Production of superoxide by neutrophils

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Summary. Oxygen uptake by neutrophils has been stimulated by particulate serum-treated-zymosan (STZ) and soluble N-formylmethionyl-leucyl-phenylalanine (FMLP) in the presence and absence of the superoxide radical scavenger, cytochrome C. Results indicate that FMLP may stimulate neutrophils to produce superoxide but that STZ may not.

Conflicting results from 2 laboratories have cast some doubt on the formation of superoxide radicals by stimulated neutrophils[1,2]. In each case the experiments were designed to test the premise that if cytochrome C were supplied to a superoxide producing system (stimulated neutrophils) oxygen should be regenerated (eqn 1) and the net oxygen uptake should be less than in a system which did not contain cytochrome C

$$\cdot O_2^- + \text{cytochrome C } (Fe^{3+}) \rightarrow O_2 + \text{cytochrome C } (Fe^{2+}) \ldots \quad (1)$$

In 1 set of experiments there was no difference in oxygen uptake in the presence or absence of cytochrome C[1] while in a 2nd set there was a significant difference[2]. It was proposed that the difference between these 2 sets of results may have been due to a much lower neutrophil concentration in the 2nd case[2]. The current work was undertaken to test this hypothesis with a substantially lower neutrophil concentration than either of the above using both a particulate (STZ) and a soluble (FMLP) stimulus.

Materials and methods. Oxygen uptake: To neutrophil suspension (isolated from healthy female volunteers as described previously)[3] (0.1 ml) in a Clark-type oxygen electrode was added Hanks' balanced salts (supplemented with glucose 0.1% and bovine serum albumin 0.1%) (0.90 or 0.65 ml) and cytochrome C (0.0 or 0.25 ml of 60 μM) and the cells stirred at 37 °C for 15 min to establish the endogenous rate of oxygen consumption. STZ (0.02 ml of 50 mg/ml) or FMLP (0.01 ml of 10 μM) was then added and the increase in oxygen consumption followed for 5 min. Superoxide output: After 5 min the above reaction containing cytochrome C was stopped with N-ethylmaleimide (0.1 ml of 10 mM), cooled in an ice bath for 10 min, centrifuged and superoxide in the supernatant determined by increase in absorption at 550 nm (adjusted for the blank value) using the extinction coefficient of 21.1 mM^{-1} cm^{-1} for reduced cytochrome C.

In experiments to determine the reduction of cytochrome C by cellular antioxidants the initial cytochrome C concentration was 15 μM and each reaction mixture was made up to 1 ml with 0.05 M phosphate buffer pH 7.8 containing 10^{-4} M EDTA. The ascorbic acid stock solution (0.28 mM) lost 25% of its cytochrome C-reducing activity in 1 h at room temperature and all ascorbic acid values are adjusted for this.

Results and discussion. It was found (table 1) that there was no significant difference in oxygen uptake between neutrophils stimulated by STZ in the presence or absence of cytochrome C whereas in neutrophils stimulated by the chemotactic factor FMLP there was a significant difference ($p < 0.01$) between the results in the presence and absence of cytochrome C. Superoxide output (assayed as cytochrome C reduction) resulting from both stimuli was completely inhibitable by superoxide dismutase (results not shown).

Thus the cytochrome C-reducing agent resulting from FMLP stimulation of neutrophils behaves as expected of superoxide whereas that resulting from STZ stimulation does not. These STZ results argue against the suggestion that the trapping of superoxide by cytochrome C (and the consequent return of oxygen to the system) would be more efficient with a low concentration of neutrophils than with a high concentration[2] since the neutrophil concentration in the current work was much the lowest of the 3 sets of experiments. Similarly, it may not be argued that there was insufficient cytochrome C to efficiently trap the superoxide in the present work since a 3 times higher concentration ratio of cytochrome C to neutrophils produced oxygen uptake and superoxide output values which were almost identical to the present ones[2].

Other evidence also suggests that cytochrome C reduction by STZ-stimulated neutrophils may not be due to superoxide. For example, it has been shown that zymosan particles

Table 1. Oxygen uptake (in the presence and absence of cytochrome C) and superoxide output (cytochrome C-reduction) by stimulated neutrophils

Neutrophils	Oxygen uptake (nmole/5 min/10^6 cells)				Superoxide output (nmole/5 min/10^6 cells)	
	STZ stimulation Cytochrome C		FMLP stimulation Cytochrome C		STZ stimulation	FMLP stimulation
($\times 10^6$)	−	+	−	+		
0.74 ±0.16	21.9 ±1.5	23.4 ±3.3	9.9 ±1.1	4.8 ±2.5	15.1 ±2.9	10.0 ±3.0

Results are means ± SD of 4 experiments.

Table 2. Reduction of cytochrome C by cellular antioxidants in the presence or absence of divalent cations

	Ascorbic acid (5.6 μM)				Reduced glutathione (80 μM)			
	Control	+ Ca^{2+} (0.5 mM)	+ Mg^{2+} (0.3 mM)	+ Superoxide dismutase (29 U)	Control	+ Ca^{2+} (0.5 mM)	+ Mg^{2+} (0.3 mM)	Ca^{2+} (0.5 mM) + superoxide dismutase (29 U)
Cytochrome C reduction nmoles/2.5 min	3.0	3.0	2.8	2.8	0.1	2.4	2.0	2.5

which have been opsonized in serum stored at −20 °C or in serum stored at +4 °C cause the same level of oxygen uptake when exposed to neutrophils but that only the zymosan opsonized in −20 °C serum initiates significant cytochrome C reduction[3]. Thus there appears to be a thermolabile substance in serum which stimulates neutrophils to reduce cytochrome C. That neutrophils could produce a cytochrome C-reducing agent without change in oxygen uptake suggests that the compound was not superoxide since, if it were, it would be expected that a variety of reductive and dismutative reactions would return oxygen to the system causing a reduction in net oxygen uptake. Preliminary studies to determine whether this unknown cytochrome C reducing agent was one of the well-known cellular antioxidants indicated that it was not ascorbic acid or glutathione since, although both reduced cytochrome C their reactions were not inhibited by superoxide dismutase (table 2). (It was noteworthy that glutathione was a much stronger cytochrome C-reducing agent when chelated with calcium or magnesium than in their absence.) The unknown compound could however be a protein with one or more reactive sulphydryl groups.

Present results indicate that the properties of the agent responsible for cytochrome C reduction in FMLP stimulated neutrophils are consistent with those expected of superoxide. Since FMLP is a chemotactic agent these results support a previous suggestion that superoxide output may have more to do with chemotaxis than with phagocytosis[3].

It has been accepted for many years that stimulation of neutrophils by a variety of particulate and soluble agents[4-8] gives rise to the production of superoxide radicals (cytochrome C reduction). The current work suggests that further examination of such cytochrome C-reducing activity is necessary before it may be unequivocally attributed to superoxide.

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Indication of reduced doxorubicin-induced cardiac toxicity by additional treatment with antioxidative substances

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Summary. The influence of antioxidative substances on doxorubicin-induced cardiac toxicity was studied in C 57 BL mice. Tocopherol (500 mg/kg), glutathione (1000 mg/kg), cysteamine (15 mg/kg) and L-cysteine (1000 mg/kg), injected i.p. 24 h before doxorubicin treatment (15 mg/kg i.p.) were able to reduce malonaldehyde production in cardiac tissues significantly. SH-containing substances with high reducing activity, such as vitamin E, could be a useful tool in clinical trials to prevent doxorubicin induced cardiac damage.

Doxorubicin is now part of the chemotherapeutic regimen for many hematopoetic malignancies as well as for a variety of solid tumors[1]. Until now treatment with this agent is still limited by a potentially lethal and dose-dependent congestive cardiomyopathy[2]. Although the etiology of doxorubicin-induced cardiomyopathy has not been clearly elucidated, there is some evidence for increased superoxide anion and hydrogen peroxide formation in mitochondria and sarcoplasmatic reticulum after doxorubicin treatment[3]. The accumulation of drug-induced reactive oxygen radicals in heart cells may explain the increased cardiac lipid membrane peroxidation[3], and the reduced cardiac glutathione pools that have been found after doxorubicin treatment[4]. Since sulfhydryl groups play an important role in the maintenance of muscular contractile function[5] and membrane integrity[6], and also promote the non-enzymatic detoxification of hydroxyl radicals[7] and lipid peroxides[8], it was previously suggested that augmenting sulfydryl group content in the heart might enhance the ability of heart muscle to withstand doxorubicin exposure[9].

In this study we investigated the activity of some antioxidative substances on doxorubicin induced lipid peroxidation in vivo.

Material and methods. Male C 57 BL mice had continuous access to food (Altromin 1324) and water and weighed 18–22 g when used. Animals were divided into 10 groups of 15 mice each. Animals in group 1–3 (6–8 weeks old) were treated with 15 mg/kg doxorubicin hydrochloride (Farmitalia) i.p. and killed by cervical dislocation at 24 h (group 1), 48 h (group 2) and 72 h (group 3) after i.p. injection of the anthracycline. Animals in group 4–6 were untreated, but of different ages; group 4: under 6 weeks old; group 5: 6–8 weeks old; group 6: more than 24 weeks

Table 1. Influence of doxorubicin treatment (15 mg/kg i.p.) on malondialdehyde production in C 57 Bl mice (6–8 weeks old)

Hours[a]	Malondialdehyde (nmole/g wet wt)[b] Heart	Liver
Controls	2.5- 3.1 (2.74± 0.33)	2.6- 3.5 (2.85±0.40)
24	9.9-14.2 (12.42± 1.59)	3.3- 5.1 (4.2 ±0.69)
48	12.4-21.5 (17.04± 3.67)	8.0-10.2 (8.8 ±0.96)
72	51.0- 157 (90.80±43.6)	4.0-13.5 (8.1 ±3.58)

[a]Time is calculated from doxorubicin i.p. injection. [b]Range and mean ± SD (n = 5; 3 mice in each group).