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Aspects of the Release of Superoxide by Leukocytes, and a Means by Which This Is Switched Off

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Although great progress has been made in understanding the respiratory burst of leukocytes that produce superoxide (O$_2^-$), it is possible that a component, or components, might have been overlooked. Furthermore, O$_2^-$ production and its sequels, though cardinal in bactericidal action, might ultimately be damaging to the host's own cells. It is important, therefore, that a biologic mechanism exist to turn off O$_2^-$ production by stimulated leukocytes. This article offers evidence that methoxatin (PQQ), a redox-cycling orthoquinone, might be involved in O$_2^-$ production by leukocytes. This is based on the fact that inhibitors of O$_2^-$ production, such as diphenylene iodonium (DPI) and 4,5-dimethylphenylene diamine (DIMPDA), were shown to sequester PQQ in leukocytes, i.e., to form adducts with that substance. Addition of PQQ to cells blocked with the inhibitors partially restored O$_2^-$ release. With respect to turning off cellular O$_2^-$ release, a factor was observed to be released to the medium by old macrophages (14 days old, but not by those less than 7 days old). Such conditioned medium, when added to stimulated neutrophils or macrophages, blocked O$_2^-$ release. This factor was sensitive to proteases, exhibited molecular sizes of 3 and 11 kDa, and its action was independent of the nature of the stimulus applied to the leukocytes. It was partially purified by column ( sizing) chromatography and HPLC. It seems to be a general modulator of the release of reactive oxygen species by phagocytes and is irrespective of phagocytic cellular type, or species from which the cells were derived. — Environ Health Perspect 102(Suppl 10):43–44 (1994)

Key words: superoxide, leukocytes, modulation, methoxatin, deactivation, PQQ adducts, diphenylene iodonium

Introduction

Diphenylene iodonium (DPI) has been an important and potent inhibitor of O$_2^-$ release at approximately 50 μM (1). Its use has contributed to our comprehension of the mechanism by which leukocytes, stimulated by particulate or soluble agents, elaborate O$_2^-$. Cross, for example, has adduced evidence that a flavoprotein is involved by showing that such a protein is labeled when broken cells are exposed to [125I]-DPI (2,3). Other workers have indicated that the labeling is less specific (4). As described below, using somewhat different conditions and titrated DPI, we reached the conclusion that methoxatin (PQQ) might be involved. In other words, we wonder whether the "turn on" mechanism is clear yet, and in fact whether all the components have been recognized so far (5).

At the other end of the spectrum, we considered the fact that O$_2^-$ release from stimulated leukocytes could be inimical to host cells and tissues if it persisted beyond its useful stage, i.e., its microbicidal function. Is there a biological mechanism to turn O$_2^-$ release off? Such indications do exist in the literature (6–8). However, the factor we have studied differs in molecular weight and immediacy of action from factors obtained earlier (9).

Experimental

All substances used, cells employed, methods adopted, etc., have been outlined in relevant earlier papers. Thus, synthetic PQQ, titrated PQQ and DIMPDA, as well as the sizing columns and HPLC methodology used, are described (10). The cells from which the deactivating factor was obtained, the mode of harvesting, the steps of partial purification by ultrafiltration, gel filtration, and reversed-phase HPLC, etc., are described (8,11).

Results

Turning on O$_2^-$ Release by Granulocytes

DPI, DIMPDA, and diphenylene iodonium all inhibited O$_2^-$ release from stimulated guinea pig granulocytes, as expected (3). DPI was the most potent inhibitor. As reported, a 50-nM concentration resulted in a 64% inhibition. A 1.5-μM solution of inhibitor caused greater than 80% inhibition, which was relieved by addition of PQQ in increasing amounts. By 100 μM PQQ, inhibition was diminished by 50%. When 16.5 μM titrated DPI was incubated with guinea pig granulocytes, the pellet of cells retrieved and extracted, the following were found:

• A water-soluble adduct of [3H]DPI and PQQ was obtained. This was examined by HPLC and was found to cochromatograph with an adduct of authentic PQQ with [3H]DPI-PQQ. The adduct had a unique HPLC profile.
• Exposure of the putative [3H]DPI-PQQ adduct to Tiron (a detergent) in excess split out PQQ. Since this was not radioactive, it was detected by its absorbance profile and its redox characteristics. The adduct itself had different properties.
• No titrated protein bands were seen on SDS-gel of the extract of the cellular pellet after removal of the adduct of [3H]DPI and PQQ. These experiments were repeated with [3H]DIMPDA, and supported by observa-
The inhibition was concentration-dependent with respect to the inhibitor. The factor was sensitive to pronase or trypsin. It functioned against O$_2^*$ release by stimulated macrophages or neutrophils and was not specific for species (human, mouse, guinea pig cells were all inhibited). Furthermore, its action was independent of the nature of the stimulating factor used (retinal, f-Met-Leu-Phe, phorbol myristate acetate). These stimulating agents operate by different modes.

**Discussion**

The first part of this article raises the possibility that PQQ might participate in the respiratory burst of leukocytes. Against this is the fact that reconstituted systems have largely employed recombinant materials (12,13). Nevertheless, the possibility that some ubiquitous PQQ was present cannot be excluded. Furthermore, the activity of the reconstituted systems *in vitro* the whole cell is not quite apparent. Such evidence as presented here, and more fully elsewhere (10), together with the fact that PQQ has previously been suggested to have a role in the immune system, especially in white cells (14), is tempting. Our calculations from the data on adduct formation with [3H]DPI suggest that the concentration in granulocytes is about 300 times that in plasma, i.e., 20 to 30 nm/10$^8$ cells. One caveat in considering the whole question is to remind oneself that the present data derive from studies of guinea pig cells, whereas most work on the mechanism of O$_2^*$ release by stimulated leukocytes has been done on human cells.

Such concerns regarding species differences do not seem to apply in the case of the turning off of O$_2^*$ release by stimulated macrophages. It should be noted that the factor is so potent that its biological activity is evident at concentrations at which the protein involved cannot be measured. Nevertheless, with some assumptions, it can be calculated that it has been purified at least 1000-fold.