9

Oxygen and Nitrogen Reactive Metabolites and Phagocytic Cells

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The goal of this chapter is to clarify the role of phagocytic cells (mainly neutrophils) as mediators of both protection and destruction. Specifically, the linkage between the oxidative metabolic pathways and protection against bacterial invasion is contrasted with the subsequent tissue damage that ensues when these processes are not properly regulated. Historically, tissue injury, microbial killing, and many pathogenic mechanisms have been ascribed to reactive oxygen intermediates (ROI). However, with the realization of the importance of reactive nitrogen intermediates (RNI), attention has shifted somewhat from ROI. This recent understanding of the role of nitrogen radicals such as nitric oxide and peroxinitrite is compared to the oxygen radical pathways.

Following the discovery of antioxidants such as superoxide dismutase (SOD) by McCord and Fridovich in 1969 (McCord and Fridovich, 1969b), most studies published in neutrophil physiology have been related to the destructive nature of superoxide (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radical ($OH\cdot$) with the reasonable understanding that these reactive molecules were also the causative agents in tissue damage involving phagocytic cells. Neutrophils and macrophages have a tremendous capacity for production of these molecules.

Sixty million neutrophils per minute are released into the circulation through the

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normal surveillance mechanism of the reticuloendothelial system. Only erythrocytes (RBC) are produced more prolifically in the body, about 1.5 times the rate of the neutrophil (Erslev and Weiss, 1977). Thanks to their longer life-span, however, RBC outnumber neutrophils in the peripheral circulation by a factor of 10^3. Neutrophils are replaced at such a high rate because of their very short half life (several hours) and total life-span (3-4 days). A number of important factors affect the final disposition of these neutrophils, one being a substantial increase-as much as six-to eightfold—in phagocyte production due to stress (Boggs, 1967; Robinson and Mangalik, 1975), and consequently a significant number of immature neutrophils may be present in the circulation. Because some cytokines can delay apoptosis and thereby increase the life-span of neutrophils, vast quantities of these cells can accumulate at inflammatory sites. The mechanism for removal of neutrophils from inflammatory sites requires the neutrophils to progress toward an apoptotic demise rather than becoming necrotic and releasing vast quantities of granule enzymes into surrounding tissue. The potential for tissue damage is enormous, particularly if the respiratory mechanism of these cells has been activated. A delicate balance between manufacture of reactive oxygen radicals and their removal or detoxification must be maintained. Understanding the nature of this balance mechanism is the key to discerning the difference between protection and destruction in phagocytic cell function.

In this chapter the pathways of oxygen metabolism in phagocytic cells are unraveled, as are those of the parallel nitrogen metabolism, and an attempt is made to show the relationship between protective and destructive mechanisms. Of particular importance is our current understanding of the role of peroxynitrite ($ONOO^-$), a molecule formed by the union of superoxide and nitric oxide.

OXYGEN-RELATED METABOLISM

A key element in the production of ROI by phagocytes is the NADPH oxidase enzyme system, which was originally described in neutrophils (Babior et al., 1973). This enzyme system is known to consist of several components based upon the b-558 cytochrome, a heterodimer consisting of two subunits, gp22-*phox* (α unit) and gp91-*phox* (β unit), as well as a heme moiety and a flavin binding site. It is these α and β units that are missing in neutrophils of most patients with chronic granulomatous disease, in which the neutrophils fail to activate the respiratory burst. Additionally, several other crucial components of the oxidase system must be combined to make the enzyme system active. These include the cytosolic components known as gp47-*phox* (a 47 kDa protein) and gp67-*phox* (a 67 kDa protein), Rat-2 (a GTP-binding protein), and an NADPH-binding protein (Cumutte et al., 1989; Parkos et al., 1987). Phagocytic cell oxidation pathways are thought to be quite different from those traditionally associated with mitochondrial respiration, thus the terminology "phox" for oxidase-related proteins—phagocyte oxidase, implying that they are somewhat unique to phagocytic cells.

In contrast to mitochondrial respiration, where the entire pathway and all components for respiration are contained within the mitochondria, the phagocyte oxidase

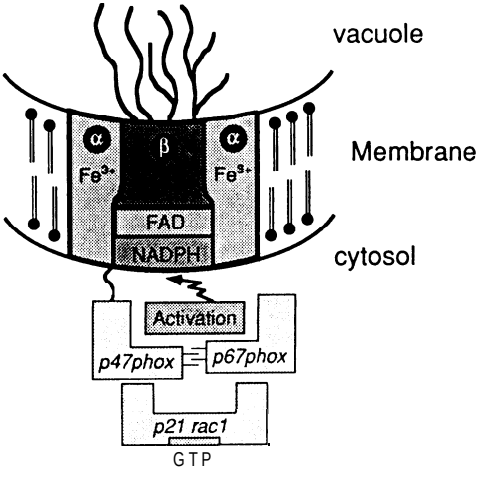

Fig. 1. Schematic representation of NADPH oxidase (after Segal and Nugent). One possible configuration which shows the association of the α and β subunits and the relationship to cytosolic p47-*phox* and p65-*phox* and p21 *rac* 1. Reprinted from Biological Oxidants: Generation and Injurious Consequences, C. G. Cochrane & M. A. Gimbrone (eds.), 1992.

system must be coupled by transporting cytosolic proteins to the plasma membrane where they are assembled into an operating complex. Figure 1 provides an overview of one possible model of the structure and function of the neutrophil oxidase complex. A series of events takes place, the exact order of which is not known. It is thought that the gp47-*phox* is first phosphorylated via PKC before being integrated into the membrane complex and further phosphorylated (Rotrosen and Leto, 1990). The exact function of these two *phox* proteins is uncertain, as is their location. However, both contain two SH3 domains, suggesting that one or both proteins may be linked to the cytoskeleton rather than to the cytoplasm (Woodman et al., 1991).

Understanding the structure of the oxidase is a key to understanding the respiratory burst in phagocytic cells. As noted above, the cytochrome in phagocytic cells is different from that in other mammalian cells. Firstly, the midpoint redox potential is very low at -245 mV (thus the alternative b_{-245} nomenclature), allowing it to reduce molecular oxygen directly to superoxide. Because the wavelength of the a band of light absorption is at 558 nm, the cytochrome is also referred to as cytochrome b-558 (Wood, 1987; Cross et al., 1981). The greatest concentration of the oxidase system is in the plasma membrane, but in the membranes of specific granules a significant amount is also present and is capable of being transferred to the membrane of the phagocytic vacuole upon fusion, subsequent to activation of the cell.

Is the oxidase system of phagocytes unique to cells such as neutrophils and monocyte/macrophages? Because O_2^- is formed during normal mitochondrial oxidation, clearly all mammalian cells contain the SOD enzyme system necessary to protect the cell from damage and also from external sources of oxidants. Some lines of evidence now suggest that other cell types may contain either the identical phagocyte NADPH oxidase system or components thereof. In most cases the activity is severely reduced

idase is the trigger for the respiratory burst. Although the exact role for each oxidase component is still in doubt, there is clear evidence that the process involves translocation of at least some components from the cytosol to the membrane. Concomitant with this translocation is the activation of several second messenger signaling pathways, including Ca^{2+}, diacylglycerol (DAG), GTP-binding proteins, and tyrosine kinase.

Electronically Excited Species of Oxygen

Singlet Oxygen. Two forms of singlet oxygen are known: Σ^1O_2, with a higher energy but short lifetime, and Δ^1O_2, with a lower energy and a lifetime of 2 μs (Keams, 1979). For many years the phenomenon of chemiluminescence associated with phagocytes has been ascribed to the production of singlet oxygen, ever since the original observation in phagocytes by Allen (Allen et al., 1972). One of the principal mechanisms for the formation of Δ^1O_2 is thought to be via the interaction of HOC1 with O_2^- (Long and Bielski, 1980) or via the myeloperoxidase-catalyzed oxidation of chloride in the presence of excess H_2O_2 (Harrison and Schultz, 1976). Although there are a number of possible mechanisms for reactions to form singlet oxygen, the evidence is still inconclusive that chemiluminescence in neutrophils actually represents singlet oxygen production.

Oxygen Radical Formation

Superoxide. Activated neutrophils are capable of making large quantities of superoxide. For instance, within 1 minute of stimulation with formyl-methionyl-leucyl-phenylalanine (fMLP) in a l-2 ml 1 volume, 2×10^6 neutrophils were shown to produce 10 n*M* O_2^-, equivalent to 5–10 m*M* in the absence of dismutation (Schraufstatter and Jackson, 1992). Superoxide either rapidly dismutates to H_2O_2 (via SOD), combines with NO· to form peroxynitrite ($ONOO^-$) (see below), or is protonated (HO_2·) in a reaction that is equally probable as SOD-catalyzed dismutation at pH 4.8 (Behar et al., 1970), but less likely at either lower or alkaline pH. Superoxide is formed as a result of respiration, since from 1 to 4% of oxygen used in the mitochondrial electron transport pathway results in the production of superoxide (Cross, 1987) (accounting for the wide distribution of SOD).

Hydrogen Peroxide. Hydrogen peroxide is generated within neutrophils primarily from superoxide dismutase-catalyzed dismutation of O_2^- anions, plus some spontaneous dismutation. The general reactions are as follows:

$$2O_2 + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

$$O_2^- + O_2^- \xrightarrow{4H^+} 2H_2O_2$$

In comparison with many other reactive species, H_2O_2 itself is relatively low in reactivity, allowing it to remain in contact with cells and pass through cell membranes

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(Frisch et al., 1983). Catalase breaks down H_2O_2 to oxygen and water, thereby removing any potential consequential damage. Interaction with halides such as Cl- via myeloperoxidase (MPO) and detoxification via the glutathione cycle are the primary mechanisms for removal of H_2O_2 within phagocytes. The glutathione cycle is coupled to hexose monophosphate shunt activity because glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase reduce $NADP^+$ to NADPH which must be reoxidized, a task performed by the glutathione cycle (see Fig. 2). Reduced glutathione (GSH), a vital component in phagocytic cells, can be easily measured using a monobromobimane fluorescent probe (Hedley and Chow, 1994). Reduced glutathione is converted to the oxidized form (GSSG) by glutathione peroxidase, and subsequently reduced back to GSH by NADPH. This cycle is an important "detoxification" system for excess H_2O_2 within the cytosolic environment (Voetman and Roos, 1980).

Hypochlorous Acid. The MPO-catalyzed reaction of H_2O_2 with chloride produces a particularly dangerous molecule, hypochlorous acid (HOCl), which acts directly on membrane protein by inactivating sulfhydryl-dependent transporter systems (Schraufstatter et al., 1990). Hypochlorous acid is the predominant species at acidic pH, such as found in activated phagocytes. It also reacts with primary amines to produce monochloramine and taurine monochloramine, ZV-chloramines recognized as very reactive oxidants. The general reaction proceeds as follows:

$$H_2O_2 \xrightarrow{\text{MPO} + 2Cl^-} 2HOCl + 2e^-$$

Neutrophils contain a very significant amount of MPO, estimated to be at least 5% of dry cell weight (Schultz and Kaminker, 1962). This enzyme, found in the azurophilic (primary) granules of neutrophils, has been well characterized biochemically (Johnson and Nauseef, 199 1). Other reactions of importance include those with nitrogen-containing compounds to form chloramines. Reactions involving MPO are both numerous and complex and beyond the scope of this chapter.

Hydroxyl Radical. For many years it has been thought that superoxide, while exercising some damaging effects on biological systems, is less important in terms of tissue injury than the more dangerous hydroxyl radical (OH'), whose production from H_2O_2 is thought to occur by the iron-catalyzed Haber-Weiss reaction:

$$H_2O_2 \xrightarrow{Fe^{2+}} Fe^{3+} + OH\cdot + OH^-$$

Although this reaction has, to date, provided the most acceptable explanation for tissue damage in oxidative systems, there are some troubling points, such as the fact that the rate constant for this reaction is somewhat lower than that for the competitive reaction of ascorbic acid with iron. It has therefore been proposed that a more realistic molecule for tissue damage is $ONOO^-$ rather than $OH\cdot$ (Beckman et al., 1990) (see below).

Fluorescent Indicators of Intracellular Oxidation

Many assay systems for measuring neutrophil function have been proposed in the literature. Some recently developed methods allow simultaneous measurement of O_2^- and H_2O_2, and NO· as well. These methods are often based upon the use of fluorescent probes. Five specific probes are discussed here: dichlorofluorescin diacetate, dihydrorhodamine 123, hydroethidine, parinaric acid, and monobromobimane. Table 1 summarizes their commonly used excitation and emission wavelengths and target molecules.

Dichlorofluorescin Diacetate. Dichlorofluorescin diacetate (DCFH-DA) has been utilized for H_2O_2 measurement ever since the first application by Keston and Brandt (1965) in a bulk cell assay. The probe was later recognized as a useful one for determination of H_2O_2 in neutrophils (Gubitz et al., 1976; Homan-Muller et al., 1975) and the technique extended to flow cytometry by Bass et al. (1983). The list of subsequent publications, particularly in flow cytometry, is substantial. Dichlorofluorescin diacetate has been used to study H_2O_2 production in human neutrophils (Robinson et al., 1994a; Himmelfarb et al., 1992; Epling et al., 1992; Stelzer and Robinson, 1988a; Wolber et al., 1987; Seeds et al., 1985; Smith and Weidemann, 1993; Vowells et al., 1995), monocytes/macrophages (Holter et al., 1987; Lepoivre et al., 1986), cultured neurons (Saez et al., 1987), renal epithelial cells (Scott et al., 1988), melanocytes (Boissy et al., 1989), chondrocytes (Tiku et al., 1990), rat endothelial cells (Carter et al., 1994b), human umbilical vein endothelial cells (HUVECs) (Niu et al., 1994; Royal1 and Ischiropoulos, 1993), and bovine aorta endothelial cells (Royal1 and Ischiropoulos, 1993).

The probe works in the following manner. Dichlorofluorescin diacetate is an esterified molecule that can freely pass through the cell membrane. Once inside the cell, DCFH-DA is deacetylated by cellular esterases to the nonfluorescent dichlorofluorescin (DCFH), which is trapped within the cell by its polar nature. Dichlorofluorescin is converted by intracellular oxidants such as H_2O_2 to the green fluorescent molecule dichlorofluorescein (DCF), a reaction significantly enhanced in the presence of peroxidase. The major difficulties with this probe are its tendency to leak from the cell and its lower sensitivity to H_2O_2 as compared to DHR 123. Many studies have used this probe successfully for intracellular H_2O_2 determination, but careful controls must be made to account for cell leakage.

TABLE 1. Useful Functional Fluorescent Probes

Dye	Excitation (nm)	Emission (nm)	Indicator
DCFH-DA	488	520	H_2O_2
DHR- 123	488	520	H_2O_2
HE	488	600	O_2^-
Parinaric acid	325-360	420-450	Lipid peroxidation
Monobromobimane	350-360	420-450	Reduced glutathione

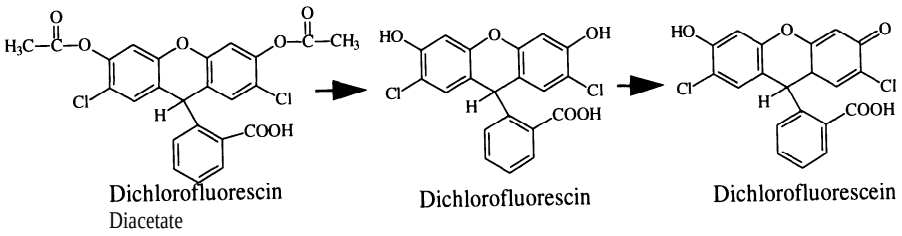

Fig. 3. Dichlorofluorescin diacetate is hydrolyzed by cellular esterases to the nonfluorescent dichlorofluorescin, which is readily oxidized to the fluorescent dichlorofluorescein.

Dihydrorhodamine 723. Dihydrorhodamine (DHR 123) is by far the most-used probe for measurement of intracellular H_2O_2. DHR 123 is oxidized directly to rhodamine 123, which is excitable at 488 nm and emits at 5 15 nm in the same emission range as DCF and FITC (Rothe et al., 1988). Publications describe its use in human neutrophils (Wenisch et al., 1996; Emmendörffer et al., 1994; Tanigaki et al., 1993; Waddell et al., 1994; Cao et al., 1993; Demaurex et al., 1996; Smith and Weidemann, 1993; Vowells et al., 1995; Rothe et al., 1988; van Pelt et al., 1996; Wenisch et al., 1995), human eosinophils (Elsner et al., 1995), HL60 cells (Kaffenberger and van Beuningen, 1994), rat mast cells (Tsinkalovsky and Laerum, 1994), guinea pig neutrophils (Tanigaki et al., 1993), cultured chondrocytes (Hayem et al., 1994), rat brain cells (LeBel et al., 1992), rat renal proximal tubular cells (van de Water et al., 1995), mesangial cells (Zent et al., 1995), and L929 cells (Goossens et al., 1995). Combinations of DHR123 with surface receptor analysis (Elsner et al., 1994), cell viability using propidium iodide (Clancy et al., 1995), and calcium indicators (Bueb et al., 1995) demonstrate how the probe can be used for simultaneous measurements.

DHR 123 enters the cells as a freely permeable dye that is converted to rhodamine 123 and subsequently localized in the mitochondria. The conversion from the nonfluorescent to the fluorescent molecule depends entirely on oxidation products and does not require enzymatic catalysis. Once oxidized, the probe is identical to rhodamine 123, a common laser dye. An example of the use of rhodamine 123 directly in endothelial cells is shown in Figure 5. One significant advantage of the DHR probe

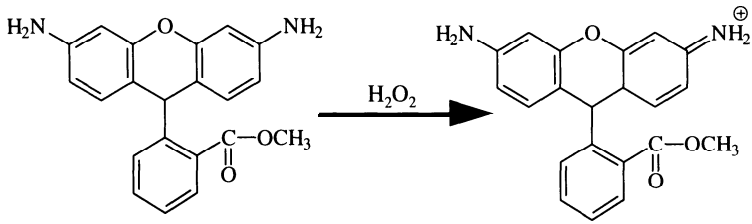

Fig. 4. Dihydrorhodamine enters the cell and is oxidized to rhodamine 123, a fluorescent molecule which emits at 520 nm, the same as FITC. The oxidation is a result of H_2O_2 production.

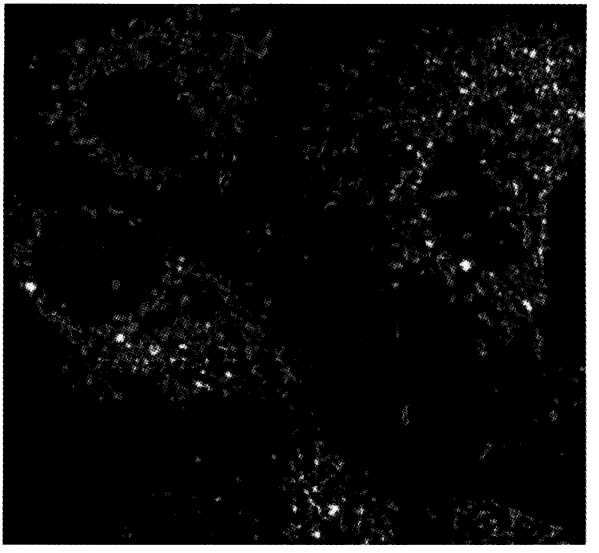

Fig. 5. Mitochondria labeled with rhodamine 123. Attached viable endothelial cells were loaded with 5 μM rhodamine 123 and imaged using a Bio-Rad MRC 1024 confocal microscope.

is that the oxidation product, rhodamine 123, remains essentially within the cell, unlike the oxidation product DCF, which has a strong tendency to leak from cells and requires careful controls to monitor leakage.

A number of publications have made direct comparisons between DCFH-DA and DHR 123 (Smith and Weidemann, 1993; Vowells et al., 1995). In summary, the advantages of using the DHR 123 probe for cellular H_2O_2 production are based upon its increased sensitivity to H_2O_2 (3-to IO-fold) and its general failure to leak from the cells.

Hydroethidine. Hydroethidine (HE) has been proposed by Rothe and Valet (1990) as a probe for measurement of O_2^-. The dye enters cells freely and is dehydrogenated to ethidium bromide (Fig. 6). A major advantage of this probe is its ability to distinguish between O_2^- and H_2O_2. As shown in Figure 7, the specificity of HE for O_2^- is significant. Studies have been performed using neutrophils (Robinson et al., 1994a; Kuypers et al., 1990; Rothe et al., 1991; Rothe and Valet, 1990) and endothelial cells (Carter et al., 1994b), as well as HL60 cells (Robinson et al., 1994b) and macrophages (Kobzik et al., 1990a, b). The probe has been used extensively with NK cell assays (Radcliff et al., 1991; Callewaert et al., 1991; Zanyk et al., 1990; Cavarec et al., 1990) and as a vital dye for identification of proliferation (Saiki et al., 1986; Bucana et al., 1986) and hypoxic cells in tumors (Olive, 1989). Fluorescence emission occurs at around 600 nm.

Parinaric Acid. The parinaric acids are the closest structural analogues of intrinsic membrane lipids among current fluorescent probes (Haugland, 1992). cis-Parinaric

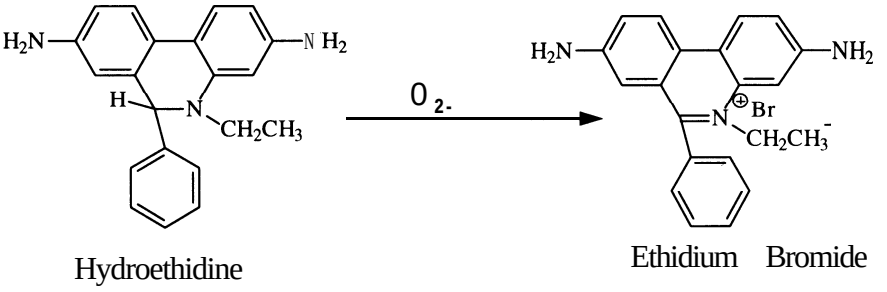

Fig. 6. Hydroethidine enters cells freely and is dehydrogenated by superoxide anion directly to ethidium bromide. Fluorescence emission occurs around 600 nm.

acid (Fig. 8), a naturally fluorescent fatty acid, loses its fluorescence over time when the four conjugated double bonds of the backbone chain become the target of lipid peroxidation reactions (Hedley and Chow, 1992). Thanks to its extensive unsaturation, parinaric acid is quite susceptible to oxidation. It is this property that is utilized in measuring lipid peroxidation. Because a 325-nm ultraviolet excitation source is required, the use of parinaric acid is restricted to spectrofluorometry and flow cytometers with helium-cadmium lasers.

Monobromobimane. Monobromobimane (Fig. 9) and monoclorobimane can be used for the determination of glutathione levels within single cells (Hedley and Chow, 1994). Although monochlorobimane is the most specific GSH probe available be-

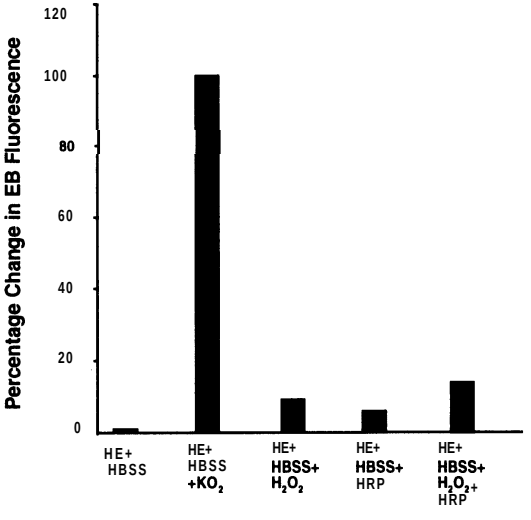

Fig. 7. Cell-free fluorescence generation using hydroethidine. Potassium superoxide oxidizes hydroethidine to ethidium bromide more efficiently than H_2O_2, as shown.

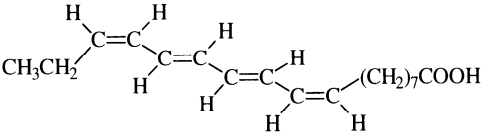

Fig. 8. Parinaric acid is a naturally fluorescent molecule whose fluorescence decreases as the conjugated double bonds are broken via oxidation.This reduction in fluorescence can be monitored kinetically using a He-Cd laser on a flow cytometer with excitation at 325 nm and emission at 420 nm.

cause its binding is catalyzed by glutathione S-transferase, human isoenzymes of glutathione S-transferase have an unacceptably low affinity for monochlorobimane at attainable physiological concentrations (Ublacker et al., 1991). For human cells (particularly neutrophils or monocytes), the most satisfactory of the probes capable of forming fluorescent adducts with GSH via the sulfhydryl group is monobromobimane. Initial trial of this probe as a tool for flow cytometry showed unacceptably high backgrounds; however, Hedley and Chow reexamined the probe and determined optimal conditions for use with human cells and flow cytometry (Hedley and Chow, 1994).

Differences between Monocytes and Neutrophils

Opinions differ as to whether neutrophils or monocytes are the more reactive. Several factors influence the result, not the least of which is the method used for measuring the particular species. For instance, measurements of external O_2^- are unlikely to provide accurate estimates of O_2^- remaining within the cell. Several early reports indicated that zymosan-stimulated neutrophils produced several times as much reactive species as monocytes based upon oxygen consumption (monocytes consuming around 40% as much as neutrophils) and H_2O_2 production (around 20%) (Roos et al., 1979; Reiss and Roos, 1978). It has been demonstrated that peripheral blood monocytes are far less reactive to soluble activation than neutrophils (Robinson et al., 1988). However, when these monocytes are able to bind to particulates such as bacteria, a significant amount of H_2O_2 is measurable. More recent work has shown that monocytes activated by an identical concentration of PMA not only produce less

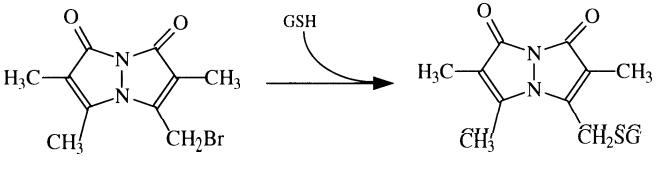

Fig. 9. Monobromobimane combines nonenzymatically with glutathione at low concentrations and is converted from a nonfluorescent molecule to a fluorescent one when so bound. The optimal excitation for flow cytometry is the 350-360 nm UV beam from an argon laser, although the 325-nm line from a helium-cadmium laser will suffice. Emission is at 420-450 nm. A maximum of 10 min incubation is needed for cell staining.

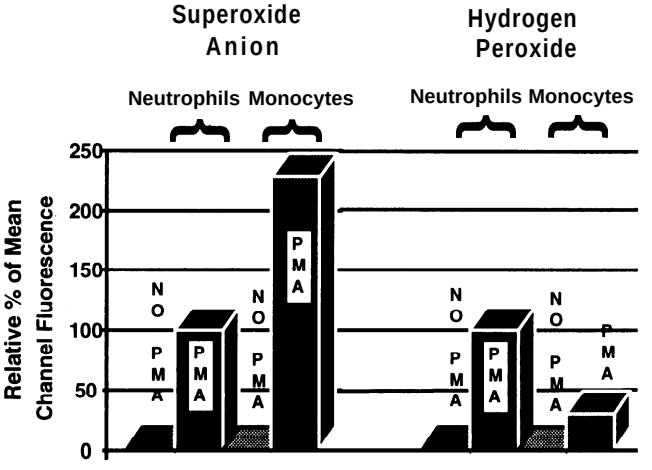

Fig. 10. Comparison between human neutrophils and monocytes of O_2^- and H_2O_2 capacity after stimulation with 10 ng/ml PMA. Data are normalized to set neutrophil production at 100%. Monocytes produce less H_2O_2 but more O_2^- than neutrophils.

H_2O_2 than neutrophils (about a third as much), but also can produce substantially ***more*** O_2^- as measured intracellularly with the hydroethidine probe (unpublished observation). These data are shown in Figure 10, normalized so that the neutrophil O_2^- or H_2O_2 production is set at 100%.

Oxygen as a Defense Mechanism-Antioxidant Systems

Peroxidases. Peroxidases remove H_2O_2 via peroxidatic mechanisms (glutathione peroxidase is an important example); a number of peroxidases are found in phagocytic cells. In the absence of oxidants, the activity of peroxidases as such is poor. The better known peroxidases within the phagocytic system are myeloperoxidase (which preferentially reacts with Cl-) in neutrophils and eosinophil peroxidase (which preferentially reacts with Br^-) in eosinophils.

SOD. Superoxide dismutases are a group of metalloproteins/metalloenzymes present in all respiring cells [including mammals, plants, fungi, and bacteria with the exception of some obligate anaerobes (McCord et al., 1971)] that catalyze the dismutation of O_2^- to H_2O_2.

There are three isoenzymes of superoxide dismutase (SOD) in mammals, the first of which was discovered by McCord and Fridovich in 1969 (McCord and Fridovich, 1969a). This CuZn SOD was isolated from cytoplasm, nucleus, and peroxisomes. The second, MnSOD, discovered by Weisiger and Fridovich in 1973 (Weisiger and Fridovich, 1973), is a cytoplasmically synthesized enzyme directed primarily toward the mitochondria. The third isoform, EC-SOD (extracellular), was discovered by Marklund (Marklund, 1982) and is a CuZn SOD with a positively charged binding

domain optimized for localization in the extracellular matrix. This isoenzyme has been shown to have particularly high expression in vascular tissue (Oury et al., 1994) and umbilical cord tissue (Sandstrom et al., 1993).

The structure of CuZn SOD in bovine erythrocytes has been determined as a homodimer of 16 kDa with the active site located within a cylinder β structure (Richardson et al., 1975), where it is well protected and is known to retain catalytic activity during isolation procedures (Forman and Fridovich, 1973).

The mechanism of action of SOD is that the copper ion at the active site is reduced by one O_2^- molecule, then reoxidized by another in a continuing cycle (Fridovich, 1981). Thus, copper oscillates between the monovalent and divalent states. Copper-containing SOD is inhibitable by cyanide but the Mn form is not (Haffner and Coleman, 1973). Azide (Misra and Fridovich, 1978) and diethyldithiocarbamate (Heikkila and Cohen, 1977) (which removes the Cu) are also able to inactivate SOD activity in cell preparations, thus rendering the cells susceptible to autodestruction. Recently Naranayan et al. (1998) showed that environmental pollutants such as PCBs reduce the effectiveness of antioxidant systems in human neutrophils, possibly by inactivating SOD activity. If this mechanism is confirmed, it represents the possibility that chronic low levels of antioxidants may be capable of causing more damage than previously understood, because depletion of SOD and/or glutathione may be responsible for a subsequent increase in apoptosis.

Catalase. Catalase is a heme protein present mostly in peroxisomes that rapidly removes H_2O_2 and reduces it to H_2O and 0; via the general reaction sequence

$$H_2O_2 + ROOH \longrightarrow H_2O + ROH + 0,$$

where R is a short chain alkyl group. The rate of H_2O_2 removal via catalase is 10^8 times faster than the dismutation of H_2O_2 to water and oxygen (Forman and Fisher, 1981), meaning that it is virtually impossible to saturate catalase activity under normal biological conditions. Phagocytic cells contain catalase, which can scavenge not only H_2O_2 produced within the neutrophil but also H_2O_2 added exogenously to cell preparations, because H_2O_2 can freely move across the cell membrane (Voetman and Roos, 1980). Being a large molecule, catalase obviously cannot penetrate the cell membrane and its use as an antioxidant in experimental situations is restricted to extracellular locations.

Glutathione. Glutathione (GSH) is a tripeptide present in most cells that acts as a general antioxidant for the removal of H_2O_2 via the general reaction

$$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$$

Glutathione is generally found in the reduced form GSH as shown in Figure 1 above and its presence in cells affords considerable antioxidant protection. In contrast to catalase, which is located primarily in peroxisomes, glutathione in the cell is ubiquitous. The role of the glutathione cycle has been described in neutrophils (Ohno and

Gallin, 1985) and neutrophil apoptosis (Robinson and Narayanan, 1996), endothelial cells (Andreoli et al., 1986; Tsan et al., 1985), hepatocytes (Keller et al., 1985), platelets (Freedman et al., 1996), and tumor cells (Goossens et al., 1995). Evidence that nitric oxide may play a regulatory role in neutrophils (and perhaps other cells) has recently been demonstrated (Forslund and Sundqvist, 1995a; Nikitovic and Holmgren, 1996; Clancy et al., 1994).

Ascorbic Acid. Although not synthesized in most cells, ascorbic acid can be present in reasonably large quantities in some tissues, where it can act as an antioxidant via the following general reaction:

$$2O_2 + 2H^+ \xrightarrow{\text{ascorbic acid}} 2H_2O_2 + \text{dehydroascorbate}$$

It should be noted that this reaction is not preferred over the dismutation of O_2^- by SOD; however, in some tissues there may be sufficient ascorbate to afford reasonable antioxidant activity.

Vitamin E. Vitamin E is generally accepted to act as an antioxidant via nonenzymatic reduction of polyunsaturated lipid oxide free radicals in the general reaction

$$\text{vitamin E} + RO\cdot \longrightarrow \text{vitamin E}\cdot + ROH$$

The vitamin E· radical must be further reduced by other reaction sequences, possibly via its interaction with ascorbic acid (vitamin C). Intracellular vitamin C can restore the vitamin E radical to reduced vitamin E, thereby limiting lipid peroxidation (Forman and Fisher, 1981).

Polyunsaturated Fats. It is possible that polyunsaturated fats acting as a trap may preferentially reduce certain ROI, preventing further damage to DNA or organelles and subsequently more serious damage to the cell. The reactions may produce conjugated dienes and other molecules that can themselves cause damage under certain conditions. The general reaction sequence for lipid peroxidation would be

$$RH + O_2^- + OH\cdot \xrightarrow{+\,H^+} ROOH + H_2O$$

It has been suggested that O_2^- in particular can cause a chain reaction whereby further lipid peroxidation takes place (Thomas et al., 1978).

Oxygen as a Pathogenic Molecule

Superoxide is produced in significant quantities in many cells; as previously stated, from 1 to 4% of all oxygen consumed in the mitochondrial electron transport chain results in production of O_2^- (Cross, 1987). As discussed above, activated phagocytes can produce a variety of oxidants that may be found both within the phagocytic cells and released into the extracellular milieu. After neutrophils have been recruited to ap-

proach, roll, and attach to the vascular endothelium, they transmigrate to the source of inflammation. Once at the site, they usually become further activated, producing additional oxidants. Any neutrophil breakdown releases large quantities of enzymes, with the potential for considerable tissue damage. There are several clear-cut situations in which activated neutrophils cause classic tissue damage, predominantly through the production of reactive oxygen species.

One such case is that of complement-mediated lung injury; invasive neutrophils have been shown to be present in high numbers where severe tissue damage occurs (Till et al., 1982). Similar damage has been observed in a model of immune complex damage to the lung. In this model, a large neutrophil infiltrate subsequent to intratracheal instillation of IgG antibody to bovine serum albumin is thought to be the cause of significant tissue injury (Johnson and Ward, 1974). In terms of free radical generation, it has been shown that alveolar macrophages from patients with asthma have a higher rate of production of superoxide (Jarjour and Calhoun, 1994).

NITROGEN-RELATED METABOLISM

The discovery of nitric oxide is attributed to Joseph Priestley, a clergyman and part-time chemist, who called his find "nitrous air." When exposed to iron, this gas was converted to nitrous oxide (laughing gas, reviewed by Gilbert, 198 1). The year of this discovery was 1772, but more than 200 years were to pass before the realization that nitric oxide has a crucial role to play in physiological systems.

The chemistry of nitrogen monoxide (NO) has been elucidated over the past several years. The most studied molecule has been the radical nitric oxide ($NO\cdot$); however, there are several redox states of NO, including nitrosonium cation (NO+), nitric oxide ($NO\cdot$), and nitroxyl anion (NO-), not dissimilar to the well-known states of oxygen (dioxygen) ($O_,$), namely, superoxide (O_2^-) and hydrogen peroxide (H_2O_2). $NO\cdot$ itself has the lowest molecular weight of any known mammalian secretory product (Nathan, 1992). The seminal discovery that L-arginine was converted to nitric oxide by macrophages and that this was involved in tumoricidal activity (Hibbs et al., 1987) created an entirely new area of research. Literally tens of thousands of reports have surfaced in the past 10 years associating nitrogen-related metabolites with what was previously an exclusive domain of oxygen metabolism.

Production and Properties of Nitric Oxide

Production. Nitric oxide is produced by oxidation of one of the terminal nitrogens on arginine via nitric oxide synthase, producing *N*-hydroxyarginine, then citrulline, and finally nitric oxide. Each molecule of nitric oxide produced requires one arginine, two oxygens, and 1.5 NADPHs, involving an overall reduction of five electrons.

There are three distinct isoforms of the synthase enzyme, two of which have been termed constitutive: one from endothelial cells (ECnos-Type III) and one from neuronal cells (NCnos-Type I) (Bredt et al., 1991). The constitutive form (*c*NOS) is Ca^{2+}-dependent, appears to be mediated by calmodulin (Pollock et al., 199 1), requires

the presence of co-factors such as tetrahydrobiopterin for activity (Förstermann et al., 1991), and is believed to be located on the cytoplasmic face of the cell membrane (Knowles and Moncada, 1992). The third isoform, calcium-independent and inducible (*i*NOS—Type II), is tightly bound to calmodulin (Nathan and Xie, 1994) and is found in most cells in the body (Stuehr and Griffith, 1992; Nussler and Billiar, 1993).

Nitric oxide ($NO\cdot$) is formed through a five-electron oxidation of L-arginine via the action of nitric oxide synthase, NADPH, and tetrahydrobiopterin (Stuehr and Griffith, 1992), which proceeds from the hydroxylation of L-arginine to form N^{ω}-hydroxy-L-arginine through subsequent hydrolysis to L-citrulline and hydroxylamine with further oxidation to nitric oxide:

$$\text{L-arginine} + \text{NADPH} \xrightarrow{\text{NOS}} N^{\omega}\text{-OH-L-arginine} \xrightarrow{\text{NOS}} \text{L-citrulline}$$

$$+ \text{NH}_2\text{OH} + \text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} \text{NO}\cdot + 2\text{H}_2\text{O} + \text{H}+$$

$$\text{L-Arg} + 30, + 1.5\ \text{NADPH} + \text{H}_4\text{-biopterin} \longrightarrow \text{NO}\cdot + \text{L-citrulline}$$

$$+ 1.5\ \text{NADP}^+ + \text{H}_2\text{-biopterin} + 2\text{H}_2\text{O} + \text{H}_2\text{O}_2$$

Hydroxylamine is oxidized by a catalase-like activity to form $NO\cdot$, utilizing H_2O_2 as a substrate (Stuehr and Griffith, 1992). It is known that $NO\cdot$ reacts rapidly with both oxyhemoglobin and deoxyhemoglobin with a final product of nitrate (Wennmalm et al., 1992):

$$\text{NO}\cdot + \text{O}_2\text{Hb} \longrightarrow \text{NO}, + \text{Hb}^+$$

As discussed below, the reaction of $NO\cdot$ with superoxide produces nitrates by way of the peroxynitrite free radical intermediate (Knowles and Moncada, 1992):

$$\text{NO-} + \text{O}_2^- \longrightarrow \text{ONOO}^- \longrightarrow \text{NO},$$

or, in the absence of superoxide and hemoglobin at pH 7.4, $NO\cdot$ can utilize other reactive species of oxygen such as singlet oxygen (Knowles and Moncada, 1992):

$$2\text{NO}\cdot + {}^1\text{O}_2 \longrightarrow \text{N}_2\text{O}_4 \longrightarrow \text{NO}, + \text{NO}_3^-$$

Thus it is highly likely that $NO\cdot$ and O_2^- are very closely interactive during the inflammatory response and that the modulation of O_2^- by $NO\cdot$ is most likely an important mechanism for regulation of ROI production.

Physiological Properties. At standard temperature and pressure, pure nitric oxide gas is approximately 40 m*M*, whereas a saturated aqueous solution is 1.9 m*M;* however, physiological levels of $NO\cdot$ range from 10 n*M* (necessary to cause vasodilation) to as high as l-10 μ*M* around activated macrophages (Beckman, 1995). Ni-

tric oxides have potentially extensive half-lives in tissue, as long as 17 h for a 10 nM concentration and around 11 min for a 1 μM concentration of NO· (Beckman and Tsai, 1994).

One of the earlier effects observed for NO· was that it prevented neutrophil aggregation (Kubes et al., 1991; McCall et al., 1988). Thus nitric oxide could regulate the buildup of inflammatory cells and reduce potential tissue damage. The mechanism of leukocyte recruitment is well understood. Leukocyte adhesion to endothelial cells is primarily mediated by the β-integrin glycoprotein complex on neutrophils (Tonnesen, 1989). NO· plays a role in P-selectin—dependent leukocyte rolling, for N^{ω}-nitro-L-arginine methyl ester pretreatment of rats with L-NAME caused increased leukocyte rolling (Davenpeck et al., 1994). It is known that once an inflammatory state is signaled (via a chemoattractant, for instance), neutrophils slow down and gently adhere to the wall of the blood vessel via L-selectin. Because the adherence is of low affinity, the effect is a rolling along the endothelial layer. The L-selectin (on neutrophils) binding via E(ELAM-1)- and P(CD62)-selectins (on endothelial cells) is essentially intermittent until the neutrophil approaches sufficiently close to the source of the inflammatory mediator. At this point, the neutrophil slows and then adheres to the endothelial surface via adhesion glycoproteins (CD 1 l/CD 18) on neutrophils and their ligands (ICAM- 1) on endothelial cells. Now the neutrophil migrates through the microvessel wall, a process that takes several minutes. Thus the regulatory role that NO· appears to play in expression of P-selectin is physiologically very significant, with the implication that NO· acts as a homeostatic regulator of neutrophil-endothelial interactions (Beckman, 1995).

Other physiological effects of nitric oxide on neutrophils include direct inhibition of superoxide production, possibly by direct action on the NADPH oxidase (Clancy et al., 1992), providing additional evidence of protection against oxidative injury. In a similar manner, nitric oxide has been shown to act against mast cells, demonstrating a diminution of mast cell reactivity. It has been shown that exvenous addition of NO· via the NO· producer nitroprusside decreased the amount of histamine released by mast cells (Salvemini et al., 199 1) as well as the amount of platelet activating factor produced by them (Hogaboam et al., 1993).

Peroxynitrite

Peroxynitrite ($ONOO^-$) is formed from a radical-radical reaction between O_2^- and NO· at a diffusion-limited rate (k = 6.7 X $10^9 M^{-1}s^{-1}$) (Huie and Padmaja, 1993). It has been known for many years that superoxide and nitric oxide could combine to form peroxynitrite, as well as the conditions under which this will occur (Blough and Zafiriou, 1985). Making accurate measurements of $ONOO^-$ in biological systems, however, proved more difficult. Beckman et al. (1990) have determined many of the properties of peroxynitrite. The pK_a is 7.49 at 37°C, and once protonated it rapidly decomposes with a half-life of 1.9 s. Macrophages are capable of producing at least lmmol/min of peroxynitrite; and the effects on bacterial survival are not altered by mannitol, ethanol, or benzoate, but are enhanced by dimethyl sulfoxide or deferoxamine (Zhu et al., 1992).

When $ONOO^-$ is protonated, the resultant peroxynitrous acid (ONOOH) decomposes to several toxic species with reactivity similar to hydroxyl radical and nitrogen dioxide (Volk et al., 1995; Radi et al., 1991; Beckman et al., 1990; Ischiropoulos et al., 1992a,b). Peroxynitrite has also been shown to have high bactericidal capacity in macrophages (Zhu et al., 1992; Ischiropoulos et al., 1992a,b) and to replace the active site of CuZn superoxide dismutase, removing its dismutation capacity for superoxide anion by blocking the O_2^- binding site (Beckman et al., 1992; Ischiropoulos et al., 1992a,b). DNA strand breakage (Inoue and Kawanishi, 1995; Szabo et al., 1996) demonstrated in macrophages (Szabo et al., 1996), smooth muscle (McCauley and Hartmann, 1984; Begley et al., 1985), and thymocytes (Salgo et al., 1995) has been attributed to $ONOO^-$. Furthermore, $ONOO^-$ can directly oxidize sulkydryl groups such as cysteine and BSA at physiological pH (7.4) at rates 1000 times faster than H_2O_2 (Radi et al., 1991a).

The situation is further complicated by the fact that transition metals can catalyze $ONOO^-$ to form OH- (hydroxyl anion) plus nitronium ion (NO,+), which can subsequently nitrate protein tyrosine residues to produce 3-nitrotyrosine in a reaction catalyzed by SOD (Beckman et al., 1992), whose normal role is to dismutate O_2^-.

It has been suggested that the toxicity of either the O_2^- or $NO\cdot$ radical is significantly enhanced when the two combine to form $ONOO^-$ (Beckman et al., 1990). Peroxynitrite has been shown to exhibit strong activity with a number of biological molecules, and a substantial amount of evidence demonstrates that $ONOO^-$ is considerably more reactive than $NO\cdot$. The strong oxidizing capability has been shown to act on peroxidation of lipids in the absence of iron (Rubbo et al., 1994; Radi et al., 199 1 a), cause formation of malondialdehyde and conjugated dienes during lipid peroxidation (Radi et al., 199 lb), and also act directly on carbohydrates (Beckman et al., 1990).

Peroxynitrite inhibits pulmonary α1-proteinase inhibitor and therefore oxidizes critical methionine residues in the active site of the enzyme (Moreno and Pryor, 1992). Peroxynitrite is also responsible for the oxidation of arachidonic acid and formation of F2-isoprostanes via oxidation of low-density lipoproteins (Moore et al., 1995) and causes inhibition of mitochondrial respiratory chain enzymes, including cytosolic aconitase (Radi et al., 1994; Hausladen and Fridovich, 1994).

Deferoxamine and glutathione are scavengers of peroxynitrite, whereas H_2O_2 has been shown to prolong its half-life (Alvarez et al., 1995). It is likely that most of the damaging effects against mitochondria result from the activity of peroxynitrite, not $OH\cdot$ or other radicals, for antioxidants against O_2^- or $OH\cdot$ provide little protection.

Under certain conditions the $NO\cdot/O_2^-$ interaction may behave as a significant antioxidant mechanism for the detoxification of O_2^-. What is clear is that the most important criteria for the role of the $NO\cdot$ and O_2^- combination may be the relative fluxes of each molecule and the nature of the tissue in which they are formed.

Fluorescent Indicators of Excited Nitrogen Species

Some confusion exists as to what particular fluorescent probes actually measure. A number of reports in the literature claim to measure one or another oxidant, when in

fact there is no clear evidence as to what molecule is actually being measured. This problem is witnessed by the vast quantity of studies described in the early 1990s purporting to measure nitric oxide, but in reality measuring an accumulation of nitrites. This has led many investigators to the conclusion that $NO\cdot$ was indeed produced and was the key biological species. Many publications have assumed that the species contributing the biological effect was $NO\cdot$ when it could well have been, and most probably was, a combination of excited molecules. Regardless, the conclusion has been drawn on many occasions that $NO\cdot$ was the key to the biological mystery being unraveled. In a similar manner, $NO\cdot$ and $ONOO^-$ measurements using fluorescent probes may have resulted in the same problem of crossreactivity already observed with probes for O_2^- and H_2O_2.

Dihydrorhodamine 123. DHR 123 has recently become a popular probe for measuring peroxynitrite. Haddad et al. (1994) have used DHR 123 to measure peroxynitrite production, claiming that the simultaneous generation of O_2^- and $NO\cdot$ resulted in the oxidation of DHR 123 to rhodamine 123. They claim further that DHR 123 is not oxidized by $NO\cdot$, O_2^-, or H_2O_2 alone (Kim et al., 1996; Haddad et al., 1994). Clearly there are contradictory publications on the nature and use of certain fluorescent probes, and the choice of such probes depends entirely on the biological system in question. Many studies have used DHR 123 as a direct measure of peroxynitrite (Szabo et al., 1995); for cases involving noncellular systems where highly defined reactions can be studied, the efficacy of the probe is acceptable. In one such controlled system the authors concluded that in the presence of oxygen, nitric oxide induces a relatively slow oxidation of dihydrorhodamine owing to the formation of nitrogen dioxide and consequently that dihydrorhodamine was a sensitive and efficient trap for peroxynitrite (Miles et al., 1996; Kooy et al., 1994).

When using these probes for more complex biological systems such as cell suspensions or for in vivo use, care must be exercised in drawing and interpreting conclusions from the data. Experiments such as injecting DHR 123 directly into rats and measuring subsequent plasma levels of rhodamine 123 spectrofluorometrically are not straightforward cases of cause and effect. In that instance, the conclusions that the resulting measurement was a direct measure of peroxynitrite (Szabo et al., 1995) must be interpreted with great caution, and in the case of peroxynitrite measurements, are only relative to NOS-inhibitable changes in fluorescence as discussed by Szabo (Szabo et al., 1995).

Dichlorofluorescin Diacetate. Evidence exists that DCFH-DA under some circumstances may actually measure $NO\cdot$ in addition to H_2O_2. Such evidence includes the observation for human neutrophils that a calmodulin inhibitor (W-13) inhibits O_2^- production as measured by cytochrome c and nitroblue tetrazolium assays but enhances the formation of fluorescent DCF (Rao et al., 1992). Furthermore, this reaction was inhibitable by NMMA (N^{ω}-monomethyl-L-arginine), an L-arginine analogue that inhibits production of $NO\cdot$. Additionally, it was demonstrated that pure $NO\cdot$ gas could directly oxidize DCFH to the fluorescent DCF (Rao et al., 1992).

It has also been observed that DCFH-DA-loaded neutrophils incubated with a

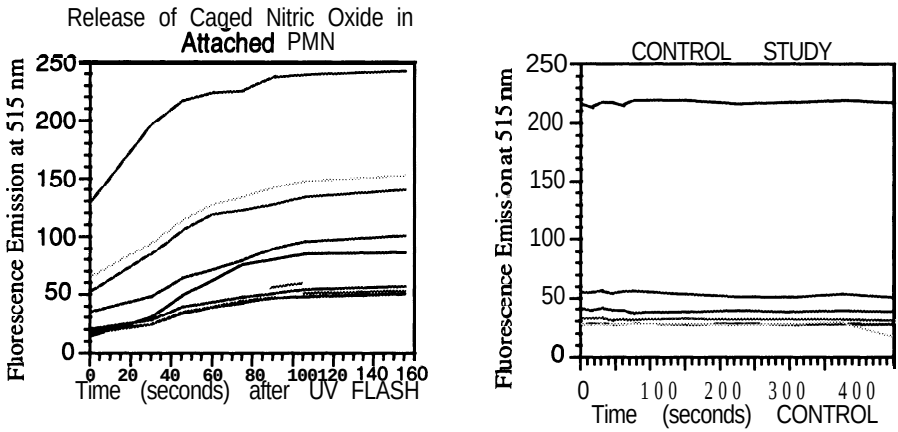

Fig. 11. The change in fluorescence over time after a controlled UV flash (360 nm excitation) on a small population of neutrophils previously loaded with DCFH-DA. Neutrophils attached to coverslips coated with polylysine were loaded with DCFH-DA (20 μM) and incubated at 37°C on the heated stage of a Bio-Rad MRC 1024 confocal microscope for 20 min to hydrolyze the probe. Cells were then incubated with potassium nitrosylpentachlororuthenate (PNPCR, a "caged nitric oxide" compound). Control cells not flashed with UV light but given equal exposure to 488-nm excitation demonstrated no fluorescence shift. Neither did cells incubated with DCFH-DA alone and given the same UV flash.

caged nitric oxide showed an increase in DCF fluorescence after the cage was released by ultraviolet light. In these (unpublished) studies it appears that the DCFH-DA probe was measuring the released NO·, for the cells were otherwise not stimulated to produce reactive oxygen species (Fig. 11). Ultraviolet light alone did not appear to change the fluorescent nature of the DCFH probe.

Nitric Oxide Production in Neutrophils

Although there is no doubt that neutrophils can produce staggering quantities of oxygen radicals, their ability to produce nitric oxide has been disputed. Before the biological properties of nitric oxide had been elucidated, reports of the impact of rat neutrophils on relaxation of smooth muscle (Rimele et al., 1988), elevated cGMP levels (Lee et al., 1988), and platelet antiagreggation factor (McCall et al., 1989) would suggest involvement of NO· based upon present-day knowledge. Subsequently, the "factor" present in these neutrophils was shown to be nitric oxide (Schmidt et al., 1989; Wright et al., 1989). The controversy, however, has continued.

The question as to whether the presence of nitrites indicates intracellular NO· production or results from alternative metabolic pathways has been addressed by a number of groups. Klebanoff and Nathan (1993) measured nitrite production in human neutrophils in the presence of azide and catalase; nitrite production was unaffected by addition of either SOD or monomethylarginine. When stimulated neutrophils were replaced with the H_2O_2-generating system glucose-glucose oxidase, nitrite was also produced, leading these authors to conclude that nitrite production did not reflect nitric oxide synthase activity, but rather the catalase-catalyzed conversion of azide in

the presence of H_2O_2 generated by stimulated neutrophils. Another study by Padgett and Pruett (1995) found that rat, mouse, and human neutrophils were able to produce very small amounts of nitrite, far less than would be required for antimicrobial activity. They conclude that the small amount of NO· produced by these cells may be related to intercellular signaling rather than playing a role as a defense mechanism.

These data are contrasted with a significant literature confirming the presence of both *i*NOS and subsequent production of significant amounts of NO·. Identification of *i*NOS expression has been confirmed in human neutrophils by measuring reverse transcription polymerase chain reaction (RT-PCR) products (Kolls et al., 1994; Cook et al., 1994; Evans et al., 1996). In one neutrophil sudy, PCR (polymerase chain reaction) products to both the endothelial constitutive (756 bp) and neuronal constitutive (629 bp) NOS isoforms were identified (Chen and Mehta, 1996). Confirmation of *i*NOS or NO· production has been by Southern blot (Chen and Mehta, 1996), spectrophotometric measurement of nitric oxide-dependent methemoglobin formation from oxyhemoglobin (Larfars and Gyllenhamrnar, 1995), and antibodies against *i*NOS in rat neutrophils (Clark et al., 1996). Detailed immunohistochemistry with a specific anti-nitrotyrosine antibody showed intense staining in both macrophages and neutrophils in mouse lung (Akaike et al., 1996) and rat macrophages and neutrophils (Goldman et al., 1996), and reports of the conversion of ^{3}H-arginine to ^{3}H-citrulline in human monocytes and neutrophils (Laffi et al., 1995) have been published.

Secondary evidence exists for the effect of NOS inhibitors on neutrophil function. For example, NOS inhibition attenuated chemotaxis of both unstimulated and primed neutrophils, suggesting a role for NO· synthesis in neutrophil emigration (Wildhirt et al., 1995) as well as in monocyte chemotaxis (Belenky et al., 1993), and exogenous NO· has been shown to induce chemotactic locomotion in human neutrophils (Beauvais et al., 1995). Other evidence of NO· production is based upon nitrite production alone (Ahmed and Weidemann, 1996; Dias-Da-Motta et al., 1996; Carreras et al., 1994a; Biswas et al., 1993) and chemiluminescence (Forslund and Sundqvist, 1995a; Catz et al., 1995; Carreras et al., 1994b).

Wright et al. (1989) showed that human neutrophils were able to generate nitric oxide at a rate of 1.8 nmol/2 $\times 10^6$ cells/30 min. Direct evidence of NO· through detection in the gas phase of the specific chemiluminescence resulting from the reaction of nitric oxide with ozone showed categorically that neutrophils produce NO·.

It is becoming increasingly clear that NO·, present either exogenously or intracellularly, exerts serious influence on the physiology of neutrophil function. Production of O_2^- is reduced by the direct effects of extracellular NO· on the neutrophil membrane-bound oxidase (Forslund and Sundqvist, 1995b). Augmentation of bacterial phagocytosis by human neutrophils in the presence of L-arginine (but not D-arginine or glycine) has been demonstrated (Moffat et al., 1996). NO· has been shown to reduce neutrophil adherence (Clancy et al., 1995; Egdell et al., 1994; McCall et al., 1988) and cause depletion in intracellular glutathione (Clancy et al., 1994), but not to affect neutrophil-mediated killing by fMLP-activated rat neutrophils (Wagner et al., 1996). Anesthetics such as lidocaine stimulate NO· production in human neutrophils (Mamiya et al., 1995).

Both O_2^- and H_2O_2 production decrease in neutrophils pre-incubated with L-argi-

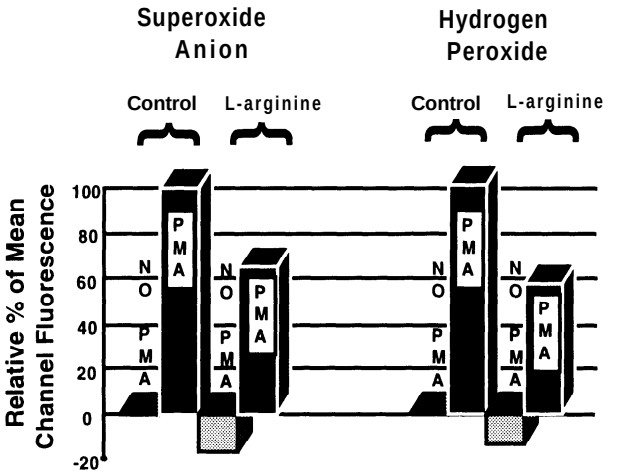

Fig. 12. Relative percent of O_2^- and H_2O_2 in human neutrophils with and without added L-arginine. 2 ng/ml PMA was used for cell stimulation. Both basal and stimulated O_2^- and H_2O_2 levels decreased after preincubation of PMN with 1 m*M* L-arginine.

nine and stimulated with low concentrations of PMA (2 ng/ml) (Fig. 12). In one set of experiments, reductions were 35 and 43% for O_2^- and H_2O_2, respectively. In unstimulated cells, L-arginine also caused a reduction in the basal O_2^-- and H_2O_2-induced fluorescence by approximately 15% each. These data provide evidence that L-arginine can modulate both O_2^- and H_2O_2 production in (human) neutrophils.

The effect of nitric oxide modulators on rat neutrophil O_2^- activity is demonstrated using the fluorescent indicator hydroethidine in Figure 13, which shows the rela-

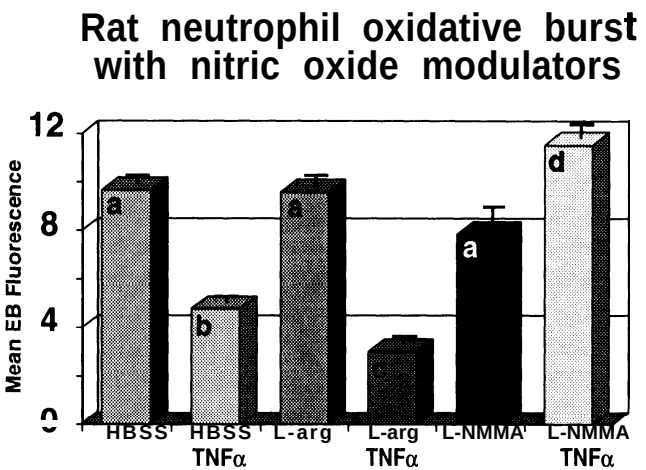

Fig. 13. O_2^- in rat neutrophils measured by flow cytometry after 15 min incubation with 4 ng/ml PMA. NOS inhibitor L-NMMA significantly reduced intracellular O_2^- of neutrophils.

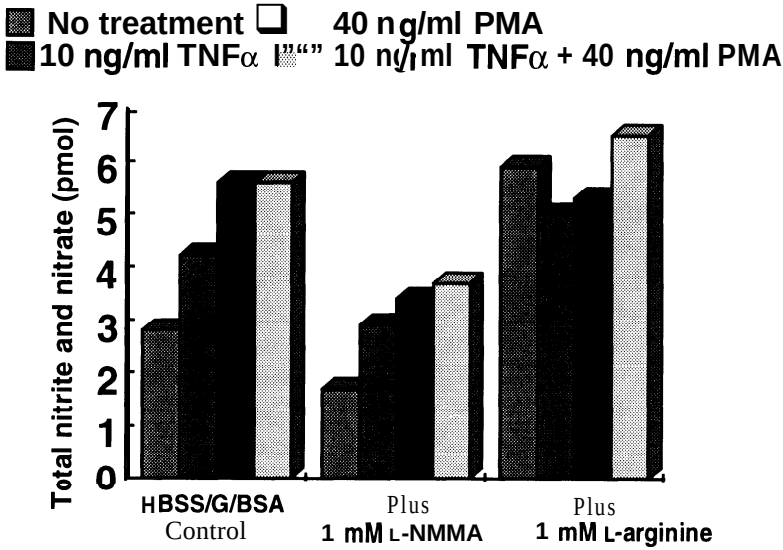

Fig. 14. Total nitrite and nitrate production in NO·-modulated rat peripheral blood neutrophil. Measurements after the treatments shown indicate significant NO· production.

tive change in ethidium bromide (EB) fluorescence (reflecting O_2^- production) in rat neutrophils incubated with L-NMMA (N^ω-monomethyl-L-arginine), an inhibitor of *i*NOS. A low concentration of phorbol myristate acetate (4 ng/ml, approximate V_{max} for the rat oxidative burst) was sufficient to elicit measurable changes in O_2^- production, which was significantly decreased by stimulating NO· production with 1 m*M* L-arginine and TNF-α (10 ng/ml). Upon addition of L-NMMA to inhibit NO· production, O_2^- was significantly increased. Confirmation of these data came from measuring total nitrites and nitrates in the same neutrophils. Figure 14 shows the differences in three groups: control, addition of L-NMMA, and L-arginine. A significant difference was observed between addition of L-NMMA to inhibit *i*NOS and all other treatments. This suggests the production of NO· in significant quantities by neutrophils and supports the hypothesis that the interactions between ROI and RNI play an important role in the activity of both systems.

Nitric Oxide, Peroxynitrite, and Disease

Nitric oxide is a free radical that has recently received considerable attention as an important messenger molecule in the body. In reviewing the physiological roles of the molecule, attention should first be directed to its function as a vasodilator through its production in vascular endothelial cells and action on underlying smooth muscle (Palmer et al., 1987). Confirmation that the effect was mediated by NO· was obtained by utilizing an inhibitor of the synthase enzyme for NO·, namely N^ω-monomethyl-L-arginine (L-NMMA) (Moncada et al., 199 1).

It should be noted that NO·, being a gas, can freely diffuse throughout the cell, and

indeed from one cell to another, unless bound by protein. The molecule can survive for about 10–30 s in typical physiological environments, during which time it has been hypothesized to travel between 200 and 600 μm (based upon diffusion coefficient of oxygen in tissue sections) (Knowles and Moncada, 1992).

The recognition that nitric oxide regulation can affect some disease processes has brought about an interest in the therapeutic use of high L-arginine diets (Becker et al., 1993; Saito et al., 1987; Gianotti et al., 1993) or L-arginine infusion in traumatized patients. Studies in L-arginine–treated rats have demonstrated significant alterations in the translocation of gut bacteria. In one study (Becker et al., 1993), the amount of NO· was substantially increased in L-arginine–fed rats after bum. In another (Gianotti et al., 1993), the authors concluded that one of the more significant effects of L-arginine administration was influence on immune function, most likely the macrophage. Current dogma suggests that increases in intracellular and extracellular nitric oxide may be beneficial to the appropriate microenvironment since the nitric oxide can act as an O_2^- scavenger, essentially being converted to nitrite and nitrate and forestalling the conversion of O_2^- via SOD to H_2O_2 and OH·.

A similar situation might exist in ischemia/reperfusion injury, in which the role of neutrophils has been demonstrated, particularly in remote injury such as seen in the lung (Mulligan et al., 1992). In addition, it has recently been demonstrated that endotoxin can cause a two-to fivefold increase in arginine transport by pulmonary artery endothelial cells (Lind et al., 1993), suggesting that vast amounts of NO· can be made, at least in the lung, which consists of approximately 50% endothelial cells (Crapo et al., 1978), creating significant quantities of potentially dangerous molecules. Administration of a recombinant human TNF soluble receptor type 1 to lipopolysaccharide-treated rats significantly reduced the damaging effects of endotoxin in lung tissue, probably by preventing the upregulation of IL-6 by TNF (Ulich et al., 1993).

Thus we have a curious situation. On the one hand, superoxide and its dismutation products, hydrogen peroxide and subsequent hydroxyl radical, have been considered to be the dangerous species of oxygen-mediated tissue damage. On the other hand, it has been hypothesized that SOD, by removal of superoxide anions, creates a protective barrier by directly preventing the reaction of O_2^- with NO· to produce peroxynitrite (Beckman et al., 1990) in a reaction known to be very rapid with a rate constant of at least $3.7 \times 10^7\ m^{-1}s^{-1}$ (Zhu et al., 1992).

Nitrogen as a Defense Mechanism

The concept that NO· can act as a defense mechanism is based upon the knowledge that NO· can remove superoxide from a system by a diffusion-limited reaction that forms peroxynitrite ($ONOO^-$):

$$O_2^- + NO\cdot \longrightarrow ONOO^-$$

Furthermore, this reaction occurs faster than the dismutation of O_2^- by SOD and therefore is highly likely to take place in biological systems. It is thought that NO· can react with transition metals, especially Fe^{2+} (which may also be the most available met-

al), thus competing with the Haber-Weiss reaction between H_2O_2 and Fe^{2+}. If this is true, NO· may be acting as an antioxidant.

Nitric oxide has been identified as playing a protective role by a number of studies, such as that of Wink et al. (1993), who showed that in the presence of NO·, the cytotoxicity of H_2O_2 and O_2^- against Chinese hamster lung fibroblasts was abrogated in a dose-responsive manner. Nitric oxide has been shown to promote ADP ribosylation of actin, thus inhibiting cytoskeletal assembly in neutrophils. The result is the regulation of neutrophil adhesion in margination, adhesion, and diapedesis (Clancy et al., 1995). Suppression of NO· in arteriolar and venular endothelium resulted in an increase in adhesion of leukocytes, but also a significant increase in oxidative stress. Furthermore, this L-NAME-induced enhancement of endothelial adhesiveness was mediated by intracellular oxidative stress rather than by direct action of NO· suppression (Hausladen and Fridovich, 1994).

Prolonged NOS inhibition in HUVECs caused an oxidative- and platelet activating factor (PAF)-associated rise in adhesion of neutrophils on the surface of endothelial cells (Niu et al., 1994), activated mast cells in the mucosa (Kanwar et al., 1994), and mast cells in rat mesenteric postcapillary venules (Kubes et al., 1993). Inhibition of nitric oxide synthesis increased leukocyte and endothelial interaction in rat mesenteric venules as measured by an increase in P-selectin expression (Davenpeck et al., 1994). Likewise Gauthier et al. (1994) demonstrated a reduction in adhesion and decreased P-selectin expression following infusion of exogenous nitric oxide. Volk et al. (1995) have demonstrated that extracellularly produced H_2O_2, but not O_2^-, enhanced the toxicity of NO· against endothelial cells. Decreased basal release of NO· after myocardial ischemia/reperfusion preceded enhanced neutrophil adherence to the coronary endothelium, leading to neutrophil-induced myocardial injury (Ma et al., 1993).

Nitric oxide overproduction was shown to be a major protective mechanism in the T cell-dependent shock induced by staphylococcal enterotoxin B in mice, and NOS inhibition might have detrimental consequences in T cell-mediated inflammatory disorders by enhancing both production and toxicity of inflammatory cytokines (Florquin et al., 1994). These data indicated that NO· production was able to exert a direct effect on the production of some cytokines (TNF-α and IFN-γ). Additionally, there is a significant amount of evidence that the presence of NO· regulates the adhesion of neutrophils, and in the absence of NO·-producing systems, a potentially damaging accumulation of inflammatory cells is likely.

Nitrogen as a Pathogenic Molecule

Direct tissue damage by NO· has also been demonstrated, but usually at higher than physiological concentrations. Administration of high concentrations of NO· (via nitroprusside, 10–40 μg/kg/min for 15 min) was shown to cause rat mucosal damage, which was attributed to excessive formation of peroxynitrite and subsequent formation of superoxide and hydroxyl radicals (Lamarque and Whittle, 1995).

The primary evidence for a role of nitric oxide in pathogenesis must come from the effect of peroxynitrite, a potent and reactive oxidant (Beckman and Tsai, 1994).

Peroxynitrite has been shown to damage endothelial cells directly (Palmer et al., 1992; Kooy and Royall, 1994; Kooy et al., 1994), and to impair vascular permeability (Villa et al., 1994) and play a role in the pathogenesis of atherogenesis (White et al., 1994).

CONCLUSIONS

It is clear that many of the observations previously considered to be related solely to reactive oxygen species are more likely associated with both ROI and RNI and the interactions between the two. There is a strong body of evidence that peroxynitrite is a mediator of serious consequences, and that NO· plays a powerful role in regulating many of the inflammatory responses, particularly those involving interaction between neutrophils and endothelial cells.

The benefits of using NOS inhibitors or NO· donors are yet to be made clear. If peroxynitrite is a particularly dangerous molecule, then prevention of its formation by NOS inhibitors is logical. However, the consequences of blocking NO· formation are themselves quite serious, leading to increased neutrophil adhesion and simultaneous removal of a significant pathway for elimination of superoxide.

Neutrophils and macrophages are particularly reactive cells. Neutrophils know but the one role, which they play to perfection: they are designed with powerful protective mechanisms for antioxidant defenses. They produce reactive oxygen and nitrogen species as a means of destroying invading microorganisms, while attempting at the same time to stay alive, at least until they have completed their disinfection task. It is during this period—between the attachment phase and destruction of microbes, and their subsequent removal from inflammatory sites-that significant tissue damage can occur. Alternatively, when vast numbers of these cells respond, as in ischemia/reperfusion injury, regulation of their reactive nature is necesasary to prevent rapid and destructive tissue injury. How exactly this regulation can be achieved without further compromising the host is a question still seeking answers.

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