Vol. 175, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1991 Pages 143-151

INHIBITION OF $O_2^{\cdot-}$ GENERATING OXIDASE OF NEUTROPHILS BY IODONIUM BIPHENYL IN A CELL FREE SYSTEM: EFFECT OF THE REDOX STATE OF THE OXIDASE COMPLEX

Jacques DOUSSIERE and Pierre V. VIGNAIS

Laboratoire de Biochimie, Département de Biologie Moléculaire et Structurale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble cedex, France

Received January 11, 1991

Summary. The conditions of inhibition of neutrophil $O_2^{\cdot-}$ generating oxidase by iodonium biphenyl (IBP) were studied. In a cell free system of oxidase activation consisting of neutrophil membranes and cytosol, GTP-γ-S, Mg^{2+} and arachidonic acid, the inhibitory effect of IBP depended on the redox conditions of the medium. Inhibition was observed when the medium was supplemented with dithionite or NADPH. When the cell free system was incubated with IBP in the absence of reducing agents, full oxidase activity was recovered after removal of free IBP by gel filtration. Bovine neutrophil membranes, but not cytosol, contained component(s) sensitive to IBP. Upon treatment of neutrophil membranes by IBP followed by reduction, the spectrum of reduced cytochrome b_{558} was modified, suggesting that cytochrome b_{558} is a target site for IBP.

A number of diaryl iodonium salts, including diphenylene iodonium (DPI), iodonium thiophen and iodonium biphenyl (IPB), have been shown to strongly inhibit mitochondrial respiration (1, 2) and $O_2^{\cdot-}$ production by the oxidase complex of activated neutrophils (3, 4). In the latter case, it has been claimed that a 45 kDa flavoprotein, thought to belong to the oxidase complex, is covalently labeled during reaction of human neutrophil extracts with [^{125}I]DPI (5). The chemical reactivity of iodonium salts is well documented (6). Recently, it has been shown that diaryliodonium salts oxidize Fe^{II} tetraphenyl porphyrin to give the corresponding ferric porphyrin complex (7). In the presence of an excess of reducing agent, σ aryl complexes are formed. Interestingly, cytochrome P450-Fe^{II} in the presence of NADH or sodium dithionite was found to behave as ferroporphyrin does with respect to diaryliodonium salts, and to give rise to a complex of the type

Abbreviations: **IBP,** iodonium biphenyl ; **DPI,** diphenylene iodonium ; **SOD,** superoxide dismutase ; **PBS,** phosphonate buffer saline consisting of 2.7 mM KCl, 136.7 mM NACl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4.

0006-291X/91 $1.50

cytochrome P450-Fe^{III} phenyl absorbing at 480 nm (7). The redox components of the oxidase complex in neutrophils consists of an NADPH dehydrogenase with FAD as prosthetic group and a *b* type cytochrome called cytochrome b_{558} ; during the course of activation, cytosolic proteins are translocated to the membrane-bound complex (for review, see 8). Because of the high reactivity of diaryl iodonium compounds with respect to ferroporphyrin derivatives, it was of interest to determine whether cytochrome b_{558} could be a target site for diaryliodonium compounds. We report here that inhibition of the neutrophil oxidase system by iodonium biphenyl (IBP), C_6H_5-I^+-C_6H_5, depends on treatment of neutrophil membranes by reducing agents, and that the resulting spectral modifications of the membrane redox components are consistent with binding of IBP to cytochrome b_{558}.

MATERIAL AND METHODS

Material. NADPH and GTP-γ-S were purchased from Boehringer ; superoxide dismutase, ferricytochrome *c* (horse heart grade VI), nitroblue tetrazolium and arachidonic acid from Sigma ; iodonium biphenyl and neutral red from Aldrich. Arachidonic acid was dissolved in absolute ethanol and stored at -80°C under N_2 until used. KO_2 (Pierce) was used as a source of $O_2^{\cdot-}$.

Biological preparations. Neutrophils were obtained from bovine blood and suspended in PBS (9). Cytosol and membrane fractions were isolated from homogenates of resting neutrophils as described (10), and used to make up a cell-free system of oxidase activation. In this system, elicitation of oxidase activity was performed in two steps (11). In brief, during the first step 100 µg of membrane protein and 200 µg of cytosol protein were incubated for 10 min at 22°C in 100 µl of PBS with 10 µM GTP-γ-S, 5 mM $MgSO_2$ and an optimal amount of arachidonic acid which was determined as a function of the concentration of membrane and cytosolic proteins (12). The elicited oxidase activity was assayed spectrophotometrically for production of $O_2^{\cdot-}$ either directly, or after filtration-centrifugation on Sephadex G50 column in a 1-ml syringe equilibrated with PBS (13). The photometric cuvette used for the assay contained 2 ml of PBS supplemented with 5 mM $MgCl_2$, 100 µM ferricytochrome *c* and 200 µM NADPH. Reduction of ferricytochrome *c* was followed at 550 nm. The absorption coefficient used was 21.1 $mM^{-1}.cm^{-1}$. After a linear phase of a few min, 50 µg of superoxide dismutase was added, and recording continued for another 3 min. The difference between the two slopes, i.e. the SOD-inhibitable reduction of cytochrome *c*, corresponded to the oxidase activity.

Spectral determinations. Spectra were recorded with a double-beam Perkin Elmer 557 spectrophotometer, at the temperature of liquid nitrogen, with a 2 mm optical path cuvette. Reduction was achieved with a few grains of sodium dithionite (9).

Protein measurement. Protein concentration in cytosol was measured by the method of Bradford (14). Protein of the membrane fraction was determined by the biuret method as previously described (9).

RESULTS AND DISCUSSION

Inhibition of oxidase activity by IBP depends on the reducing power of the medium

In the cell free system of oxidase activation used to test the inhibitory effect of IBP on the elicited oxidase activity, cytochrome *c* was reduced at a rate of 226 nmol/min/mg membrane protein. (Figure 1, Trace 1). Following addition of SOD, a scavenger of $O_2^{\cdot-}$ the rate of reduction of cytochrome *c* was strongly inhibited (1 nmol/min/mg membrane protein), indicating that cytochrome *c* was reduced essentially by $O_2^{\cdot-}$. In the same system, IBP behaved as a powerful inhibitor of cytochrome *c* reduction (Figure 1, Trace 2). Half inhibition required 1.5 μM IBP or 20 nmol IBP/mg membrane protein (Insert Figure 1). The mechanism of inhibition of cytochrome *c* reduction by IBP differed from that caused by SOD, whose effect is to dismutate $O_2^{\cdot-}$ into H_2O_2. In fact, using KO_2 dissolved in dimethyl sulfoxide as a source of $O_2^{\cdot-}$, and nitroblue tetrazolium to measure the concentration of $O_2^{\cdot-}$, no scavenging effect of IBP was detected. A control carried out with SOD in this system showed the strong scavenging effect of this enzyme on $O_2^{\cdot-}$. It was therefore concluded

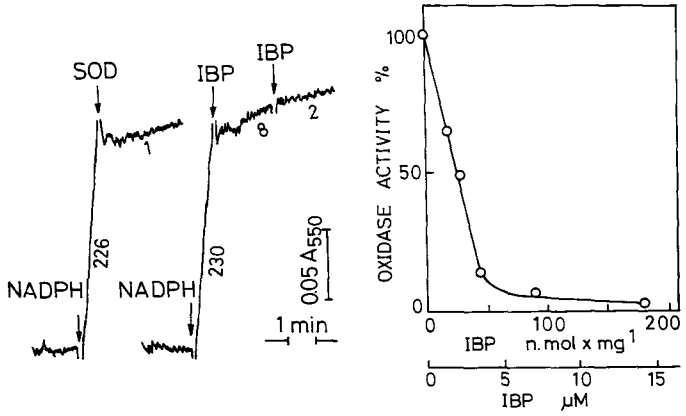

Figure 1. Effect of IBP on the oxidase activity elicited in cell free system. The cell free system for oxidase activation is described in Materials and Methods. The oxidase activity was assayed after transfer of 100 μl of the activated oxidase medium into a photometric cuvette containing 100 μM cytochrome *c* and 5 mM $MgCl_2$ in 2 ml PBS. Reduction of cytochrome *c* was initiated by addition of 100 μM NADPH. **First trace:** After about 1 min of reduction of cytochrome *c*, 50 μg SOD was added. This addition resulted in nearly full inhibition of cytochrome *c* reduction, indicating that cytochrome *c* reduction was caused by $O_2^{\cdot-}$. **Second trace:** The NADPH-dependent reduction of cytochrome *c* by activated oxidase was inhibited with 100 μM IBP. The numbers on the slopes correspond to the rate of reduction of cytochrome *c* in nmol x min^{-1} x mg membrane $protein^{-1}$. On the right hand side is shown the titration curve illustrating the inhibition of oxidase activity by increasing amounts of IBP.

that IBP inhibits the activity of the oxidase complex by interacting with a component of this complex.

In the following experiment, IBP was incubated for 10 min at 22°C in the cell free system of oxidase activation. A control incubation was performed without IBP. Additional incubations were carried out, in which the medium was supplemented with NADPH or sodium dithionite as reducing agents and KO_2 as a generator of $O_2^{\cdot-}$ to test the effect of different redox conditions on the IBP-dependent inhibition. After centrifugation-filtration (cf. Materials and Methods) the different mixtures were assayed for oxidase activity (Table I). It was checked that KO_2 and dithionite have no deleterious effect *per se* on the enzyme. The results show that in the absence of reducing agents, oxidase activity escapes inhibition when free IBP is removed from the incubation medium. In contrast, in the presence of low redox potential agents, inhibition of the oxidase complex by IBP is apparently irreversible, since inhibition is observed even after removal of free IBP from the medium. A plausible hypothesis for the strong inhibitory effect on the generation of $O_2^{\cdot-}$ by the cell-free system under the above mentioned conditions is that the IBP target site is a redox component of the oxidase complex, which is able to bind firmly IBP under its reduced state.

Table I

Effect of the oxidation-reduction state of the cell free system of oxidase activation on the inhibition of oxidase activity by IBP

Additions to the cell free system of oxidase activation	Oxidase activity (nmol $O_2^{\cdot-}$/min/mg membrane prot.)	
none	236	
NADPH	248	
Dithionite	385	
KO_2	177	
IBP	237	(0%)
IBP + NADPH	19	(92%)
IBP + dithionite	71	(82%)
IBP + KO_2	182	(0%)

The cell free system of oxidase activation (see Materials and Methods) consisted of 150 µg of membrane protein, 300 µg of cytosol protein, 5 mM $MgCl_2$, 10 µM GTP-γ-S and 147 nmol arachidonic acid in 100 µl PBS. NADPH, Na dithionite and KO_2 were present at the final concentrations of 200 µM, 2 mM and 1 mM, respectively. IBP was added at the concentration of 200 µM to the cell-free system as indicated, and let to react for 15 min at 22°C. After centrifugation-filtration (cf. Materials and Methods), aliquots of the filtrate corresponding to 100 µg membrane protein were assayed for oxidase activity as described in Materials and Methods. Values in brackets refer to the percentages of oxidase inhibition caused by IBP, determined with respect to the oxidase activity in the absence of IBP.

Localisation of target site(s) for IBP in the membrane fraction

To localize this IBP sensitive component of the activated oxidase complex, membranes and cytosol from bovine neutrophils were treated separately either with dithionite or dithionite plus IBP. After centrifugation-filtration to remove the non-reacted IBP and dithionite, membranes and cytosol were mixed, and the mixture was supplemented with GTP-γ-S, Mg^{2+} and arachidonic acid for oxidase activation. As shown in Table II, incubation of either membranes or cytosol with dithionite in the absence of IBP did not affect the oxidase activity elicited in the cell free system. However, when the membrane fraction treated with both dithionite and IBP was combined after centrifugation-filtration with non-treated cytosol (control cytosol), GTP-γ-S, Mg^{++}, and arachidonic acid, the oxidase activity

Table II

Subcellular localization of the IBP target protein

Combinations	Oxidase activity (nmol $O_2^{\cdot-}$/min/mg membrane protein)	
Control membrane + control cytosol	254	
Dithionite treated membrane + control cytosol	258	
Dithionite and IBP treated membrane + control cytosol	38	(85%)
Control membrane + dithionite treated cytosol	256	
Control membrane + dithionite and IBP treated cytosol	240	
Dithionite and IBP treated membrane + dithionite and IBP treated cytosol	42	(84%)
NADPH and IBP treated membrane + control cytosol	194	(24%)
Control membranes + NADPH and IBP treated cytosol	260	

A 100 µl sample of cytosol (4.7 mg protein) was filtered on Sephadex G50 as described in Materials and Methods (control cytosol). Another sample of cytosol (200 µl) was reduced with a few grains of dithionite and divided in two fractions of 100 µl. To one fraction, IBP was added at the final concentration of 200 µM and let for 10 min at 22°C. The other fraction was incubated without IBP. The two fractions were filtered on Sephadex G50. They corresponded to fractions denoted dithionite and IBP treated cytosol and dithionite treated cytosol, respectively. Membrane aliquots of 100 µl (1.1 mg protein) and 200 µl (2.2 mg protein) were treated similarly to cytosol. The cytosolic and membrane fractions recovered from gel filtration were combined as indicated in the Table, using for each assay 100 µg membrane protein and 200 µg cytosolic protein. The different mixtures were supplemented with 10 µM GTP-γ-S, 5 mM $MgCl_2$ and 120 nmol arachidonic acid for oxidase activation. The same experiment was performed with NADPH as reducing agent. After 10 min at 22°C, the elicited oxidase was assayed as described. Numbers in brackets are percentages of inhibition of the elicited oxidase activity with respect to that determined with control membrane plus control cytosol (line 1).

was inhibited. Substitution of dithionite by NADPH also resulted in stable inhibition of oxidase by IBP, although to a lesser extent (24 % *vs* 85%). This is probably due to the fact that in the absence of cytosol the oxidase complex in the membrane fraction is not activated, and that the electron flux is not high enough to allow complete reduction of the IBP-sensitive component. When the reverse combination was used, i.e. cytosol treated by dithionite (or NADPH) plus IBP combined with non-treated membranes (control membranes), followed by addition of GTP-γ-S, Mg^{++} and arachidonic acid, no inhibition of oxidase activity ensued. This result clearly shows that the IBP sensitive redox target is located in the membrane fraction.

Spectral modification of reduced cytochrome b_{558} by IBP

The absorption spectra of the redox components of bovine neutrophil membranes shown in Figure 2 were taken at 77°K. The difference spectrum (dithionite reduced minus oxidized) (Trace A) showed peaks at 635 nm and 468 nm which belongs to myeloperoxidase.

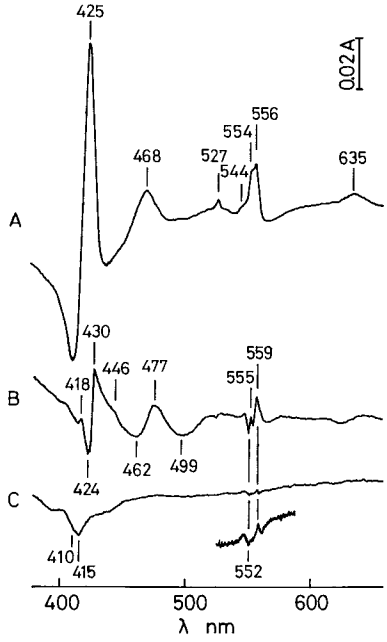

Figure 2. **Spectral modifications of bovine neutrophil membranes incubated with IBP.** Conditions are described in Materials and Methods. The neutrophil membranes in PBS were used at the concentration of 7 mg/ml. They were treated with Na dithionite and IBP as indicated. The spectra were recorded at 77°K.
Trace **(A)**: Dithionite treated membranes minus oxidized membranes ;
Trace **(B)**: Difference spectrum (Dithionite, IBP minus dithionite) ;
Trace **(C)**: Difference spectrum (oxidized, IBP minus oxidized).

Reduced cytochrome *b* could be identified by the two α bands at 556 nm and 554 nm, the β band at 527 nm and the γ band at 425 nm.

The effect of IBP on the spectrum of dithionite-treated membranes was explored with a concentration of IBP of 70 nmol/mg membrane protein, resulting in about 90% inhibition of oxidase activity. Immediately after addition of IBP, a transient increase in the peak at 425 nm was observed, together with a decrease in the peak at 556 nm. Then the peak at 425 nm was red-shifted by a few nm. In the stabilized spectrum, recorded after 15 min, the peaks of both reduced cytochrome *b* and myeloperoxidase underwent shifts of a few nm which resulted in the difference spectrum (reduced, IBP minus reduced) (Figure 2, Trace B) in the occurrence of throughs at 424 nm and 552 nm and peaks at 430 and 559 nm for cytochrome *b*, and throughs at 462 nm and 499 nm and peak at 477 nm for myeloperoxidase. When addition of IBP preceded treatment of neutrophil membranes by dithionite, the same difference spectrum was obtained, but the transient modification of cytochrome *b* mentioned above was not observed. The difference spectrum (IBP, oxidized minus oxidized) (Trace C) shows that IBP *per se* was able to induce a significant change in the γ band of the oxidized cytochrome *b*, but was ineffective on oxidized myeloperoxidase. However, these changes were small in size and they differ from those revealed after addition of dithionite. These results, and those reported by Battioni et al. (7) in the case of cytochrome P450, indicate that IBP is a general reagent for reduced hemoproteins.

Absence of effect of IBP on the diaphorase activity of the cell free system of $O_2^{\cdot-}$ generation

As recently shown (15) the intrinsic diaphorase activity of the $O_2^{\cdot-}$ generating oxidase can be unmasked in a cell free system through the use of low molecular weight electron acceptors with low mid-point potential. In the experiment illustrated in Table III, bovine neutrophil membranes were tested in the absence and presence of IBP for their capacity to reduce cytochrome *c* and neutral red, two redox components differing widely in their mid-point redox potential (+0.22 V and -0.32 V, respectively) and their molecular masses. The rates of reduction of cytochrome *c* and neutral red catalyzed by the activated membranes, i.e. membranes preincubated with cytosol, GTP-γ-S, Mg^{2+} and arachidonic acid were similar. They, however, differ by their sensitivity to IBP, the reduction of neutral red being only partly sensitive to IBP in contrast with that

Table III

Effect of the nature of the electron acceptor on the extent of inhibition caused by IBP

Electron acceptor	Conditions	Electron transfer (nmol/min/mg prot.)
Cytochrome *c*	Activated membrane	255
	Activated membrane + IBP	26
	Resting membranes	22
	Resting membranes + IBP	22
Neutral red	Activated membranes	292
	Activated membranes + IBP	243
	Resting membranes	102
	Resting membranes + IBP	102

Neutrophil membranes in PBS were supplemented with cytosol, GTP-γ-S, $MgCl_2$ and arachidonic acid for oxidase activation as described in Materials and Methods (activated membranes). Membranes in the absence of cytosol, GTP-γ-S, $MgCl_2$ and arachidonic acid are termed resting membranes. The oxidase was assayed at 22°C in PBS pH 7.8 in the presence of 200 μM NADPH, 5 mM $MgCl_2$ and either 100 μM cytochrome *c* (E_o' + 0.22 V) or 50 μM neutral red (E'_o - 0.32 V). Rates of reduction in the absence or presence of 100 μM IBP were calculated, using ε_{550nm} values of 21.1 $mM^{-1}.cm^{-1}$ for reduced cytochrome *c* and 10 $mM^{-1}.cm^{-1}$ at 459 nm for neutral red.

of cytochrome *c* which was nearly totally inhibited (Table III, lines 1, 2, 5 and 6). This suggested that neutral red might be reduced by direct electron transfer from NADPH via the dehydrogenase component of the oxidase complex, corresponding to a diaphorase activity. In the absence of cytosol, GTP-γ-S and arachidonic acid, neutrophil membranes (resting membranes) exhibited a 10-fold lower electron transfer activity from NADPH to cytochrome *c* than in the full system, and cytochrome *c* reduction was insensitive to IBP (Table III, lines 3 and 4). Reduction of neutral red by resting membranes was 4 to 5-fold faster than that of cytochrome *c*, and insensitive to IBP (Table III, lines 7 and 8), corroborating the idea of a by-pass of electrons at the level of the NADPH dehydrogenase component of the oxidase complex. An interesting result concerns the 3-fold enhancement of the electron transfer activity with neutral red as electron acceptor during the transition from resting membranes to activated membranes. It is noteworthy that, in the absence of the oxidase system, the rate of spontaneous reduction of neutral red by NADPH was very low (less than 5% compared to that measured in the presence of the oxidase). Two conclusions can be drawn from the results summarized in Table III.

1. Although the 3-fold increase in the rate of reduction of neutral red by a diaphorase activity during the transition from resting

membranes to activated membranes is modest, compared to the 10-fold increase of oxidase activity detected by cytochrome *c* reduction, it indicates that the NADPH dehydrogenase component of the oxidase complex exerts a significant control over the respiratory burst activity of neutrophils. 2. Whereas IBP strongly inhibits the SOD-sensitive reduction of cytochrome *c*, i.e. $O_2^{\cdot-}$ production, it hardly affects neutral red reduction, which suggests that the component of the oxidase complex which transfers electrons to neutral red is not a strategic site of action for IBP.

In conclusion, the results reported here demonstrate that IBP inhibits the bovine neutrophil oxidase in the presence of reducing agents by combining with membrane-bound redox component(s) of the oxidase complex, a likely candidate being cytochrome b_{558}. They also corroborate the recent finding that the NADPH dehydrogenase component of the oxidase complex plays a central role in the regulation of the electron flux from NADPH to O_2 during oxidase activation (15).

Acknowledgements. We thank J. Bournet for excellent secretarial assistance. This work was supported by grants from the Centre National de la Recherche Scientifique (Unité de Recherche Associée 1130), the Institut National pour la Santé et la Recherche Médicale and the Faculté de Medicine, Université Joseph Fourier de Grenoble.

REFERENCES

1. Gatley, S. J. and Sherratt, H. S. A. (1976) Biochem. J. **158**, 307-315.
2. Ragan, C. A. and Bloxham, D. P. (1977) Biochem. J. **162**, 605-615.
3. Cross, A. R. and Jones, O. T. G. (1986) Biochem. J. **237**, 111-116.
4. Cross, A. R. (1987) Biochem. Pharmacol. **36**, 489-493.
5. Yea, C. M., Cross, A. R. and Jones, O. T. G. (1990) Biochem. J. **265**, 95-100.
6. Banks, D. F. (1966) Chem. Revs **66**, 243-266.
7. Battioni, J.-P., Dupré, D., Delaforge, M., Jaouen, M. and Mansuy, D. (1988) J. Organometallic Chem. **358**, 389-400.
8. Segal, A. W. (1989) Biochem. Soc. Trans. **17**, 427-434.
9. Morel, F., Doussière, J., Stasia, M.-J. and Vignais, P. V. (1985) Eur. J. Biochem. **152**, 669-679.
10. Doussière, J. and Vignais, P. V. (1985) Biochemistry **24**, 7231-7239.
11. Ligeti, E., Doussière, J. and Vignais, P. V. (1988) Biochemistry **27**, 193-200.
12. Pilloud, M.-C., Doussière, J. and Vignais, P. V. (1989) Biochem. Biophys. Res. Commun. **159**, 783-790.
13. Penefsky, H. S. (1977) J. Biol. Chem. **252**, 2891-2899.
14. Bradford, M. M. (1976) Anal. Biochem. **72**, 248-254.
15. Laporte, F., Doussière, J. and Vignais, P. V. (1990) Eur. J. Biochem. **194**, 301-308.