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MEASUREMENT OF FREE RADICAL OXYGEN GENERATION BY CYROCHROME C REDUCTION REQUIREMENT FOR CYTOCHROME C OXIDASE BLOCKADE[1]

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Data is presented showing that one commercial preparation of cytochome c, used to trap and measure free radical superoxide anion, can be contaminated with cytochrome c oxidase activity. This activity can vary from lot to lot, can introduce variability into the measurement of superoxide anion and can result in falsely low estimations of free radical formation. This cytochrome c oxidase activity can be inhibited by low (0.2 mM) concentrations of KCN. Blockade of the cytochrome c oxidase activity allows reproducible measurement of superoxide anion formation at low levels by red cells.

The reduction of cytochrome c (cyto c) by free radical superoxide anion (0_2^-) is a technique frequently used to measure the formation of this activated oxygen species by a variety of cell types (1-9). The amount of cyto c reduced is a function of the amount of 0_2^- formed, the amount scavenged by other species in competition with cyto c and the stability of reduced cyto c in the assay system. Any change in these factors will alter the amount of 0_2^- measured. In a series of experiments to measure 0_2^- formation by red cells using scavenging by cyto c, I found that the addition of 0.2 mM KCN to the reaction mixture improved measurement reproducibility and allowed the demonstration of low level 0_2^- formation.

Methods:

Type III ferricytochrome c from equine heart and other reagents were obtained from Sigma Chemicals (St. Louis, Mo). Red cells (rbcs) were obtained from normal healthy volunteers with normal (AA) hemoglobin and anticoagulated with heparin (10 units/ml). The cells were washed free of plasma and white cells then saturated with oxygen by bubbling 0_2 through a rbc suspension.

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Abbreviations used: 0_2 : Superoxide Anion
Cyto C: Cytochrome C
SOD: Superoxide Dismutase

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Cytochrome c at 5 mM in water was reduced by adding trace amounts of powdered sodium dithionite. The dithionite was then degraded by bubbling 0_2 through the mixture for 5 minutes. The amount of reduced cytochrome c was measured at 550 nM using the molar extinction coefficient of 2.2x104. Change in the amount of reduced cyto c was measured after incubation of 50 μM cyto c in phosphate buffered saline containing 1 gm/liter of glucose at 25 and 37° for various periods of time in the presence of different concentrations of KCN.

Superoxide anion formation by washed red cells was measured via the reduction of cyto c and change in optical density at 550 nM. All incubations were carried out in replicates of six. In one set of six, only cells and 50 μM cyto c were present. In a second set 50μg/ml of bovine erythrocyte Superoxide Dismutase (SOD EC 1.15.1.1) was also added to prevent reduction of cyto c by 0_2^-. In a third set, cyto c, rbcs and 0.2 mM KCN were present. Incubations were carried out at a cell concentration equivalent to 2.5 mg Hemoglobin/ml for 2 hours at 37° . Control samples without cells but containing all the other reagents were handled in an identical manner.

Results:

Relative amounts of reduced and oxidized cyto c were determined in 50 μM solutions by optical density readings at 528 and 550 nM. Measurements were made before and after dithionite reduction and after 0_2 bubbling. Exposure of 50 μM cyto c to trace amounts of dithionite changed absorbtion at 550 nM from about 400 units to over 900. This change was clearly visible as the solution changed from dark red to light pink.

Incubation of the reduced cyto c solution at 37° resulted in a time dependent decrease in the reduced cyto c (Table 1). No such change was seen at 25°. Nor did the addition of SOD alter this "spontaneous" decline in the amount of reduced cyto c. In contrast, the addition of KCN blocked the drop in reduced cyto c in a dose dependent manner (Table 2). Three different batches of Type III cyto c from Sigma were tested for the presence of oxidase activity. The decrease in the amount of reduced cyto c after 2 hours at 37° ranged from 20 to 32%. Thus, not only is cyto c oxidase activity present in this preparation of cyto c, but the amount of activity varies between lots.

TABLE 1

Time course of cytochrome c oxidation in the absence of KCN

Time at 37°	% Decrease OD at 550 nM	nM Reduced Cyto C[1]
0	0	40nm/ml
15	10	37
30	13	35.8
60	18	33.6
120	49	20.8
180	65	14.5
240	65	14.2

1: Calculated using 2.2 x 10^4 as extinction coefficient.

TABLE 2

Effect of KCN concentration on the change in the amount of reduced cyto c after incubation at 37° for 2 hours

KCN Concentration	% Decrease in OD at 550 nM of a 50 uM solution of reduced cytochrome c
None	20%
0.001 mM	23
0.005 mM	20
0.01 mM	14
0.02 mM	5
0.1 mM	1
0.2 mM	1

The effect of cyto c oxidase was apparent when 0_2^- formation by rbcs was measured. Formation of 0_2^- by rbcs from a single donor was measured on 8 different occasions. In the absence of KCN, no free readical formation could be shown in 4 assays while in the other 4 tests from 0.117 to 0.785 nm of 0_2^- per mg hemoglobin (HgB) was measured. In contrast, when 0.2 mM KCN was present, 0_2^-formation could be demonstrated in each assay and ranged from 0.306 to 0.837 nM/mg HgB. In the absence of KCN mean 0_2^- formation was 0.194 nM/mg HgB with a standard deviation of 0.261. With KCN added, mean 0_2^-formation was 0.518 nM/mg HgB with a standard deviation of 0.189.

Discussion:

Some authors use Type VI cyto c from Sigma to measure 0_2^- formation (1-3), while others use Type III (4-6) and still others do not specify the type used (7,8). Testing for cyto c oxidase activity is not routinely reported. Although I have not attempted to survey a large number of commercial cyto c preparations for oxidase activity, it is important to note that at least one preparation of cyto c used to measure 0_2^- formation has significant amounts of oxidase activity. This finding means those using cyto c reduction to assay 0_2^- formation must consider the possibility of cyto c oxidase contamination. Cytochrome c oxidase contamination is of particular importance when low levels of 0_2^- formation are being measured. Problems with cyto c oxidase can be minimized by testing for its presence or blocking its activity with KCN. Another approach is the use of acetylated cyto c to stabilize any reduced cyto c formed as described by Nasrallah et al. (9).

Measurement of low levels of 0_2^- formation by cells, through cyto c reduction, requires

the inhibition of cyto c oxidase by KCN, stabilization of reduced cyto c through acetylation or the rigorous exclusion of cyto c oxidase activity from the system.

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