

Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase

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Diphenyleiodonium (DPI) and its analogues have been previously shown to react via a radical mechanism whereby an electron is abstracted from a nucleophile to form a radical, which then adds back to the nucleophile to form covalent adducts [Banks (1966) *Chem. Rev.* **66**, 243–266]. We propose that the inhibition of neutrophil NADPH oxidase by DPI occurs via a similar mechanism. A reduced redox centre in the oxidase could serve as electron donor to DPI, and inhibition would occur after direct phenylation of the redox cofactor, or of adjacent amino acid groups by the DPI radical. In the absence of an activatory stimulus, human neutrophil NADPH-oxidase was not inhibited by DPI. The K_i for time-dependent inhibition by DPI of human neutrophil membrane NADPH oxidase was found to be 5.6 μM .

Inhibitory potency of DPI was shown to be directly related to rate of enzyme turnover, indicating the need for a reduced redox centre. Adducts were formed between photoreduced flavin (FAD or FMN) and inhibitor (DPI or diphenyliodonium). These were separated by h.p.l.c. and characterized by absorbance spectroscopy, $^1\text{H-n.m.r.}$ and fast-atom-bombardment m.s. and found to have properties consistent with substituted 4a,5-dihydroflavins. After incubation of pig neutrophil membranes with DPI, the quantity of recoverable intact flavin was greatly diminished when NADPH was present to initiate oxidase turnover, indicating that the flavin may be the site of DPI activation. These results may provide a common mechanism of action for iodonium compounds as inhibitors of other flavoenzymes.

INTRODUCTION

Reactive oxygen species such as superoxide, H_2O_2 and hydroxyl radicals formed by neutrophils play a primary role in host defence against disease (Babior, 1978). They are also implicated in the pathogenesis of many disease states, e.g. rheumatoid arthritis, ischaemia–reperfusion injury and acute respiratory distress syndrome (Halliwell, 1987). Therefore there is great interest in the development of methods for control of their production.

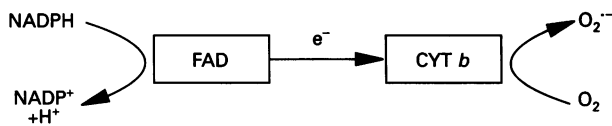
The enzyme responsible for neutrophil-derived oxygen-radical production is a membrane-bound NADPH oxidase which catalyses the one-electron reduction of oxygen to superoxide. Other reactive species are formed as a result of dismutation and by reactions of other enzymes, such as myeloperoxidase. Neutrophil NADPH oxidase is an enzyme complex with FAD and low-potential cytochrome *b* as redox centres, possibly contained on one protein subunit (Segal et al., 1992), along with other membrane-bound and cytosolic proteins (Smith and Curnutte, 1991). Electrons are donated from NADPH to the cytochrome via FAD and ultimately reduce oxygen to form superoxide (Cross & Jones, 1991), as shown in Scheme 1.

In the present paper we describe investigations on the mode of action of a group of iodonium compounds which directly inhibit NADPH oxidase activity, leading to a time-dependent inhibition of superoxide production (Cross and Jones, 1986). Iodonium compounds inhibit the activity of a variety of flavoproteins and at least one haem protein. Diphenyleiodonium (DPI) causes time-dependent inhibition of mitochondrial NADH dehydrogenase (Ragan and Bloxham, 1977), whereas its analogue diphenyliodonium (IDP) has been reported to inhibit NO synthase of macrophages (Stuehr et al., 1991), bacterial nicotine oxidase (Brandsch and Bichler, 1987), xanthine oxidase (V. B. O'Donnell,

unpublished work; Doussiere and Vignais, 1992) and cytochrome *P*-450 reductase (D. G. Tew, unpublished work). Battioni et al., (1988) showed that IDP can interact with reduced cytochrome *P*-450, leading to spectral changes strongly suggestive of covalent modification of the haem prosthetic group.

Iodonium halides are potent arylating agents (they mediate addition of phenyl rings), undergo thermal and photochemical cleavage under relatively mild conditions and react readily with organometallic compounds (Banks, 1966). Arylation of nucleophiles by iodonium compounds has been proposed (Banks, 1966) to occur by a radical mechanism, whereby the inhibitor abstracts an electron from the nucleophile to form a radical which then can add on to the donor molecule forming phenylated products. As shown in Figure 1, radical formation from IDP is associated with a splitting of the molecule to give a phenyl radical and the loss of iodobenzene in solution. In contrast, due to the presence of an extra cross-bridge, DPI forms an intact radical (Berlinger et al., 1958). Previous studies in this laboratory have shown that DPI inhibits NADPH oxidase of both pig (Cross and Jones, 1986; Cross, 1987) and human (Ellis et al., 1988) neutrophils and rat peritoneal macrophages (Hancock and Jones, 1987). Cross and Jones (1986) showed that when DPI was added to NADPH oxidase during turnover there was a loss of reduction of both flavin and cytochrome *b*, accompanied by labelling by DPI of a 45 kDa protein. This suggested that the flavin was the site of attack and that 45 kDa protein was associated with FAD.

In this paper we describe an investigation of the interactions of both DPI and IDP with free flavins in aqueous solution, showing the production of covalent adducts. The formation of these adducts was shown to be dependent on the presence of reduced flavin. We describe kinetics of inhibition of NADPH/oxidase by DPI which indicate the mechanism of inhibition to be similar to the interactions observed with free flavins. We also show that DPI



Scheme 1 Suggested arrangement of electron carriers in neutrophil NADPH oxidase

Abbreviation: CYT, cytochrome.

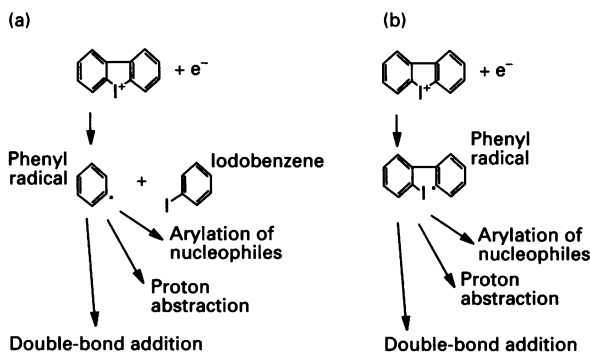


Figure 1 Reaction pathways of (a) IDP and (b) DPI

treatment of NADPH oxidase during turnover leads to loss of recoverable FAD. Following this study on the reactivity of iodonium compounds towards free flavins and NADPH oxidase, we postulate a mechanism of action whereby the inhibitor abstracts an electron from the reduced redox centre to form a radical, which then adds back directly on to the prosthetic group or adjacent protein groups in or near the active site to form adducts.

METHODS AND MATERIALS

Materials

DPI sulphate was a gift from Mr. T. Reid of the Department of Biochemistry, University of Bristol. It was dissolved in dimethyl sulphoxide to give a 10 mM stock solution. [^{125}I]DPI was synthesized as described by Gatley and Sherratt (1976). Na- ^{125}I was obtained from Amersham International, Amersham, Bucks., U.K. IDP was obtained from Fluka Chemica A.G., Buchs, Switzerland. FAD and FMN were obtained from Sigma Chemical Co., St. Louis, MO 63178, U.S.A.

Preparation of neutrophils and activated neutrophil membranes

Human neutrophils were isolated from buffy coats obtained from the South West Regional Blood Transfusion Service, Southmead Hospital, as described by Segal and Jones (1979). Pig neutrophils were isolated from whole pig blood obtained from the local abattoir, as described by Cross et al. (1984). Activated membranes from phorbol myristate acetate (PMA)-treated neutrophils (1 $\mu\text{g}/\text{ml}$) were prepared by nitrogen cavitation and differential centrifugation and were resuspended in phosphate-buffered saline (PBS) (137 mM NaCl/2.68 mM KCl/8.1 mM $\text{Na}_2\text{HPO}_4/1.47$ mM KH_2PO_4) before assay (Cross et al., 1985). Although human neutrophil membranes were used for most experiments, it was necessary to use pig neutrophil membranes where greater enzyme concentrations were needed.

Determination of superoxide production

NADPH-dependent superoxide production was determined as the superoxide dismutase-sensitive rate of reduction of cytochrome *c*, as previously described by Cross et al. (1982). The incubation mixture for assay of isolated membranes consisted of 50 μg of membrane protein, 100 μM horse heart cytochrome *c* (Sigma type III) and 500 μM NADPH in 1 ml of PBS, pH 7.4, at 20 $^\circ\text{C}$. Whole neutrophil superoxide production was determined after addition of PMA to the assay cuvette, which contained 4.8×10^6 cells and 100 μM cytochrome *c* in 1 ml of Krebs-Ringer buffer, at 37 $^\circ\text{C}$ (Cross et al., 1984). Superoxide dismutase (375 units) was added at end of the assay to establish the superoxide-dependent rate of cytochrome *c* reduction. The kinetics of inhibition of human neutrophil membrane NADPH oxidase were determined by using Enzfitter (R. J. Leatherbarrow, Elsevier-Biosoft, 1987). DPI (1–25 μM) was present in the assay mixture before activity was initiated by the addition of activated membranes.

The effects of DPI on NADPH oxidase in the absence of enzyme turnover were investigated by preincubating whole neutrophils in DPI (1–10 μM) in the absence of PMA (37 $^\circ\text{C}$, 5 min). Neutrophil NADPH oxidase activity was assayed after diluting the suspension by adding 20 μl of incubation mixture to 1 ml of 100 μM cytochrome *c* in Krebs-Ringer buffer, pH 7.4 (Cross et al., 1984). The cells were activated by using PMA.

Labelling of proteins with [^{125}I]DPI

PMA-activated human neutrophil membranes were solubilized as described previously (Cross et al., 1985). Samples (40 μl ; 120 μg of protein) of solubilized enzyme were incubated for 5 min at 37 $^\circ\text{C}$ or 4 $^\circ\text{C}$ respectively in the presence of [^{125}I]DPI with or without activation of NADPH oxidase turnover (2 mM NADPH). After incubation, 10 mM unlabelled DPI was added to block protein reaction with radioactive DPI during the procedures for SDS/PAGE.

SDS/PAGE

Samples were solubilized in SDS buffer and separated by electrophoresis on 12% (w/v)-polyacrylamide slab gels in the Laemmli (1970) system of buffers. Gels were fixed in 20% (v/v) methanol/5% (v/v) glycerol for 30 min and dried at 80 $^\circ\text{C}$ for 2 h. Dried gels were autoradiographed against flashed Fuji-Rx film for 1 week at -80°C . After autoradiography the gels were rehydrated and stained with Coomassie Brilliant Blue.

Anaerobic photoreduction of flavins

FAD (85 μM) in PBS, pH 7.4 at 20 $^\circ\text{C}$, was deoxygenated in a stoppered 3 ml cuvette by flushing with oxygen-free argon for 15 min before and during assay. EDTA and IDP were similarly deoxygenated before use (Merkel and Nickerson, 1954). Additions to the anaerobic cuvette were made with a gas-tight SGE syringe which had been flushed with argon before use, to maintain anaerobiosis. FAD (85 μM) reduction to approx. 10% was achieved by addition of EDTA (1 mM final concn.) to the cuvette and subsequent illumination by a 1.0 kW tungsten-filament lamp for 10 s. The cuvette was placed in a water bath during illumination to prevent heating. Reduction of flavin was monitored by scanning between 300 and 500 nm and using $\Delta\epsilon_{450-500} = 11.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. Maximum reoxygenation was mediated by flushing the cuvette with air. Percentage reoxidation was calculated as percentage of reduced FAD recovered on reoxygenation.

Determination of protein content

Concentration of protein was determined by the method of Bradford (1976).

Determination of flavin and cytochrome *b* content of NADPH oxidase preparations

Flavins were assayed by oxidized-minus-reduced difference spectroscopy, by using $\Delta A_{450-500} = 11.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$. Cytochrome *b*₋₂₄₅ was determined from reduced-minus-oxidized difference spectra by using $\Delta A_{559-540} = 21.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Cross et al., 1982).

H.p.l.c. of flavin adducts

Flavin adducts were separated on either a C₈ (Beckman; 4.6 mm × 15 cm) or a C₁₈ (Hichrom; 5 mm × 20 cm) h.p.l.c. column using a water (Milli-Q) to methanol (h.p.l.c. grade) gradient (0–100%) over 20 min (flow rate 1 ml/min) with 20 mM sodium acetate (AnalaR) as buffer and ion-pairing agent; 1 ml fractions were collected for analysis. The h.p.l.c. system consisted of an LDC Gradient Master and Constametric pumps, a 250 μl Rheodyne injection loop, Cecil Instruments u.v.-detection monitor and LKB 1 channel recorder. Absorbance was monitored at 225 nm. Samples were then pooled and freeze-dried before being reconstituted in Milli-Q water for removal of sodium acetate by h.p.l.c. (conditions as before but without sodium acetate). Fast-atom-bombardment spectroscopy (f.a.b.-m.s.) was kindly performed by Dr. N. Haskins at SmithKline Beecham, The Frythe, Welwyn Garden City, Herts., U.K. ¹H-n.m.r. (500 MHz) was performed in ²H₂O (reference H₂O) by Dr. G. Bloomberg and Dr. M. Murray at the Molecular Recognition Centre, University of Bristol, Bristol BS8 1TD, U.K.

Extraction of flavin from pig neutrophil membranes

Flavins were extracted by a modification of the method of Faeder and Siegel (1973). Briefly, samples of pig neutrophil membrane isolated as described above (1.45 mg of protein in 0.5 ml of PBS, in duplicate) were incubated for 30 min at 20 °C with or without NADPH (900 μM) and DPI (50 μM or 500 μM). They were then boiled in foil-covered glass tubes for 10 min. An equal volume of 0.3 M HClO₄ was added, and samples were centrifuged at 200 000 *g* for 2 h to remove precipitated protein. After this, the concentration of HClO₄ was raised to 1 M by the addition of 118 μl of 7.2 M HClO₄ stock to the supernatants, and the samples were boiled for 10 min to facilitate conversion of FAD into FMN (Chappelle and Picciolo, 1971). The pH of the supernatant was raised to 7.7 by addition of small volumes of NaOH (10 M). Fluorescence spectra were recorded with a Perkin-Elmer LS5B scanning fluorescence spectrophotometer. Emission was monitored at 525 nm with excitation at 450 nm. Control samples of FMN (40 μM) were treated similarly in order to detect any degradation of flavin by this method.

RESULTS

Preincubation experiments

In whole resting neutrophils, preincubation with DPI had almost no inhibitory effect on subsequent PMA-stimulated activity. Figure 2 shows percentage of initial activity remaining after

whole neutrophils were preincubated for 5 min at 37 °C with various concentrations of DPI or buffer. DPI was diluted out before PMA stimulation of the cells. The strong inhibitory effect of DPI added after PMA stimulation is shown.

Inhibition kinetics

At saturating [NADPH], the inhibition of NADPH oxidase in isolated human neutrophil membranes by DPI is clearly both time-dependent and concentration-dependent (Figure 3) and closely resembles the inhibition of mitochondrial NADH-ubiquinone reductase by DPI described by Ragan and Bloxham (1977). For an irreversible enzyme-inactivation process, the rate of enzyme inhibition (*k*) can be shown to be given by

$$k = -dE/dt + e^{-kt}$$

where *E* is active enzyme concentration. Thus for a reaction

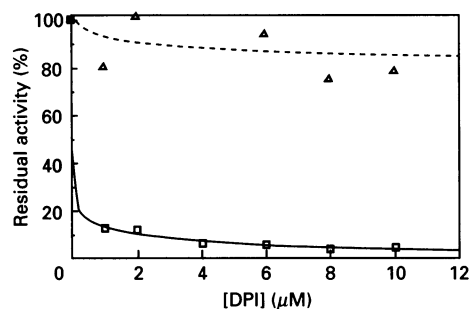


Figure 2 DPI inhibition of NADPH oxidase is less effective when oxidase is not turning over

DPI was added to PMA-activated whole neutrophils (4.8×10^6 cells in 1 ml of cytochrome *c* in Krebs buffer) at 37 °C (—), or DPI was added for 5 min (4.8×10^6 cells in 20 μl of Krebs buffer) and then diluted out before PMA activation and assay as described in the Materials and methods section (----). Superoxide generation was determined as described in the Methods and materials section.

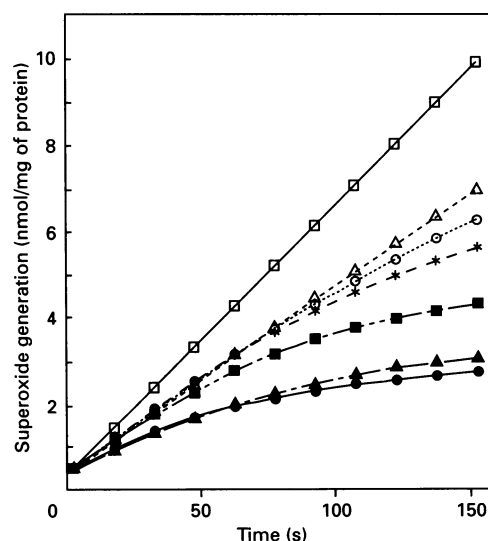


Figure 3 Inhibition of NADPH oxidase activity by DPI

Superoxide generation by disrupted human neutrophil membranes was measured as described in the Methods and materials section in the absence (\square) and presence of DPI: \triangle , 0.5 μM ; \circ , 1.0 μM ; $*$, 5 μM ; \blacksquare , 10 μM ; \blacktriangle , 20 μM ; \bullet , 25 μM . To each assay 50 μg of membrane protein was added (pH 7.4, 20 °C).

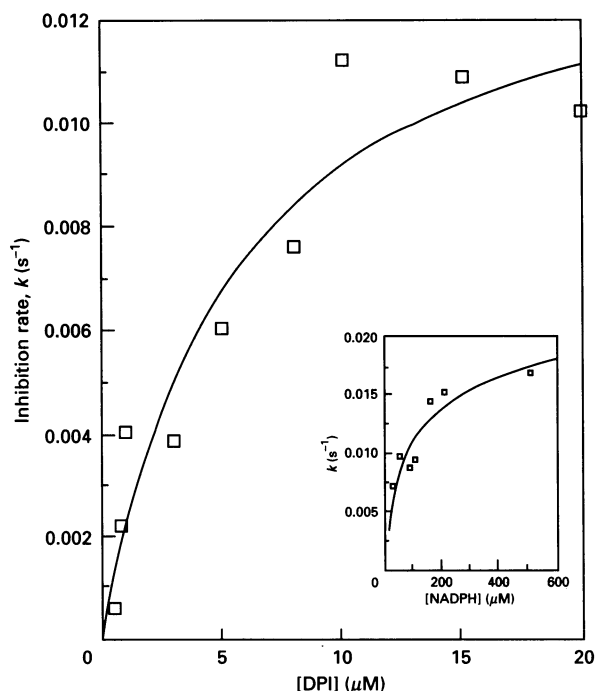


Figure 4 Inhibition of NADPH oxidase by DPI: calculation of K_i

Inhibition rate (k) is derived from the rate of decrease in superoxide production after addition of enzyme (6.2 μg of membrane protein) to the assay mixture. DPI was added to the assay before enzyme. Assay was performed at pH 7.4, 20 °C. $k = k_{\text{cat}}[I]/(K_i + [I])$. See the Results section for more details. Insert: effect of [NADPH] on inhibition of NADPH oxidase by DPI. Inhibition rate was determined as described for the main Figure. [DPI] was kept constant at K_i (5.6 μM), whereas [NADPH] was varied around K_m . Note: maximum inhibition rate was double that observed when [NADPH] = 50 μM (K_m). See the text for details.

proceeding with an initial rate A , it can be seen that the rate (v) at any time (t) is given by:

$$v = Ae^{-kt}$$

Integrating with respect to t , between $t = 0$ and $t = \infty$ gives the total turnover of substrate (V), as:

$$V = A(1 - e^{-kt})/k$$

By fitting the observed absorbance-versus-time traces to this equation (Figure 3), values of k can be derived. Replotting k against DPI concentration (Figure 4) shows that, at high concentrations of DPI, the inactivation rate k approaches a maximum value, k_{cat} (= 0.014 s^{-1}). This indicates that an enzyme-inhibitor complex is formed before the inactivation process, since a simple one-step inactivation process would give a linear k -versus- I (inhibitor concn.) plot. The plot of k versus I may also be used to give a value of the apparent K_i for DPI by using the equation (Kitz and Wilson, 1962):

$$k = k_{\text{cat}} \cdot I / (K_i + I)$$

This gives a value of 5.6 μM for K_i .

On increasing the NADPH concentration at constant inhibitor concentration (5.6 μM), the rate of inactivation increases to a maximum value (Figure 4, insert). This shows that DPI is an uncompetitive inhibitor with respect to NADPH. Because NADPH oxidase is a membrane-bound enzyme, it is not possible to vary the concentration of other components in the reaction (e.g. cytochrome b). Hence a full kinetic analysis of the enzyme is not possible. However, the fact that increasing NADPH concentration causes k to increase (as opposed to either decreasing

or remaining unchanged) can only be accounted for if DPI is, at least qualitatively, uncompetitive with respect to NADPH. This strongly suggests that DPI reacts with reduced NADPH oxidase only. This result was in contrast with those previously reported (Cross and Jones, 1986), where a less direct assay was used, involving the reactions of labelled DPI and oxidase, followed by SDS/PAGE (see below).

Reactions of flavin and iodonium compounds monitored by anaerobic spectroscopy

Where large quantities of inhibitor were necessary, IDP was used, as this is commercially available. Key experiments, such as isolation of adducts, absorbance spectroscopy and mass spectrometry, were repeated with DPI.

The effect of IDP on photoreduction and reoxidation of free flavins in buffered aqueous solution (pH 7.4) was investigated. Flavins can be specifically photoreduced by using EDTA as electron donor; light excites the flavin to a triplet state, which can abstract an electron from EDTA (Heelis, 1990). Reoxidation can then be achieved by aeration of the anaerobic cuvette. When examining reoxidation of photoreduced FAD, IDP was added after illumination to ensure that direct reduction of the inhibitor was not taking place. Dithionite was not used as flavin reductant, since this might directly reduce IDP, allowing phenyl-radical formation without the need for reduced flavin as electron donor.

IDP (50 μM) had no effect on the extent or rate of photoreduction of FAD (100 μM); however, a significant decrease in recovery of oxidized FAD on reoxidation was noted (Figure 5). At a ratio of IDP:FAD of 1:1, only 62% of the FAD was recovered on reoxidation, indicating that some reduced FAD had been modified by reaction with IDP.

Detection of flavin adducts by h.p.l.c.

To elucidate whether low recovery of FAD was due to covalent adduct formation, a sample of reaction products was examined by h.p.l.c. EDTA, FAD and IDP were injected separately to standardize retention times. Separation into expected peaks was found on injection of mixtures of EDTA with either FAD or IDP, or FAD and IDP together. Retention times for EDTA, FAD and IDP were 2, 10 and 17 min respectively.

To form adducts with IDP, FAD (5 mM) was first reduced with light and EDTA (10 mM). An equal volume of IDP (10 mM) was then added and allowed to react at 20 °C before samples were removed for analysis by h.p.l.c. An additional peak with a retention time of 15.5 min was observed, indicating the formation of a novel product. This fraction was collected and its u.v. absorbance spectrum recorded. In order to collect enough product for structural analysis, dithionite was used as reductant in place of EDTA and light, so that complete flavin reduction could be achieved. As found for photoreduced FAD, the new peak appeared only in the presence of reductant. Its retention time and u.v. spectrum were identical with those observed on photoreduction, and it was assumed to be the same derivative. Samples were desalted as described in the Methods and materials section, and chromatograms from the desalting gradient showed the product to be present as a single peak, with u.v. spectrum as before.

Reaction of dithionite-reduced flavin with IDP appeared rapid and complete, since no increase in yield of the suggested adduct was observed if samples were allowed to stand before h.p.l.c. separation. With FAD:IDP at 1:2, approx. 56% of the FAD was modified, as estimated from the chromatogram peak height. However, when DPI was substituted for IDP, with FAD:DPI at

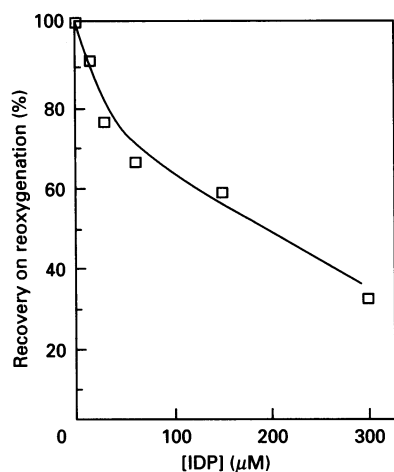


Figure 5 Effect of IDP on recovery of oxidized FAD after EDTA-mediated photoreduction

FAD (85 μM) was photoreduced by EDTA (1 mM) in a 3 ml anaerobic cuvette. Degassed buffer (control) or IDP (to 300 μM) was then added. Reoxidation was mediated by flushing the cuvette with air. Oxidation state was monitored spectrophotometrically at 450–500 nm. Percentage reoxidation refers to proportion of FADH₂ which was recoverable as reoxidized after aeration.

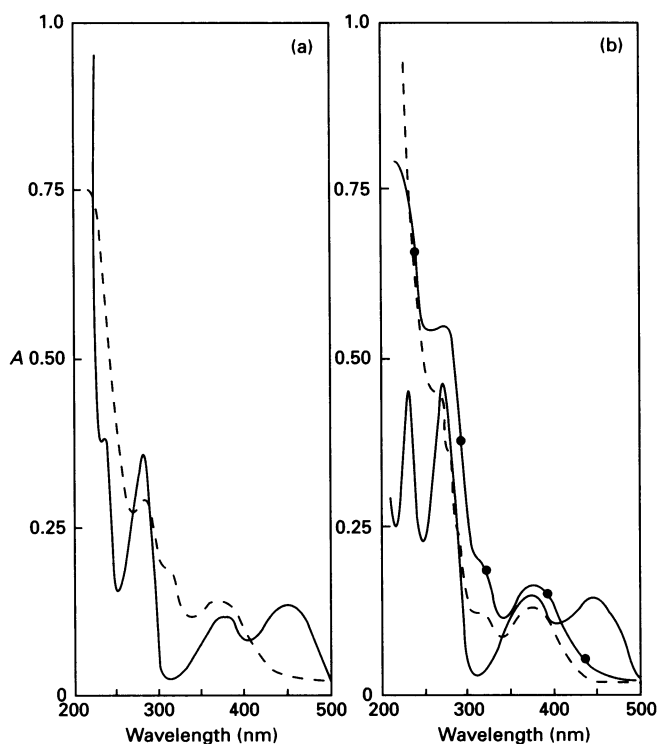


Figure 6 Absorbance spectroscopy of flavins and h.p.l.c.-purified flavin-inhibitor adducts

(a) —, Free FMN; ----, FMN-IDP adduct. (b) —, Free FAD; ----, FAD-IDP adduct; —●—, FAD-DPI adduct.

1:2, more than 90% of FAD was modified after reduction. Similar results were obtained when dithionite-reduced FMN was substituted for FAD. Figure 6 shows the absorbance spectra of the h.p.l.c.-purified adducts of dithionite-reduced FMN or FAD with either DPI or IDP. Spectra of FAD and FMN are provided

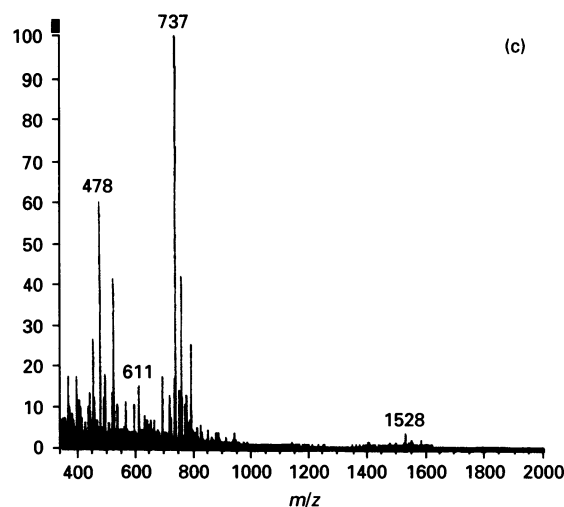
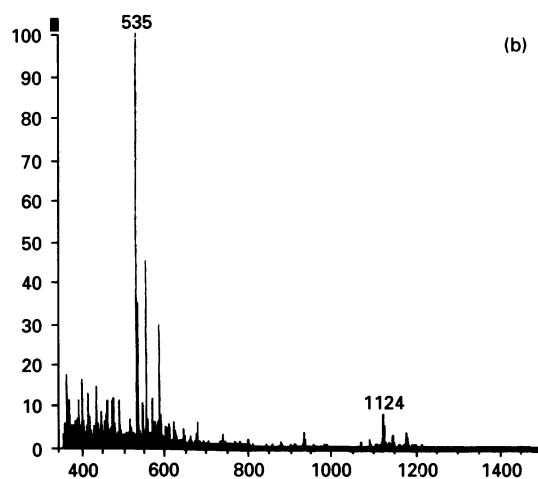
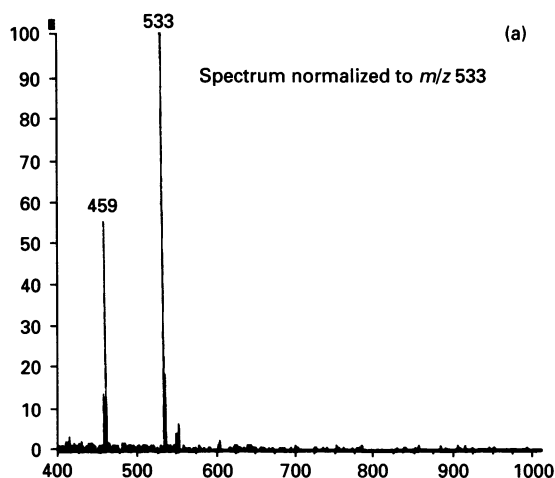


Figure 7 F.a.b.-m.s. of h.p.l.c.-purified flavin-inhibitor adducts

(a) Spectrum of FMN-IDP adduct (negative-ion mode); molecular ion at m/z 534. (b) Spectrum of FAD-IDP adduct (positive-ion mode); molecular ion at 1123. (c) Spectrum of FAD-DPI adduct (positive-ion mode); molecular ion at 1527.

for comparison. The loss of the 450 nm peak on adduct formation is indicative of a reduced flavin chromophore, quite similar to those described by Ghisla et al. (1974) and Schonbrunn et al. (1976) for C4a- and N5-substituted dihydroflavins and also that

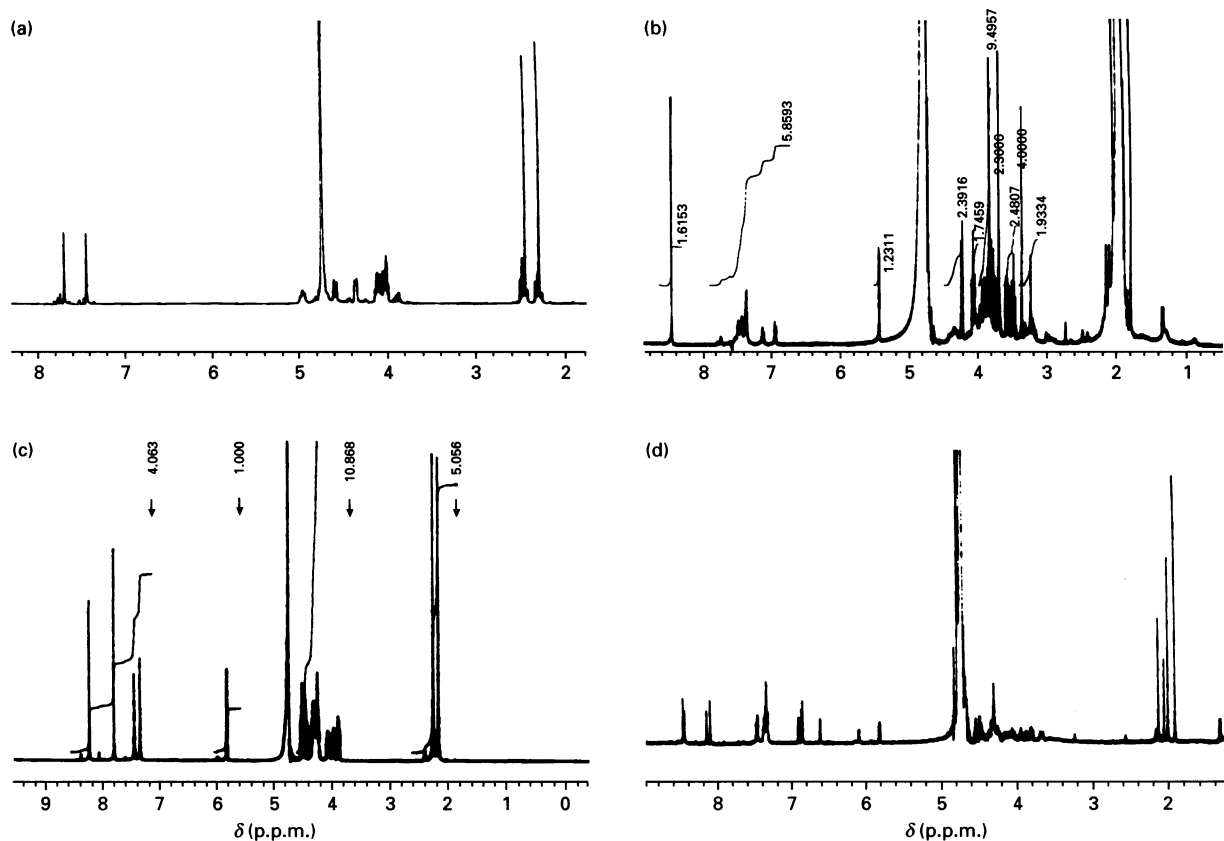


Figure 8 ^1H -n.m.r. spectroscopy of flavins and h.p.l.c.-purified flavin-inhibitor adducts

(a) Free FMN; (b) FMN-IDP adduct (note: sodium acetate at 2 p.p.m.); (c) free FAD; (d) FAD-IDP adduct.

shown by Kemal and Bruce (1976) for C4a substitution. This may possibly indicate C4a and/or N5 to be the sites of attack on adduct formation.

Approx. 3–8 mg of each adduct shown in Figure 6 was isolated for further analysis. All were non-fluorescent and on freeze-drying appeared pale yellow, indicating that a bleaching of colour of the native flavins had occurred.

Mass spectrometry of the flavin adducts

Molecular masses of the h.p.l.c.-purified adducts of reduced flavins with IDP and DPI were determined by f.a.b.-m.s. (Figure 7). On reduction, IDP and DPI form phenyl radicals. Radical formation by IDP is also associated with the loss of iodobenzene in solution (Figure 1). The FMN-IDP adduct which we isolated had a molecular mass of 534, which exactly corresponds to FMN with one phenyl ring attached (Figure 7a). FAD adducts, however, did not give such clear results. On reaction with either IDP or DPI, the reduced FAD or its products appear to be hydrolysed to give the phenylated FMN adducts. These can be seen at 534 and 736 on the mass spectra for IDP and DPI respectively (Figures 7b and 7c). However, other peaks are apparent, notably a higher-molecular-mass peak which appears in both cases, but does not correspond to any expected adducts.

N.m.r. spectroscopy of flavin adducts

^1H -n.m.r. spectroscopy was performed on the h.p.l.c.-purified FAD-IDP and FMN-IDP adducts. In comparison with the

standard FMN spectrum (Figure 8a), the FMN-IDP adduct spectrum (Figure 8b) showed the appearance of signals at 7–7.5 p.p.m., indicating addition of a phenyl group. Integration of these signals confirmed the mass-spectroscopy result, indicating addition of 1 phenyl group per flavin molecule. It is unclear whether one of the isoalloxazine proton peaks has disappeared or shifted.

Although integration of the n.m.r. spectrum of FAD-IDP confirmed the presence of phenyl groups and flavin, complete structural interpretation was not possible (Figure 8d). Four protons are seen at 8 p.p.m. as with the FAD standard (Figure 8c), and four methyl groups are seen at 2 p.p.m. instead of the expected two. Numerous signals arose in the region of 5.8–7 p.p.m., which could not be assigned. Evidently, as with FMN, further rearrangement takes place. However, possibly owing to the greater complexity of the FAD, not only is intramolecular rearrangement observed, but association of fragmentation products may occur.

Radiolabelling of neutrophil membranes

During inhibition of oxidase, the generation of reactive radicals may be associated with labelling of protein groups adjacent to the site of inhibitor activation. We therefore examined protein labelling in the presence and absence of enzyme turnover, using SDS/PAGE followed by autoradiography of enzyme incubated with [^{125}I]DPI. [^{125}I]DPI labelling of membrane proteins was increased by addition of NADPH during incubation. Non-

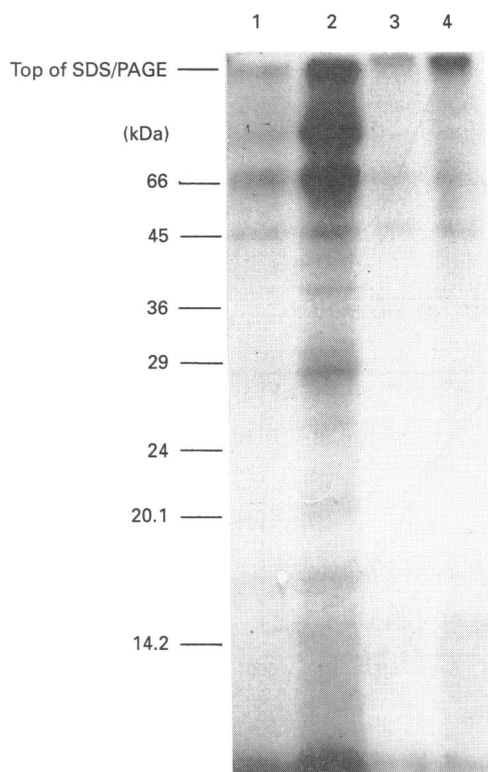


Figure 9 Labelling of human neutrophil membranes with [^{125}I]DPI

Labelling of proteins with [^{125}I]DPI, autoradiography, and protein staining were performed as described in the Methods and materials section. NADPH was added during incubation with [^{125}I]DPI to tracks 2 and 4. Unlabelled DPI (10 mM) was added after incubation with [^{125}I]DPI to tracks 3 and 4.

specific labelling induced by sample preparation after incubation was greatly decreased by the addition of unlabelled DPI (10 mM) after incubation (Figure 9).

Flavin recovery from pig neutrophil membranes

Having shown that reduced flavin could be modified by reaction with iodonium compounds, we investigated whether levels of extractable flavin from neutrophil membranes were affected by DPI inhibition of oxidase activity. In order to extract detectable flavin, pig neutrophils were used, as greater enzyme concentrations could be obtained in this way. Fluorescence of total flavin recovered is shown in Figure 10(a), with free FMN (100 nM) as comparison. Since the use of HClO_4 (0.15 M) to facilitate protein precipitation may lead to hydrolysis of FAD to the more fluorescent FMN, all supernatants from the centrifugation step were boiled in 1 M HClO_4 for 10 min. In this way, all FAD was converted into FMN before fluorescence analysis. There was no apparent degradation of standard FMN when treated as above, as judged by fluorescence spectroscopy. In order to recover detectable flavin, high enzyme concentrations were necessary. The inhibitor concentration was therefore raised to ensure that the ratio of enzyme to inhibitor was similar to that used for kinetic assays.

The addition of DPI, with NADPH as electron donor in the incubation mixture to promote oxidase turnover, resulted in a significant loss of recoverable flavin. After incubation of enzyme with 34.5 nmol of DPI/mg of membrane protein (50 μM DPI) and 900 μM NADPH, only 50% of the total membrane flavin

was recovered (Figure 10b). When DPI concentration was raised to 345 nmol/mg of protein (500 μM DPI), flavin recovery was further decreased to 24.2% of that after incubation with NADPH alone. In the absence of NADPH, there was no loss of recoverable flavin associated with the presence of 50 μM DPI (Figure 10c), showing that enzyme turnover was essential for this process.

DISCUSSION

DPI is a potent inhibitor of NADPH oxidase when added to stimulated neutrophils. However, when whole neutrophils were preincubated in DPI, the activity observed after dilution and activation was found to be almost unaffected, compared with that seen when DPI was added to activated neutrophils (Figure 2). This indicated either a need for electron flow through the enzyme complex, or that a configuration change involved in the change from resting to active oxidase exposed a DPI-sensitive site. Our results now suggest that the change in enzyme configuration on activation is unlikely to be the factor governing inhibitory activity, since we have shown that inhibition rate may be related to rate of enzyme turnover (see Figure 4 insert).

DPI and its analogue IDP appear to inhibit a variety of electron-transport systems by similar mechanisms. The observation that the rate of inhibition of NADPH oxidase by DPI increases on increasing NADPH concentration indicates that inhibition is uncompetitive with respect to NADPH. D. G. Tew (unpublished work) found that IDP caused a time-dependent inhibition of cytochrome *P*-450 reductase which was directly proportional to substrate concentration, whereas Ragan and Bloxham (1977) found no effect on the inhibitory potency of DPI on increasing the incubation time of resting NADH:ubiquinone reductase with DPI, although inhibition during turnover was time-dependent. These results support the view that enzyme turnover is a necessary requirement for inhibition.

NADPH oxidase contains two redox centres, FAD ($E_{m,7} = -280$ mV; Kakinuma et al., 1986) and a *b*-type cytochrome ($E_{m,7} = -245$ mV; Cross et al., 1981). During catalysis, these two centres alternate between reduced and oxidized redox states. From the known reactivities of iodonium compounds (Banks, 1966) and the nature of the inhibition observed, we have postulated a mechanism of inhibition involving the need for a reduced redox centre. This reduced centre could serve as an electron donor to the iodonium inhibitor, allowing phenyl-radical formation (Figure 1). The radical could then inhibit the enzyme by addition to the flavin or haem cofactor directly, or to amino acid side chains adjacent to the active site. NADPH in the absence of oxidase preparations is incapable of activating IDP to form radicals in solution (demonstrated spectrophotometrically; result not shown), suggesting that during enzyme inhibition a reduced redox centre might mediate electron donation to the inhibitor.

It has previously been shown that IDP is capable of modification of reduced haem cofactors. Spectral changes indicative of phenylation of haem of cytochrome *P*-450 by IDP have been shown to occur only in the presence of reductant (dithionite or NADPH) by Battioni et al. (1988). Recently, Doussiere and Vignais (1991) suggested a similar mechanism of action of IDP during inhibition of bovine NADPH oxidase. However, in this case dithionite was added as reductant. This would lead directly to formation of phenyl radicals without the need for reduction of the IDP by a reduced redox centre. Although NADPH oxidase has two redox centres, either of which may be the site of inhibition, we suggest that the flavin may be the preferred site of attack. During steady-state electron transfer from NADPH to O_2 the cytochrome *b* is typically around 10% reduced and the

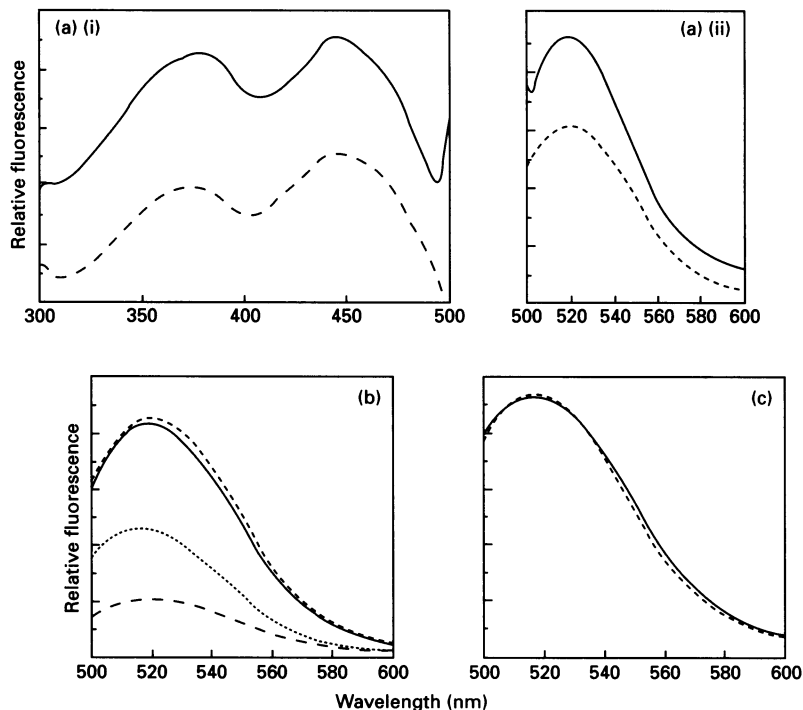


Figure 10 Recovery of intact flavin from pig neutrophil membranes

Flavins were extracted as described in the Methods and materials section (1.45 mg in 0.5 ml of buffer). (a) (i) Excitation (emission at 525 nm) and (ii) emission (excitation at 450 nm) spectra of flavin extracted from pig neutrophil membranes (—). Standard FMN fluorescence spectra (100 nm) are provided for comparison (----). (b) Fluorescence of flavins extracted from pig neutrophil membranes after incubation with NADPH with or without DPI as indicated (30 min at 20 °C): —, control, no additions; ----, NADPH (1 mM) only; ·····, NADPH, plus DPI (50 μ M); - - - - - , NADPH, plus DPI (500 μ M). (c) Fluorescence of flavins extracted from pig neutrophil membranes after incubation with (----) or without DPI (50 μ M) (—) alone for 30 min at 20 °C as indicated.

FAD 40% reduced (Cross et al., 1985; Parkinson, 1985). Using the Nernst equation it can be calculated that during turnover the cytochrome *b* is poised at -189 mV, and the flavin at -270 mV. Since DPI has a midpoint potential of -420 mV (Beringer, 1972), FAD would be a much more effective reductant of DPI than the cytochrome *b*. Iodonium compounds are inhibitors of such flavoenzymes as xanthine oxidase (V. B. O'Donnell, unpublished work; Doussiere and Vignais, 1992), mitochondrial NADH dehydrogenase (Ragan and Bloxham, 1977) and macrophage NO synthase (Stuehr et al., 1991), none of which contains haem prosthetic groups, indicating that these cofactors are not necessary for the formation of the inhibitory radical.

Controlled anaerobic reduction and reoxidation of FAD in the presence of IDP indicated that reduced, but not oxidized, FAD is attacked by the inhibitor, leading to loss of reoxidizable flavin. Mass spectrometry and n.m.r. spectroscopy of FAD and FMN adducts formed showed this loss to be due to direct phenylation of the flavin molecule. Although we could not elucidate the site of attachment, absorbance spectroscopy indicated this to be either C4a or N5 of the isoalloxazine ring. FMN gave much clearer results than FAD, possibly owing to its simpler structure. Although we were able to demonstrate FAD-IDP and FAD-DPI adduct formation, we were not able to characterize the products formed, since considerable hydrolysis of FAD to FMN appeared to occur on phenylation. It was clear from this work that reduced flavin, but not oxidized flavin, could react with iodonium compounds. During inhibition of cytochrome *P-450* reductase (an enzyme with FMN and FAD prosthetic groups) by IDP, one of us (D. G. Tew, unpublished work) demonstrated the formation of phenylated FMN, but not

FAD. This may indicate that in the system, too, on phenylation the FAD molecule becomes degraded, leading to a loss of detectable altered flavin.

In order to examine the labelling of neutrophil membrane proteins with DPI, radiolabelled inhibitor was incubated with samples of enzyme, with or without oxidase turnover. Inclusion of NADPH in the incubation mixture was associated with increased labelling of a number of bands, particularly those of 45 kDa and 66 kDa, along with increased background labelling. Since NADPH cannot activate DPI directly, this observation may indicate that the mobility of the radicals generated was such that they were able to diffuse from their site of generation before reacting with protein. The labelling of all protein bands was appreciably decreased by adding a saturating dose of unlabelled inhibitor immediately after the inhibition incubation. This may indicate that DPI labelling in the absence of turnover (Cross and Jones, 1986) may arise during preparation of samples for SDS-PAGE.

We were unable to isolate phenylated FAD associated with inhibition during turnover of NADPH oxidase of pig membranes; however, significantly less intact flavin was recovered after incubation with DPI and NADPH, but not with DPI or NADPH alone. If inhibition of enzyme occurred after attack of phenyl radicals on protein side chains within the active site, little loss of flavin would be seen. Our results suggest that flavin rather than protein may be the most sensitive site attacked by DPI in the oxidase complex. Also, since not all pig neutrophil membrane flavin will be associated with NADPH oxidase, or indeed be NADPH-reducible, not all would be attacked by DPI where NADPH is used as reductant. The kinetics of inhibition of

neutrophil NADPH oxidase by DPI support the hypothesis of a radical-mediated mechanism of action proposed previously (Banks, 1966). Using the free flavin coenzymes FAD and FMN, we have demonstrated reductant-dependent adduct formation with both DPI and IDP. This may provide a common mechanism of flavoenzyme inhibition for these iodonium compounds and lead to design of analogues specific for certain flavoprotein active sites.

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