolished the burst in CO_2 generation and H^+ secretion induced by opsonized zymosan (Fig. 2 and Table I), in agreement with the known inhibitory effect of 2-deoxyglucose on the respiratory burst (4, 17, 24, 25). As seen in Fig. 4, CO_2 generation stops when the O_2 is exhausted by the stimulated neutrophils. These results, therefore, indicate

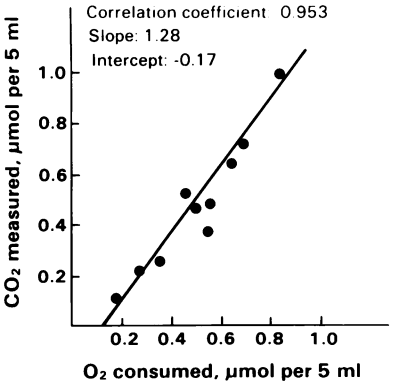

Figure 3. O_2 consumption and CO_2 generation. Oxygen consumption was measured in 10 ml of a neutrophil suspension, 0.5–1.0 × 10^7 cells/ml. Phagocytosis was initiated by opsonized zymosan, 1.8 mg/ml. After variable length of time (1–6 min), 5-ml samples were removed and the CO_2 content measured (as described in Methods) and correlated to the amount of oxygen consumed. The line defined by regression analysis is drawn and specified. It is not significantly different from a line with a slope of 1 and an intercept of O ($P > 0.5$).

that glucose is the only quantitatively significant source of CO_2 produced by the cells during phagocytosis. Previous reports have amply demonstrated that decarboxylation of glucose by phagocytosing neutrophils quantitatively occurs only in the HMPS (4, 10, 11, 14, 26). Therefore, the demonstration here of a close

Table I. H^+ Secretion by Stimulated Neutrophils

	Maximal rate	Lag period
	fmol/cell/min	*s*
No addition	5.53±1.05 (11)	90±6.2 (17)
Carbonic anhydrase, 300 µm/ml	5.47±0.41 (3)	91±5.0 (3)
Dimethyl sulfoxide, 10 mM	5.39±0.61 (3)	94±9.3 (3)
Ethoxzolamide, 0.2 mM		
Dimethyl sulfoxide, 10 mM	5.93±0.42 (3)	91±6.8 (3)
2-Deoxyglucose, 5 mM	0.03 (3)	ND

H^+ secretion was measured in 10 ml of cells, 0.5–1.0 × 10^7 cell/ml. The maximal rate was calculated from the slope of the titration curve during phagocytosis of opsonized zymosan, 1.8 mg/ml, minus the slope of the titration curve of the cells before phagocytosis was initiated. In experiments with ethoxzolamide and dimethyl sulfoxide, the cells were preincubated in the presence of these agents for 30 min on ice before assay. ND, not determined. The number of experiments are given in parentheses.

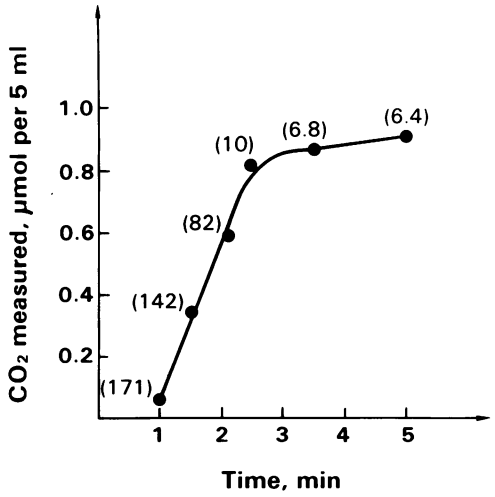

Figure 4. Kinetics of CO_2 generation. Oxygen consumption was measured in 10 ml of a neutrophil suspension, 2 × 10^7 cells/ml. Phagocytosis was initiated by opsonized zymosan, 1.8 mg/ml. At variable periods of time after addition of zymosan, the experiment was stopped by withdrawing 5 ml of the cell suspension and injecting this into lactic acid (Methods). Numbers in parenthesis are the concentration of O_2 (µM) in the cell suspension at the time when the experiment was stopped.

stoichiometric association between O_2 consumption and CO_2 generation during phagocytosis shows that the HMPS is activated to give CO_2 production equivalent to the O_2 consumed. The ordinate intercept in Fig. 3, although not statistically significantly different from zero ($P > 0.5$), might reflect that the initial O_2 consumed by neutrophils uses a pool of NADPH before $NADP^+$ accumulates to activate the HMPS. In accordance with this hypothesis, we observed a longer lag period for H^+ secretion (Table I) than for O_2 consumption (55±7 s, $n = 9$, data not shown).

Discussion

The observed stoichiometry of H^+ secretion, O_2 consumption, and CO_2 generation is in close agreement with that expected from the overall stoichiometry of the HMPS, the NADPH-oxidase, and associated reactions:

HMPS: 1 Glc-6-P + 12 $NADP^+$ →

6 CO_2 + 12 NADPH + 12 H^+ + P_i (27); (1)

NADPH-oxidase: 12 NADPH + 24 O_2 → 24 O_2^-

+ 12 $NADP^+$ + 12 H^+ (28); (2)

O_2^- dismutation: 24 H^+ + 24 O_2^- → 12 H_2O_2 + 12 O_2; (3)

Catalase: 12 H_2O_2 → 6 O_2 + 12 H_2O. (4)

Thus, overall (Eq. 1 + Eq. 2 + Eq. 3 + Eq. 4):

1 Glc-6-P + 6 O_2 → 6 CO_2 + 12 H_2O + 1 P_i. (5)

Degradation of H_2O_2 by the glutathione cycle will not change the overall stoichiometry (28, 29). If H_2O_2 is converted to hypochlorous acid by myeloperoxidase:

12 H_2O_2 + 12 H^+ + 12 Cl^- → 12 HOCl + 12 H_2O, (6)

or if H_2O_2 accumulates, the overall reactions would be, respectively:

1 Glc-6-P + 12 O_2 + 12 Cl^- →

6 CO_2 + 12 OCl^- + 12 H_2O + P_i (7)

or

1 Glc-6-P + 12 O_2 → 6 CO_2 + 12 H_2O_2 + P_i. (8)

Thus, in any case, there is no net generation or consumption of protons, and depending on which route prevails in H_2O_2 catabolism, the stoichiometry between CO_2 generation and O_2 consumption would vary between 0.5 and 1.0 assuming complete dismutation of O_2^-. It should be mentioned that the same stoichiometry holds if H_2O_2 is produced directly by the oxidase. Our results indicate that H_2O_2 is predominantly converted to H_2O in agreement with previous reports (11, 30). Since CO_2 is hydrated to H_2CO_3, the overall stoichiometry of the respiratory burst is:

1 Glc-6-P + 6 O_2 → 6 HCO_3^- + 6 H^+ + 6 H_2O + 1 P_i. (9)

This equation is in agreement with the stoichiometry found in Fig. 2 and 3 which are based on end point values, but is also in agreement with the maximal rates of H^+ secretion and O_2 consumption measured as 5.53±1.05 fmol/cell per min (n = 11) vs. 5.23±0.61 fmol/cell per min (n = 9), respectively.

Thus, the basis for the respiratory burst associated proton secretion is hydration of CO_2 to H_2CO_3. This may occur by catalysis with carbonic anhydrase, or uncatalysed (rate constant = 0.04/s) (31). If uncatalysed, the lag period between achievement of steady state CO_2 generation and H_2CO_3 generation would then be 1/0.04 per s = 25 s. Therefore, addition of exogenous carbonic anhydrase might decrease the lag period of H^+ secretion by up to 25 s depending on how much unhydrated CO_2 escapes from the cell to react with the extracellular carbonic anhydrase. Conversely, if CO_2 hydration was catalyzed, inhibitors of carbonic anhydrase would increase the lag period by 25 s. For each case, no effect on maximal H^+ generation would be expected. However, we found no effect of carbonic anhydrase or its inhibitor ethoxzolamide on the lag of H^+ secretion (Table I). CO_2 hydration was therefore either catalyzed by an inhibitor-insensitive carbonic anhydrase (32), or nonenzymatic hydration occurred before CO_2 escaped the cell. We measured carbonic anhydrase activity in homogenates of neutrophils and found 6.33±0.68 units/mg protein (n = 4). This activity is only 2% of that found in erythrocytes (33). In the presence of 0.1 mM ethoxzolamide, the neutrophil carbonic anhydrase was almost totally inhibited (0.1±0.01 units/mg protein, n = 4). This activity is therefore not likely to be relevant to the CO_2 hydration in stimulated neutrophils.

In summary, we have confirmed the findings of van Zweiten et al. (17) that neutrophils secrete protons in association with the respiratory burst. We have identified the source of protons as carbonic acid generated in the HMPS and have shown that HMPS activity accounts for the reducing equivalents consumed by the NADPH-oxidase. Protons are generated inside the cell both from carbonic acid dissociation and in the reduction of $NADP^+$, but the protons are consumed in the plasma membrane or outside the cell in the superoxide dismutase reaction; therefore, a highly efficient proton translocating mechanism must be operative in the membrane, since the net result is secretion of protons.

Acknowledgments

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