ELSEVIER

European Journal of Pharmacology 401 (2000) 115–120

ejp

www.elsevier.nl/locate/ejphar

The inhibition of flavoproteins by phenoxaiodonium, a new iodonium analogue

Paul Moulton [a], Hannah Martin [c], Adele Ainger [a], Andrew Cross [b], Catherine Hoare [c], Justin Doel [c], Roger Harrison [c], Robert Eisenthal [c], John Hancock [a,*]

[a] *Department of Biological and Biomedical Sciences, University of the West of England, Coldharbour Lane, Bristol, BS16 1QY,UK*
[b] *Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, USA*
[c] *Department of Biology and Biochemistry, University of Bath, Bath UK*

Received 9 March 2000; received in revised form 15 June 2000; accepted 22 June 2000

Abstract

Iodonium compounds, especially diphenylene iodonium and iodonium diphenyl are used extensively as inhibitors of NADH–ubiquinone reductase and NADPH oxidase activity. Here, the use of a new iodonium compound, phenoxaiodonium is reported. The IC_{50} of neutrophil superoxide production, measured using the superoxide dismutase inhibitable rate of cytochrome *c* reduction, was approximately 0.75 μM, while 50% inhibition of mitochondrial respiration, measured by the rate of oxygen uptake using a Clark type oxygen electrode, was at approximately 20 μM. The inhibition of oxidation of xanthine to urate by xanthine oxidase was also studied, giving a K_i of 0.2 μM. Inhibition of nitric oxidase synthase (NOS: from rat brain) by 0.2 μM phenoxaiodonium was equivalent to 1 mM N^G-nitro-L-arginine methyl ester HCl (L-NAME), that is total abolition of activity. We conclude that phenoxaiodonium is an extremely good inhibitor of flavo-enzymes, but like diphenylene iodonium and iodonium diphenyl, will be of limited use as a pharmacological tool for the elucidation of the involvement of such enzymes in specific cellular functions.

Keywords: Iodonium; NADPH oxidase; Xanthine oxidase; Nitric oxidase (NO) synthase; NADH–ubiquinone reductase

1. Introduction

The release of free radicals, particularly superoxide (O_2^-) and nitric oxide (NO) by cells has been a subject of interest for many years. In particular, such free radicals have been shown to be beneficial in host defence systems, but can also cause harmful effects if their release is not controlled (Marx, 1987), as in inflammatory disease such as arthritis (Halliwell, 1987). Therefore, the ability to inhibit unwanted free radical release is important as a pharmacological tool for investigating their involvement in biological systems, and in elucidating the role of specific free radical producing enzymes in disease mechanisms, as well as potential therapeutic agents. More recently, free radicals and their associated non-radical by-products, such as hydrogen peroxide, have been shown to be important signalling molecules (Finkel, 1999) and the modulation of their production is therefore desirable.

Iodonium compounds have been used widely as inhibitors of flavin-containing enzymes, particularly the superoxide producing enzyme, NADPH oxidase. Most commonly, diphenylene iodonium or iodonium diphenyl is used, as first reported by Cross and Jones (1986). Previously, iodonium compounds were also reported to be useful inhibitors of NADH–ubiquinone reductase (complex 1) from mitochondria (Ragan and Bloxham, 1977; Li and Trush, 1998). Over the years, many reports have described the use of iodonium compounds to provide evidence of NADPH oxidase involvement in biological systems, for example, to investigate the role of NADPH oxidase in the antimicrobial role of macrophages (Robertson et al., 1990) or in the tumour killing of macrophages (Hancock et al., 1991). A recent search of the PubMed database returned 158 references to the use of iodonium compounds in biomedical research. The presence of NADPH oxidase activity in plant cells has been supported

* Corresponding author. Tel.: +44-117-3442475; fax: +44-117-9763871.
E-mail address: john.hancock@uwe.ac.uk (J. Hancock).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00454-4

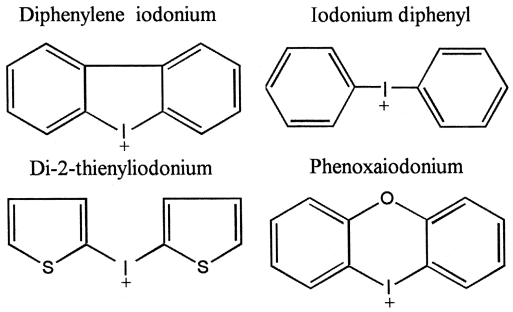

Fig. 1. The structure of iodonium compounds commonly used for flavoprotein inhibition, along with the structure of phenoxaiodonium.

by the use of iodonium compounds (Desikan et al., 1996; Dwyer et al., 1996; see review by Bolwell and Wojtaszek, 1997). However, it is now clear that iodonium compounds such as diphenylene iodonium and iodonium diphenyl inhibit other free radical producing enzymes such as nitric oxide synthase (NOS) (Stuehr et al., 1991) and xanthine oxidase (Doussiere and Vignais, 1992), as well as other flavin-containing enzymes such as bacterial nicotine oxidase (Brandsch and Bichler, 1987).

Here, we describe the inhibition of four flavoproteins by a new iodonium compound, phenoxaiodonium chloride (Fig. 1). Its differential inhibition of NADPH oxidase and NADH–ubiquinone reductase suggests that it could be a useful tool to distinguish the activities of flavoproteins, but like diphenylene iodonium and iodonium diphenyl, it is also a potent inhibitor of NOS and xanthine oxidase, limiting its value in this respect. We conclude that the results drawn when using iodonium compounds should be considered carefully, as such compounds appear to be good general inhibitors of enzymes which produce free radicals, but are not specific to any one enzyme activity.

2. Methods and materials

2.1. Phenoxaiodonium

Phenoxaiodonium chloride was obtained from Glaxo Wellcome plc, UK. Before use, stock solutions were prepared in dimethyl sulphoxide (DMSO) or 50% ethanol. Chemical analysis of the compound was carried out by Warwick Analytical Service, University of Warwick.

2.2. Preparation of neutrophils and measurement of superoxide release

Neutrophils were isolated from whole pig blood obtained from the local abattoir, as described by Cross et al. (1984).

Superoxide generation from neutrophils was measured using the superoxide dismutase inhibitable rate of cytochrome *c* reduction, using a dual wavelength spectrophotometer, with a sample wavelength of 550 nm and a reference wavelength of 540 nm (Cross et al., 1984). Briefly, cells were suspended in phosphate buffered saline (PBS containing 2 mM glucose), pH 7.4 at 37°C, containing 100 μM horse heart cytochrome *c*. Cells were stimulated to produce superoxide with 1 μM phorbol myristate acetate (PMA) and phenoxaiodonium, or superoxide dismutase added after the establishment of a linear rate.

2.3. Preparation of mitochondria and measurement of oxygen uptake

Mitochondria were isolated from pig liver using the following method. The liver was chopped into small pieces and homogenised, using a Potter homogenizer, in isolation medium (0.25 M sucrose, 0.02 M HEPES, 0.001 M Na_2EDTA, pH 7.4). The homogenate was centrifuged ($1000 \times g$, 10 min) and the supernatant subsequently recentrifuged ($10\,000 \times g$, 10 min). The resulting pellet was washed twice in isolation medium before being resuspended in isolation medium.

Oxygen uptake from mitochondria was measured using a Clark type oxygen electrode. Mitochondria were suspended and briefly sonicated, to cause uncoupling and allow inhibitor entry in buffer (100 mM KCl, 30 mM HEPES, 1 mM KH_2PO_4) containing 2.4 mM NADH. Inhibitor was added during the linear phase of oxygen uptake.

2.4. Determination of xanthine oxidase activity

For the determination of xanthine oxidase inhibition, xanthine oxidase was isolated from bovine milk as described by Sanders et al. (1997). Xanthine (Sigma, UK) was dissolved in 0.2 M NaOH before dilution in buffer. Xanthine oxidation was determined by the following the rate of production of urate at 295 nm (extinction coefficient 9.6 M^{-1} cm^{-1}) using a Cary 100 Bio UV-Vis spectrophotometer.

2.5. NOS activity

The effect of phenoxaiodonium on NOS activity was determined using a commercial kit (Calbiochem-Novabiochem, UK) that uses the conversion of arginine to citrulline as a measure of NOS activity. The activity of the NOS of a rat cerebellum homogenate was assayed as described in the manufacturer's instructions, in the absence or presence of varying concentrations of phenoxaiodonium. The NOS inhibitor, N^G-nitro-L-arginine methyl ester, HCl (L-NAME, HCl), provided with the kit was used as a control. An incubation time of 30 min at room temperature was used. ^{14}C-labelled arginine was used as

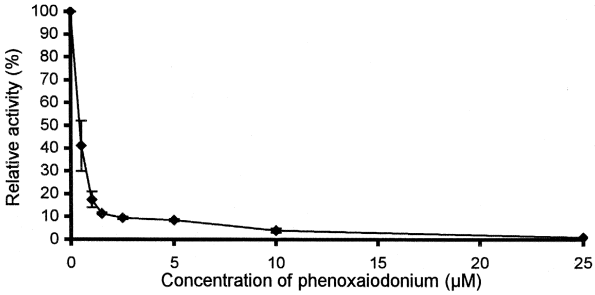

Fig. 2. Inhibition of neutrophil superoxide generation by phenoxaiodonium. Neutrophils were isolated from whole pig blood as described by Cross et al. (1984). Superoxide generation from neutrophils was measured using the superoxide dismutase inhibitable rate of cytochrome *c* reduction, using a dual wavelength spectrophotometer, with a sample wavelength of 550 nm and a reference wavelength of 540 nm (Cross et al., 1984). Results are presented as mean ± SE. Final protein concentrations used were approximately 0.6 μg ml^{-1}.

the substrate and radio-labelled citrulline was measured using scintillation counting.

2.6. Protein determinations

Protein concentrations of samples were determined by the method of Bradford (1976).

3. Results

Iodonium compounds have been widely used as inhibitors of the NADPH oxidase complex of neutrophils and other phagocytes. Therefore, the ability of phenoxaiodonium to inhibit the superoxide generation of such cells was assessed using isolated neutrophils and the superoxide dismutase-inhibitable rate of cytochrome *c* reduction (Jones and Hancock, 1994). As shown in Fig. 2, phenoxaiodonium inhibited neutrophil superoxide release at relatively low concentrations; 50% inhibition of maximal PMA-stimulated activity required approximately 0.75 μM.

Iodonium compounds are also known to inhibit other flavin-containing enzymes, and if to be of use as a tool to modulate free radical release from cells in tissue culture,

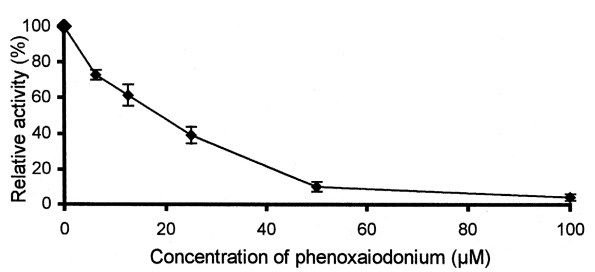

Fig. 3. Inhibition of mitochondrial oxygen uptake by phenoxaiodonium. Mitochondria were isolated from pig's liver and oxygen uptake mitochondria was measured using a Clark type oxygen electrode, in the presence of varying concentrations of phenoxaiodonium. Results are presented as mean ± SE.

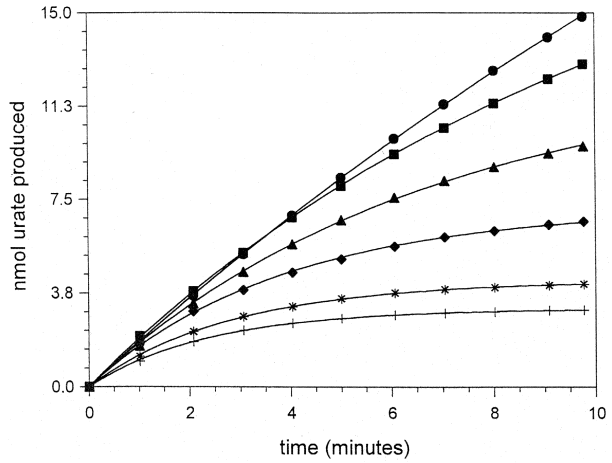

Fig. 4. Inhibition of xanthine oxidase by phenoxaiodonium. Xanthine oxidase was isolated from bovine milk as described by Sanders et al. (1997). Xanthine oxidation was determined by following the rate of production of urate at 295 nm using a Cary 100 Bio UV-Vis spectrophotometer. Concentration of phenoxaiodonium used were: ● 5 nM; ■ 20 nM; ▲ 50 nM; ♦ 80 nM; ∗ 150 nM; + 200 nM.

the effect of phenoxaiodonium on mitochondrial respiration is important. As seen in Fig. 3, phenoxaiodonium was an effective inhibitor of oxygen uptake by isolated uncoupled mitochondria, using NADH as a respiratory substrate and determining oxygen consumption with a Clark type oxygen electrode. The IC_{50} for oxygen uptake by mitochondria required approximately 20 μM, considerably higher than the concentrations needed to inhibit neutrophil superoxide release.

To study the inhibitory effects of phenoxaiodonium on other flavin-containing enzymes, xanthine oxidase was isolated from bovine milk. Here, the rate of production of urate from xanthine in the presence of oxygen was followed as a measure of enzyme activity. Inhibition was found to be both time- and concentration-dependent; 50% inhibition appeared to require less than 1 μM phenoxaiodonium (Fig. 4). Plotting the data as described by O'Donnell et al. (1993), fitted using the Kitz and Wilson equation (Kitz and Wilson, 1962) gave a value for the K_i of approximately 0.2 μM.

The final flavin-containing enzyme used in this study was NOS. To assess the inhibition by phenoxaiodonium of NOS, NOS activity was measured using a commercial kit based on the conversion of arginine to citrulline. Using rat cerebellum homogenate as a source of NOS, 0.2 μM phenoxaiodonium resulted in inhibition equivalent to 1 mM L-NAME, that is, approximately total abolition of activity.

4. Discussion

Iodonium compounds have been widely used and characterised over a relatively long period of time. Synthesis of cyclic iodonium compounds was reported in 1956 (Collette

et al., 1956) while their use as an inhibitor of neutrophil superoxide release was first reported in 1986 (Cross and Jones, 1986). Since then, these compounds have been shown to inhibit NADPH oxidase activity in other phagocytic cells, such as macrophages (Hancock and Jones, 1987), and the mechanism by which they act has been investigated. Cross and Jones (1986) and Ellis et al. (1989) both showed that reduction of the haem of flavocytochrome b_{558} was inhibited by these compounds, and suggested that the point of action of iodonium compounds was at or before the flavoprotein involved in electron transport in NADPH oxidase function. O'Donnell et al. (1993) investigated the inhibitory mechanism of iodonium compounds with regard to NADPH oxidase and showed that adducts were formed between FAD or FMN and diphenylene iodonium or iodonium diphenyl. Covès et al. (1999) have recently shown that sulphite reductase from *Escherichia coli* is inhibited by iodonium compounds in a very similar manner to NADPH oxidase and were also able to demonstrate the formation of phenylated FAD adducts generated during enzyme inhibition. However, others have shown that the haem group of the cytochrome may also be a target for iodonium compounds (Doussiere et al., 1999).

Regardless of its exact mode of inhibition of NADPH oxidase, iodonium compounds are clearly not specific for this enzyme complex. Previous work has shown that NADH–ubiquinone reductase (complex 1) is also susceptible to inhibition by diphenylene iodonium (Ragan and Bloxham, 1977). Furthermore, oxygen uptake due to respiration in whole macrophages was shown to be inhibited by such compounds (Hancock and Jones, 1987). Here, phenoxaiodonium was shown to inhibit both NADPH oxidase activity of neutrophils and oxygen uptake from mitochondria. The concentration of phenoxaiodonium needed to cause 50% inhibition of NADPH oxidase was favourably low when compared to that reported for other iodonium compounds, while the data in Table 1 indicates that there is a larger differential in the inhibition profiles of these two flavin-dependent activities with phenoxaiodonium, than there is with either diphenylene iodonium or iodonium diphenyl. A similarly large differential was reported previously for di-2-thienyliodonium (Hancock and Jones, 1987), suggesting that iodonium compounds can be produced, which preferentially inhibit NADPH oxidase over respiratory electron transport chain activity. However, 50% inhibition of NADH–ubiquinone reductase activity has also been reported to require only 2.5 μM di-2-thienyliodonium (Ragan and Bloxham, 1977).

Since the first reports of diphenylene iodonium and iodonium diphenyl inhibition of neutrophil NADPH oxidase activity, these compounds have been used as a diagnostic tool investigating the presence of NADPH oxidase activity in many cell types, from both animals and plants (Desikan et al., 1996; Dwyer et al., 1996; see review by Bolwell and Wojtaszek, 1997). They have also been used to investigate the role of NADPH oxidase in killing mechanisms, of both microbes such as *Toxoplasma gondii* (Robertson et al., 1990) and tumour cells (Hancock et al., 1991). However, it is now clear that iodonium compounds such as diphenylene iodonium and iodonium diphenyl inhibit other free radical-producing enzymes such as NADH–ubiquinone reductase (Li and Trush, 1998), NOS (Stuehr et al., 1991) and xanthine oxidase (Doussiere and Vignais, 1992) as well as other flavin-containing enzymes such as bacterial nicotine oxidase (Brandsch and Bichler, 1987) and sulphite oxidase (Covès et al., 1999). Therefore, to investigate the specificity of phenoxaiodonium towards NADPH oxidase further, its inhibition of both xanthine oxidase and NOS was investigated. Fifty per cent inhibition of xanthine oxidase required less than 1 μM phenoxaiodonium. The use of isolated xanthine oxidase enabled a more thorough investigation of the inhibition of this enzyme. The inhibition, like that of both NADPH oxidase (Cross and Jones, 1986) and NADH–ubiquinone reductase (Ragan and Bloxham, 1977) was found to be both concentration- and time-dependent. Kinetic analysis using the method described by O'Donnell et al. (1993), essentially using the Kitz and Wilson equation (Kitz and Wilson, 1962) gave a K_i of 0.2 μM for phenoxaiodonium inhibition of xanthine oxidase. Using the same concentration (0.2 μM) of phenoxaiodonium caused approximately total inhibition of NOS from rat cerebellum. Further studies on the inhibition of xanthine oxidase by phenoxaiodonium using a fixed phenoxaiodonium concentration of 5 μM with a range of substrate concentrations (0.75–10 μM) showed that the inhibition was uncompetitive with respect to xanthine (data not shown).

Although diphenylene iodonium and iodonium diphenyl have been the iodonium compounds most extensively used

Table 1
Concentrations of iodonium compounds required for 50% inhibition of NADPH oxidase activity and mitochondrial oxygen uptake

Compound	Concentration needed for 50% inhibition of NADPH oxidase activity (μM)	Concentration needed for 50% inhibition of oxygen uptake (μM)
Phenoxaiodonium	0.75	20
Diphenylene iodonium	0.90[a]	13[a]
Iodonium diphenyl	80[a]	120[a]

[a] Data taken from Hancock and Jones (1987).

as inhibitors of NADPH oxidase and other flavoprotein activities, other analogues have also been investigated. Here, we report data using a new analogue, phenoxaiodonium. Clearly, the iodine carrying a positive charge is important for the reactivity of the iodonium compounds towards their target, whether that is the flavoprotein (O'Donnell et al., 1993) or the haem group (Doussiere et al., 1999). Concentrations needed for inhibition of NADH–ubiquinone reductase by compounds without the iodine, or with the iodine substituted by either sulphur or oxygen were considerably higher than those reported for diphenylene iodonium or iodonium diphenyl (Ragan and Bloxham, 1977). Compounds where the electropositivity of the iodine is increased, such as di-2-thienyliodonium, on the other hand, are far better inhibitors of these enzymes. The same would be predicted for phenoxaiodonium, and indeed we find this is true for its activity towards NADPH oxidase.

Although these studies using phenoxaiodonium to inhibit different flavin-dependent enzymes are not directly comparable, they indicate that this compound is not specific for any particular flavin-dependent enzyme. Clearly, we are here comparing inhibition studies using whole neutrophils, where access of the phenoxaiodonium compound to the inner face of the intact neutrophil membrane is required for action, assuming that the flavin centre is the target, with studies using isolated enzymes, such as xanthine oxidase. However, even though comparison of the inhibition of NADPH oxidase and mitochondrial oxygen uptake show that phenoxaiodonium appears to show some specificity, when inhibition studies with xanthine oxidase and NOS are also carried out, it becomes apparent that this compound is not specific for any particular flavoprotein. Its use would be limited to the inhibition of free radical generation perhaps, as most if not all sources of free radicals are flavin-containing enzymes, but this compound will not be useful as a tool to dissect the exact nature of the enzyme involved in such activity. Unfortunately, the same is true of diphenylene iodonium, iodonium diphenyl and undoubtably di-2-thienyliodonium, casting doubt on the future use of iodonium compounds as truly useful pharmacological tools, or as theurapeutics, and also casting doubt on the validity of many studies, where such compounds have been used to show the presence and functioning of NADPH oxidase.

Acknowledgements

We would like to acknowledge receipt of phenoxaiodonium chloride from Glaxo Wellcome plc and in particular the help of Dr Simon Lister. Financial support for PJM was provided by the University of the West of England, Bristol and help acknowledged from Dr R Desikan. Financial support for ARC is acknowledged from the NIH grant AI24838. We also acknowledge the University of Bath for studentship support and The Wellcome Trust.

References

Bolwell, G.P., Wojtaszek, P., 1997. Mechanisms for the generation of reactive oxygen species in plant defence — a broad perspective. Physiol. Mol. Plant Pathol. 51, 347–366.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brandsch, R., Bichler, V., 1987. Covalent flavinylation of 6-hydroxy-D-nicotine oxidase involves an energy requiring process. FEBS Lett. 224, 121–124.

Collette, J., McGreeer, D., Crawford, R., Chubb, F., Sandin, R.B., 1956. Synthesis of some cyclic iodonium salts. J. Am. Chem. Soc. 78, 3819–3820.

Covès, J., Lebrun, C., Gervasi, G., Dalbon, P., Fontecave, M., 1999. Overexpression of the FAD-binding domain of the sulphite reductase flavoprotein component from *Escherichia coli* and its inhibition by iodonium diphenyl chloride. Biochem. J. 342, 465–472.

Cross, A.R., Jones, O.T.G., 1986. The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils. Biochem. J. 237, 111–116.

Cross, A.R., Parkinson, J.F., Jones, O.T.G., 1984. The superoxide generating oxidase of leukocytes: NADPH-dependent reduction of flavin and cytochrome *b* in solubilised preparations. Biochem. J. 223, 337–344.

Desikan, R., Hancock, J.T., Coffey, M.J., Neill, S.J., 1996. Generation of active oxygen in elicited cells of Arabidopsis thaliana is mediated by a NADPH oxidase-like enzyme. FEBS Lett. 382, 213–217.

Doussiere, J., Vignais, P.V., 1992. Diphenylene iodonium as an inhibitor of the NADPH oxidase complex of bovine neutrophils: factors controlling the inhibitory potency of diphenylene iodonium in a cell free system of oxidase activation. Eur. J. Biochem. 208, 61–71.

Doussiere, J., Gaillard, J., Vignais, P.V., 1999. The heme component of the neutrophil NADPH oxidase complex is a target for aryliodonium compounds. Biochemistry 38, 3694–3703.

Dwyer, S.C., Legendre, L., Low, P.S., Leto, T.L., 1996. Plant and human neutrophil oxidative burst complexes contain immunologically related proteins. Biochim. Biophys. Acta 1289, 231–237.

Ellis, J.A., Cross, A.R., Jones, O.T.G., 1989. Studies on the electron-transfer mechanism of the human neutrophil NADPH oxidase. Biochem. J. 262, 575–579.

Finkel, T., 1999. Signal transduction by reactive oxygen species in non-phagocytic cells. J. Leukocyte Biol. 65, 337–340.

Halliwell, B., 1987. Oxidants and human disease: some new concepts. FASEB J. 1, 358–364.

Hancock, J.T., Jones, O.T.G., 1987. The inhibition by diphenylene iodonium and its analogues of superoxide generation by macrophages. Biochem. J. 242, 103–107.

Hancock, J.T., White, J.I., Jones, O.T.G., Silver, I.A., 1991. The use of diphenylene iodonium and its analogues to investigate the role of the NADPH oxidase in the tumouricidal activity of macrophages in vitro. Free Radical Biol. Med. 11, 25–29.

Jones, O.T.G., Hancock, J.T., 1994. Assays of plasma membrane NADPH oxidase. Methods Enzymol. 233, 222–229.

Kitz, R., Wilson, I.B., 1962. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. J. Biol. Chem. 237, 3245–3249.

Li, Y.B., Trush, M.A., 1998. Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production. Biochem. Biophys. Res. Commun. 253, 295–299.

Marx, J.L., 1987. Oxygen free radicals linked to many diseases. Science 235, 529–531.

O'Donnell, V.B., Tew, D.G., Jones, O.T.G., England, P.J., 1993. Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. Biochem. J. 290, 41–49.

Ragan, C.I., Bloxham, D.P., 1977. Specific labelling of a constituent polypeptide of bovine heart mitochondrial reduced nicotinamide-adenine dinucleotide reductase by the inhibitor diphenyleneiodonium. Biochem. J. 163, 605–615.

Robertson, A.K., Cross, A.R., Jones, O.T.G., Andrew, P.W., 1990. The use of diphenylene iodonium, an inhibitor of NADPH oxidase, to investigate the antimicrobial action of human monocyte derived macrophages. J. Immunol. Methods 133, 175–179.

Sanders, S.A., Eisenthal, R., Harrison, R., 1997. NADH oxidase activity of human xanthine oxidoreductase: generation of superoxide anion. Eur. J. Biochem. 245, 541–548.

Stuehr, D.J., Fasehun, O.A., Kwon, O.A., Gross, N.S., Gonzales, S.S., Levi, J.A., Nathan, R., 1991. Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenylene iodonium and its analogs. FASEB J. 5, 98–103.