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A Reappraisal of Xanthine Dehydrogenase and Oxidase in Hypoxic Reperfusion Injury: the Role of NADH as an Electron Donor

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Xanthine oxidase (XO) is conventionally known as a generator of reactive oxygen species (ROS) which contribute to hypoxic-reperfusion injury in tissues. However, this role for human XO is disputed due to its distinctive lack of activity towards xanthine, and the failure of allopurinol to suppress reperfusion injury. In this paper, we have employed native gel electrophoresis together with activity staining to investigate the role human xanthine dehydrogenase (XD) and XO in hypoxic reperfusion injury. This approach has provided information which cannot be obtained by conventional spectrophotometric assays. We found that both XD and XO of human umbilical vein endothelial cells (HUVECs) and lymphoblastic leukaemic cells (CEMs) catalysed ROS generation by oxidising NADH, but not hypoxanthine. The conversion of XD to XO was observed in both HUVECs and CEMs in response to hypoxia, although the level of conversion varied. Purified human milk XD generated ROS more efficiently in the presence of NADH than in the presence of hypoxanthine. This NADH oxidising activity was blocked by the FAD site inhibitor, diphenyleneiodonium (DPI), but was not suppressible by the molybdenum site inhibitor, allopurinol. However, in the presence of both DPI and allopurinol the activities of XD/XO were completely blocked with either NADH or hypoxanthine as substrates. We conclude that both human XD and XO can oxidise NADH to generate ROS. Therefore, the conversion of XD to XO is not necessary for post-ischaemic ROS generation. The hypoxic-reperfusion injury hypothesis should be reappraised to take into account the important role played by XD and XO in oxidising NADH to yield ROS.

Keywords: Xanthine dehydrogenase, xanthine oxidase, NADH oxidase, reperfusion injury, reactive oxygen species, hypoxia

INTRODUCTION

It is clear that reactive oxygen species (ROS) formed during hypoxic reperfusion are important factors in organ transplantation[1] and in a range of human diseases[2] and, while there is no

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real consensus on the key mechanisms involved, the possible roles of xanthine dehydrogenase (XD) and xanthine oxidase (XO) have been the subject of extensive studies.

XD and XO are metalloflavoprotein enzymes, which catalyse the two-step oxidation of hypoxanthine, through xanthine, to uric acid. This is accompanied by the reduction of NAD and/or of O_2. XD prefers NAD as its electron acceptor, whereas XO will only donate electrons to O_2. XD can be converted to XO reversibly via oxidation of critical sulfhydryl groups[3] or irreversibly through limited proteolysis.[4]

An important role of XO in generating ROS during ischemic reperfusion injury was first indicated by Granger *et al.*,[5] and, on the basis of these and other studies,[6] a mechanistic hypothesis was proposed.[7] According to this hypothesis, during ischemia, endogenous XD is proteolytically converted to XO, which reduces $O_2^{\bullet-}$ rather than NAD. Concomitantly, hypoxanthine and xanthine accumulate as a consequence of ATP breakdown. Following ischaemia, O_2 is again available, allowing the newly-generated XO to oxidise accumulated hypoxanthine, producing a burst of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), which induces tissue injury.

This hypothesis depends critically on conversion of naturally-predominant XD to XO, both the extent and timing of which have been questioned.[8,9] Moreover, although the role of XO in reperfusion injury has been supported by the alleviation of injury by the specific inhibitor, allopurinol,[10–12] it has been stressed that allopurinol-based arguments in support of a role for XO or in human ischaemic reperfusion injury are not convincing.[13,14] Further complications arise from the finding that human XD/XO, at least in milk[15] and heart,[16] shows very low activity towards conventional reducing substrates such as xanthine or hypoxanthine. These observations raise questions concerning the role of human XD/XO in general and prompt consideration of alternative catalytic activities. That bovine milk XO can oxidise NADH has long been known[17,18] but not generally considered. It was recently observed that human milk XD/XO, despite its low activity to 'conventional' reducing substrates like xanthine, can catalyse the oxidation of NADH as efficiently as the bovine milk enzyme.[15] This is fully consistent with the fact that NADH donates electrons to the FAD site of the enzyme, in contrast to all other reducing substrates which donate to the molybdenum center. Thus, a high proportion of the human milk enzyme contains inactive molybdenum but an apparently normal functional FAD content.[15]

In the present study, we have examined the potential of human XD and XO to oxidise NADH and to generate ROS, by using a native gel electrophoresis assay system together with activity staining. Conventional spectrophotometric methods of determining NADH oxidoreductase activity, while appropriate for purified XO, are not applicable to cell lysates, where such activity can arise from several enzyme systems. Our procedure allowed analysis of the NADH oxidase activity of XD and XO in cells, without interference from other ROS-generating enzymes. We have also used our gel systems to examine the relative abilities of several proteases to effect XD to XO conversion, with a view to clarifying the involvement of such processes in ischemia-reperfusion injury.

MATERIALS AND METHODS

Materials

All chemicals and reagents were purchased from Sigma Chemical Co. (Poole, Dorset) unless stated otherwise. Human neutrophil elastase, thrombin and diphenylene iodonium (DPI) were purchased from Calbiochem-Novabiochem (Nottingham, UK). Human recombinant stromelysin-1 and gelatinase-A were a generous gift from Professor G Murphy.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated according to Jaffe *et al.*,[19] with modifications. Briefly, umbilical cords were stored after birth at 4°C and used within 48 hr. The umbilical veins were rinsed with PBS and incubated for 45 min at room temperature with 2 mg/ml collagenase type II in PBS. Collected cells were then seeded in HUVEC medium (M-199, 20% FCS, 15 mg endothelial growth supplement, 100 U/ml heparin, 100 U/ml penicillin, and 100 μg/ml streptomycin—Gibco, Paisley, Scotland), centrifuged for 10 min at 400 g and cultured in T-25cm^2 tissue cultured flasks in an atmosphere of 5% CO_2 and 95% air at 37°C. The next day the cells were washed with medium in order to eliminate blood cell contamination and this medium was replaced every 2–3 days. When the cells reached confluency they were subcultured into T-80cm^2 flasks and used in experiments between 1–3 passages. The purity of the isolated population, measured by immunological staining of vWF antigen (a marker for endothelial cells), was consistently over 99%.

A human lymphoblastic leukaemic cell line (CEM) was cultured in RPMI 1640 medium with 10% foetal calf serum and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Hypoxic Treatment of Cells

The aqueous O_2 concentration was determined by an O_2 electrode (Strathkelvin Instruments, Glasgow) calibrated with air-saturated (100% O_2) and N_2-saturated (0% O_2) PBS. Before carrying out the hypoxic treatment of cells, vessels containing culture medium alone were pre-equilibrated with the 95% N_2/5% CO_2 gas within a Microflow Mk1 Anaerobic Cabinet (MDH, Hampshire, UK), giving an O_2 concentration in the medium within the range 1–2%, as determined by above O_2 electrode. Prior to placing cells into the anaerobic cabinet, HUVECs were washed with sterilised PBS within flasks, whilst CEM were washed with PBS and pelleted by centrifugation. The cells were then transferred into the anaerobic cabinet, and the hypoxic treatment was carried out by replacing the PBS with the pre-equilibrated culture medium.

Preparation of Tissue or Cell Homogenates

Tissues or cells were washed three times in PBS. After the final washing step, they were homogenised in an ice-cold buffered solution (pH 7.4) containing 0.05 M potassium phosphate, 100 mM iodacetic acid, 100 mM leupeptin, 1 mM pepstatin, 1 mM PMSF, 10 mM EDTA to prevent proteolysis, and 2 mM DTT to inhibit oxidative conversion of XD to XO. The homogenate was centrifuged at 3,000 g for 10 min and supernatants derived therefrom were used for enzyme activity assays. Iodoacetic acid, a non-specific sulfhydryl reagent, had no intrinsic effect on XD and XO activities as assessed by spectrophotometric analysis.

Purification of Human Milk Xanthine Oxidase

Human XO was purified from pooled fresh human milk by a previously described method.[15] This preparation, without proteolysis, consists of a mixture of enzyme in XD and XO forms. A predominantly dehydrogenase preparation can be obtained by reduction of the isolate with DTT.

Proteolytic Conversion of Human XD to XO

A number of proteinases, including rabbit muscle calpain, porcine trypsin, human neutrophil elastase, human plasma thrombin, and human recombinant stromelysin-1 and gelatinase-A were tested for their ability to catalyse the conversion of XD to XO proteolytically. XD was incubated with each proteinase in the presence of 2 mM DTT at 37°C for a period of 1 hr. The ratios of proteinase and enzyme are detailed in the relevant figure legends.

Polyacrylamide Gel Electrophoresis (PAGE)

SDS PAGE was carried out in a Tris/glycine buffer system using 10% gels. Electrophoresis was carried out at 0.45 mA/cm^2 for periods of 2 hr. After electrophoresis, the gel was subjected to either Western blotting, silver or Coomassie blue staining.

Native PAGE with 7.5% gel was conducted in a similar manner to SDS PAGE, but SDS was omitted from both the gel mixture and the sample solution. Following electrophoresis the gel was normally cut into two portions. One portion was stained using 0.45 mM NADH as substrate in the presence of 0.245 mM nitroblue tetrazolium (NBT), and the other was stained with 1.4 mM hypoxanthine in the presence of 0.245 mM NBT.[20,21]

Bands corresponding positionally to XD and XO by a native PAGE were further analysed by SDS PAGE and Western blotting.

Western Blotting

After electrophoresis, the proteins on the SDS or native gel were blotted onto a nitrocellulose membrane. The membrane was prestained with Ponceau S (0.03% w/v) to mark the transferred molecular mass markers before immunostaining with a mouse IgG monoclonal antibody[22] raised against purified human milk XD/XO, prepared as above, and peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Denmark). The intensities of the bands were quantified using an imaging system (Bio-image, Millipore).

Spectrophotometric Assay for Activity

XD and XO activities were assayed by measurement of uric acid formation from xanthine at 295 nm in the presence and absence of 0.5 mM NAD.[23] XO activity is given by the rate of formation of uric acid in the ansence of NAD. With NAD included in the reaction mixture, the total activity (XO+XD) can be obtained. XO and XD activities are expressed as percentages of the total activity.

The ability of XD and XO to generate $O_2^{\bullet-}$ was also measured spectrophotometrically. The assay mixture contained 50 mM potassium phosphate, 0.1 mM EDTA, 10 μM horse heart cytochrome c and 100 μM NADH and XD/XO. The rate of $O_2^{\bullet-}$ production was followed by cytochrome c reduction at 550 nm.

To validate the use of NBT staining on native PAGE as described above, we monitored the production of formazan from NBT by XD/XO-generated superoxide.[20] Enzyme in phosphate buffer (50 mM, pH7.4) was incubated with 100 μM xanthine (or 1 mM NADH), 120 μM EDTA, 30 μM NBT and monitored at 560 nm.

RESULTS

NADH Oxidase Activity of XD and XO

Our purified human milk xanthine oxidase contained a mixture of XD and XO. In the presence of DTT, the XD activity in our preparation was 81.4%, as assessed spectrophotometrically by NAD-dependent uric acid formation. When this predominantly XD preparation was subjected to native PAGE, it appeared as a single activity band with either NADH or hypoxanthine as its substrate (Fig. 1, lanes 1 and 5). The protein staining with Coomassie blue also showed a single protein band, which corresponded to the enzyme activity band (data not shown).

Neither XD nor XO (up to 10 μg or 1.7 μM, the highest concentration used in this study) could directly reduce NBT to formazan, in the absence of NADH, as demonstrated by both the gel staining assay and spectrophotometric analysis. With the concentrations of enzyme used, the formation of formazan was almost completely inhibited by the addition of superoxide dismutase, suggesting that $O_2^{\bullet-}$ is an intermediate in this reaction. Flavin adenine dinucleotide (FAD) directly caused NBT reduction in the presence of NADH only at high

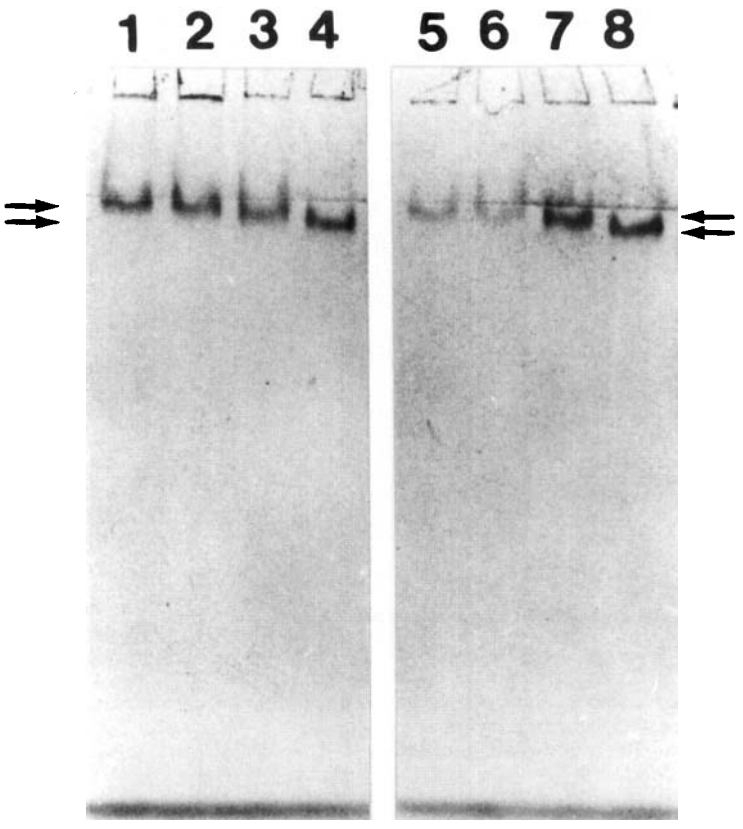

FIGURE 1 Electrophoretic comparisons of human xanthine dehydrogenase (XD) and proteolytically-induced xanthine oxidase (XO). Human XD was incubated with different proteinases at 37°C for 1 hr. 10 μg XD or XO was loaded within each lane of the gel. The Gel (7.5%) was run under non-dissociating conditions and stained by NBT using NADH (left side panel) or hypoxanthine (right side panel) as substrates. Lanes 1 and 5, purified human XD; lanes 2 and 6, rabbit muscle calpain-treated XD (the weight ratio of calpain:XD was 2:1); lanes 3 and 7, human neutrophil elastase-treated XD (elastase:XD=1:10); lanes 4 and 8, porcine trypsin treated XD (trypsin:XD=1:100).

concentrations (over 40 μM). This eliminated the possibility of non-enzymatic reduction of NBT by FAD contained within XD/XO.

Proteolytic Conversion of XD to XO

XD was incubated with a range of different proteinases for 1 hr and analysed by activity staining with NADH or hypoxanthine (Fig. 1), by SDS PAGE and silver staining (Fig. 2), and by Western blotting (Fig. 3). In agreement with previous reports, incubation of XD with trypsin resulted in the conversion of XD to XO. The spectrophotometric assay for uric acid formation showed that over 87.3% of total activity was due to XO activity.

On Western blotting, trypsin-treated XD showed an enhanced 135 kDa band and an additional 90 kDa band (Fig. 3 lane 6), whilst protein staining revealed that trypsin can cleave XD to give two additional bands (40 and 39 kDa) (Fig. 2 lane 11), which were found not to react with the monoclonal antibody. Human neutrophil elastase had the capacity to cleave both the 150 and the 135 kDa form of XD completely to a single 90 kDa band on the Western blot (Fig. 3 lane 3) together with two additional bands (40 and 38 kDa) detected by protein staining (Fig. 2 lane 5).

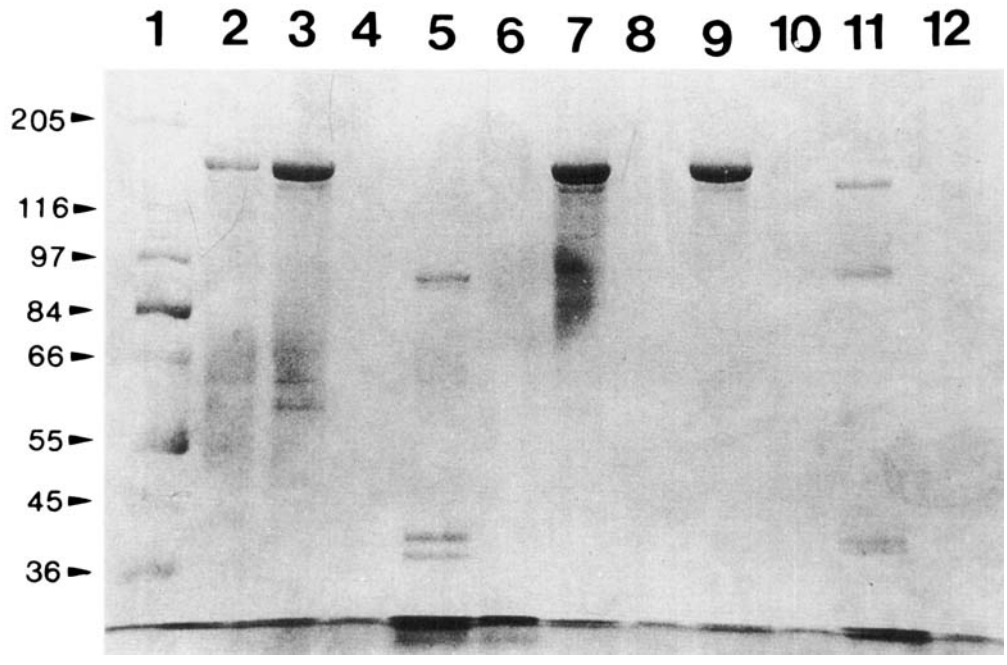

FIGURE 2 SDS PAGE profiles of human xanthine dehydrogenase (XD) and proteolytically-induced xanthine oxidase (XO). The conditions of human XD incubated with different proteinases were the same as described in Figure 1 unless stated otherwise. After electrophoresis, protein bands were silver stained. Lane 1, molecular weight markers; lane 2, human purified XD (2 μg); lane 3, rabbit muscle calpain-treated XD (4 μg); lane 4, rabbit muscle calpain; lane 5, human neutrophil elastase-treated XD (4 μg); lane 6, human neutrophil elastase; lane 7, human thrombin-treated XD (4 μg, the weight ratio of thrombin and XD was 1:10); lane 8, human thrombin; lane 9, human stromelysin-l-treated XD (4 μg, the weight ratio of stromelysin-1 and XD was 1:10); lane 10, human stromelysin-1; lane 11, trypsin-treated XD (4 μg) and; lane 12, trypsin.

However, only a slight migration was evident by the activity staining (Fig. 1, lanes 3 and 7).

Rabbit muscle calpain did not effect conversion of XD to XO, as evidenced by activity staining (Fig. 1, lanes 2 and 6), antibody detection (Fig. 3, lane 2), and protein detection (Fig. 2, lane 3). Furthermore, human stromelysin-1 and human gelatinase A and thrombin failed to cleave or convert XD to XO.

Comparison of Substrate Specificity of XD and XO

In order to allow a comparative evaluation of the substrate specificity of human XD and XO, serial dilutions of XD and trypsin-generated XO (protein loaded from 281 ng to 9 μg; 281 ng to 4.5 μg, respectively) were examined by native PAGE with enzyme activity staining. By comparing the intensities of bands as determined by the imaging system, a dose-dependent increase in the intensity of $O_2^{\bullet-}$ production was observed in response to enzyme concentration increase. When NADH was used as an electron donor, XD generated 4-fold more $O_2^{\bullet-}$ relative to hypoxanthine (Fig. 4). In contrast, XO exerted 2-fold more enzyme activity towards hypoxanthine compared with NADH. Thus, it appears that, while both XD and XO can use either NADH or hypoxanthine to generate $O_2^{\bullet-}$, under these experimental conditions, XD favours the utilisation of NADH, whilst XO favours hypoxanthine.

Inhibition of XD/XO Activity by Various Inhibitors

To examine and identify the effect of inhibitors (20 μM) on human XD/XO activity, four

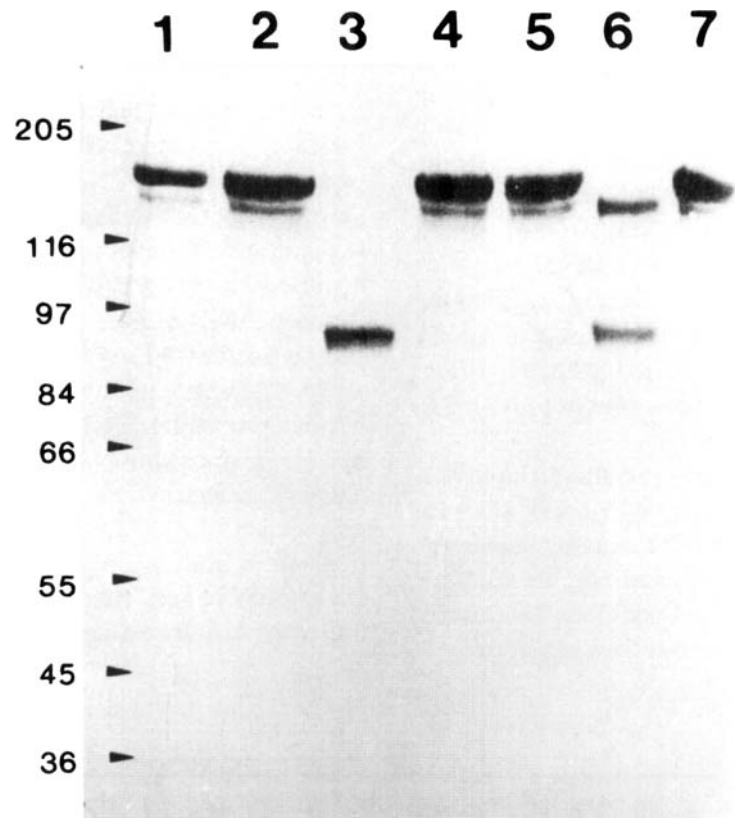

FIGURE 3 The Western blotting profiles of human xanthine dehydrogenase (XD) and proteolytically-induced xanthine oxidase (XO). The conditions of human XD incubated with different proteinases were the same as described in Figs 2 and 3 unless stated otherwise. Numbers to the left of the blot indicate the positions of molecular weight markers. Lane 1, human purified XD (2 μg); lane 2, rabbit muscle calpain-treated XD (4 μg); lane 3, human neutrophil elastase-treated XD (4 μg); lane 4, human thrombin-treated XD (4 μg); lane 5, human stromelysin-1-treated XD (4 μg); lane 6, trypsin-treated XD (4 μg); and lane 7, human gelatinase-A-treated XD (4 μg, the weight ratio of gelatinase-A and XD was 1:10).

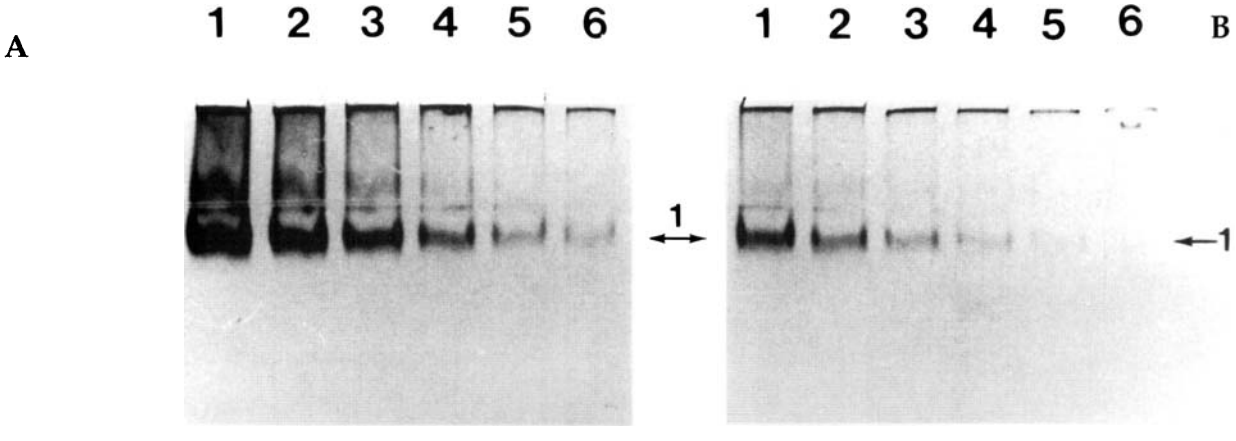

FIGURE 4 The dose response patterns of purified human xanthine dehydrogenase (XD) with NADH (left side panel) or hypoxanthine (right side panel) as its reducing substrates. In Fig 4A, equal amounts of XD were loaded on both gels, Lane 1, 9 μg; 2, 4.5 μg; 3, 2.25 μg; 4, 1.13 μg; 5, 563 ng; 6, 281 ng. In Fig 4B, the band intensities of Fig 4A with NADH on the left and hypoxanthine on the right.

compounds including allopurinol, (–)-BOF-4272, DPI and menadione, were tested in the native PAGE system with enzyme activity staining along with rat liver homogenate as a control. When hypoxanthine was used as a substrate, both allopurinol and (–)-BOF-4272 acted as potent inhibitors of human XD (Fig. 5A, lanes 3 and 7), whilst menadione and DPI had no effect on XD activity (Fig. 5A, lane 11 and Fig. 6B, lane 7). However, when NADH was used as a substrate, DPI completely inhibited XD activity (Fig. 5B, lane 5), whilst the other inhibitors (Fig. 5A, lanes 1, 5 and 9) had no effect.

The combination of DPI and allopurinol completely blocked the activity of human XD with either NADH or hypoxanthine as its substrates (Fig. 5B, lanes 1 and 3). Menadione, an inhibitor of aldehyde oxidase,[24] did not block XD activity when hypoxanthine was used as a substrate.

Cellular XD/XO Activity and Conversion Under Hypoxia

Two cell types, HUVECs and CEMs, were assessed as to their XD/XO content, and their capacities to use NADH or hypoxanthine in the generation of $O_2^{\cdot-}$. Cells subjected to ambient air or hypoxia at 37 °C for 1 hour were homogenised and analysed by native PAGE with activity staining. Activity staining of XD and XO bands from both cell types showed evidence of $O_2^{\cdot-}$ production when NADH but not hypoxanthine were used as reducing substrate (Fig. 6A). Both cell types exhibited other bands with NADH oxidase activity similar to those seen in rat liver (Fig. 5A, lanes 2 and 6). Conversion of the XD to XO was observed after hypoxia in both cell types, although the level of conversion varied. Interestingly, CEMs contain much less NADH oxidase activity than HUVECs.

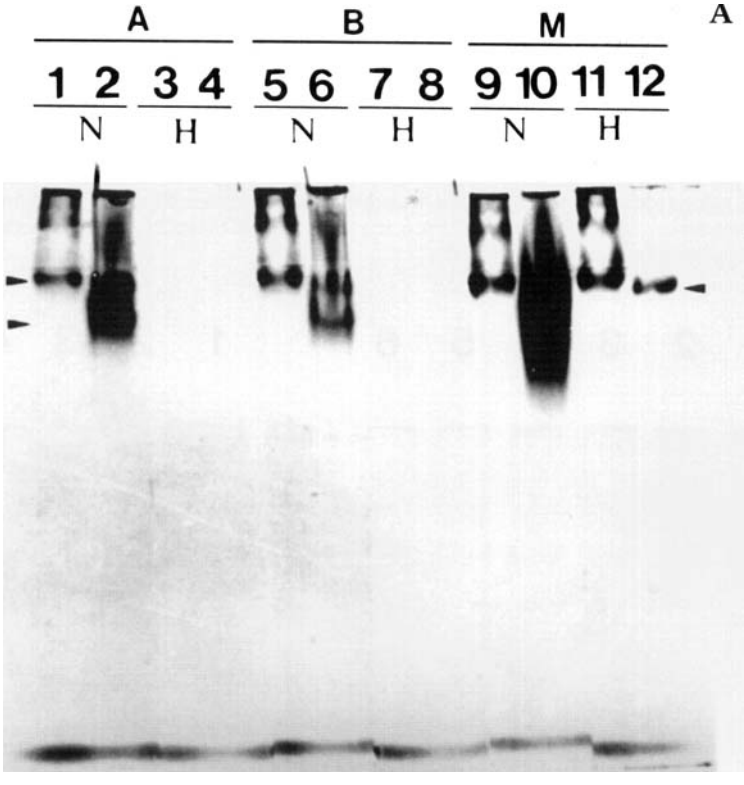

FIGURE 5A

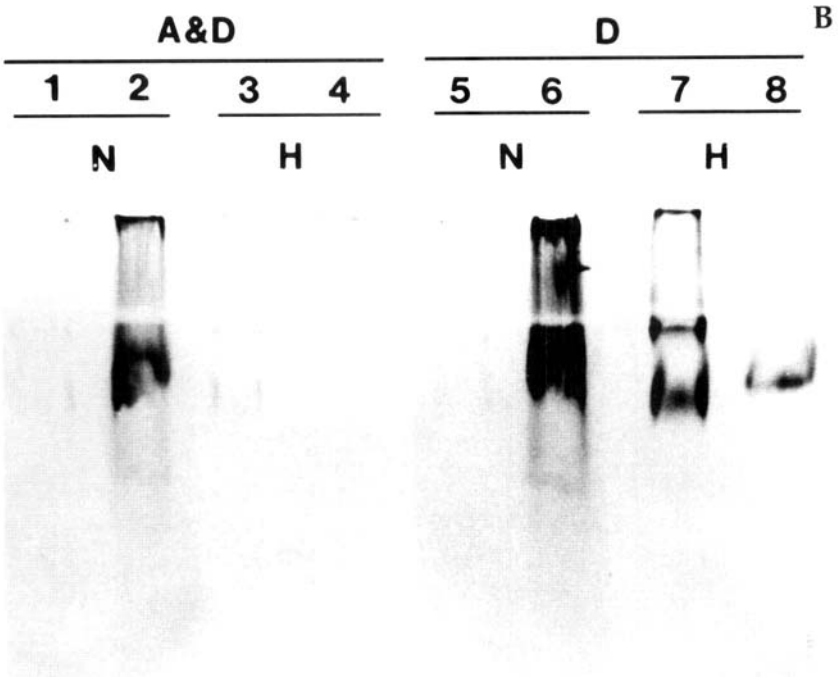

FIGURE 5 The effect of inhibitors on purified human xanthine dehydrogenase (XD) and rat liver tissue homogenase. Human XD (10 μg) and rat liver tissue homogenates (50 μg protein) were loaded onto the gel (lanes 1,3,5,7,9, and 11 of Fig 5A, and lanes 1,3,5, and 7 of Fig 5B were human XD; lanes 2,4,6,8,10, and 12 of Fig 5A and lanes 2,4,6, and 8 of Fig 5B were rat liver samples). The two lanes under the letter N were NBT-stained using NADH as a substrate, lanes under the letter H were stained using hypoxanthine as a substrate. Letter A indicates where 20 μM allopurinol was used as an inhibitor in either NADH or hypoxanthine; B, 20 μM (–)-BOF-4272; D, 20 μM diphenylene iodonium; M, 20 μM medadione.

In order to confirm that the protein detected by NADH staining of the cell homogenates was in fact XD/XO, we have employed native PAGE and Western blotting with antibody probing. The results showed that the monoclonal antibody used detected the denatured XD/XO (Fig. 3) but did not recognise the native enzyme. However, after second dimensional SDS PAGE and Western blotting of the excised portion of the native gel, which contained the NADH oxidase activity, we found a 150 kDa band in homogenates of cells which had been incubated in ambient air (Fig. 6B, lane 3), and a 90 kDa together with other bands in the molecular weight range of 50–60 kDa in homogenates from cells which had been incubated under hypoxic conditions (Fig. 6B, lane 4).

DISCUSSION

The commonly quoted mechanism involving XO in ischemia-reperfusion injury[7] is largely based on the well-known properties of bovine milk or rat liver enzymes.[17] Extrapolation to the human system, always implicit, is complicated by the low specific activity to most 'conventional' reducing substrates, of, at least the purified enzymes from breast milk[15] and heart.[16] The presence of similar "low activity" forms of XO, rather than absence of enzyme, may well also explain the low activities to be found, not only in breast milk and heart, but in many other human tissues.[25–27] Liver and intestine, in contrast, appear to contain XO with relatively high specific activity.[28,29]

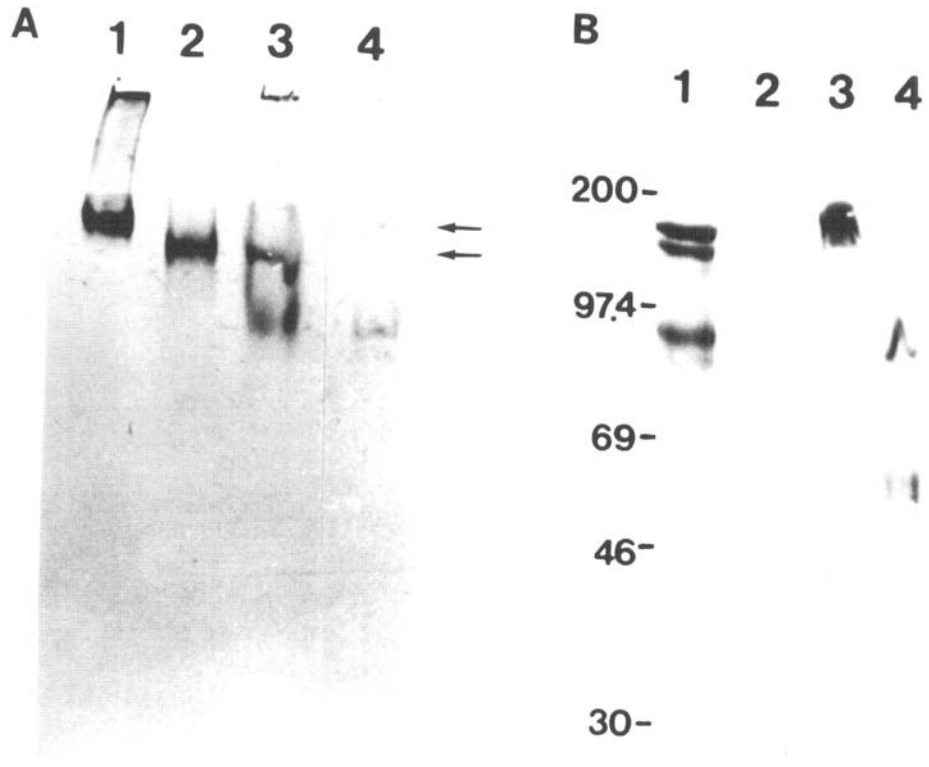

FIGURE 6 Electrophoretic patterns of human xanthine dehydrogenase (XD) and xanthine oxidase (XO) obtained from human umbilical vein endothelial cells (HUVECs) cultured in air or exposed to hypoxia at 37°C for 1 hr. Fig 6A, gel was stained by NBT using NADH as a substrate. Lane 1, purified human XD (10 μg); lane 2, trypsin-induced XO (10 μg); lane 3, hypoxia-treated HUVECs (50 μg protein); and lane 4, HUVECs cultured in air (50 μg protein). Fig 6B, second dimentinal SDS PAGE and Western blot of bands excised from first dimensional native PAGE with HUVECs exposed to hypoxia or air. Lane 1, XD control (10 μg); lane 2, XO control (1 μg); lane 3, triplet samples of excised bands from air-treated cells; lane 4; triplet samples of hypoxic treated cells.

Faced with the low activity toward xanthine and hypoxanthine of XO in at least some human tissues, we sought to explore the capacity of the human milk enzyme to generate ROS, specifically $O_2^{\bullet-}$, with NADH as reducing substrate. In our experimental system, the enzyme used either NADH or hypoxanthine as reducing substrate to generate $O_2^{\bullet-}$ which converts NBT to blue formazan.[20,21,30] When human XD and XO were examined by native PAGE, each migrated as a single band. However, XO migrated faster than XD, in good agreement with previous studies on rat liver XD/XO.[21] When, in our hands, rat liver homogenates were run under the same conditions before and after trypsin treatment, the electrophoretic patterns of XD and XO were the same as previously published.[21]

The results obtained from both native PAGE (Figs. 1 and 4) and the spectrophometric assay demonstrate that both XD and XO are able to use either hypoxanthine or NADH as their electron donors to generate $O_2^{\bullet-}$. Although our gel assay system has the advantage of allowing both XD and XO activities to be assessed directly from whole cell or tissue homogenates, it can only provide semi-quantitative kinetic information. Thus, in the presence of molecular oxygen and NADH, XD was found to generate $O_2^{\bullet-}$ approximately four times more than when hypoxanthine was the reducing substrate. The situation for XO was

reversed, with rates in the presence of hypoxanthine being two times those in the presence of NADH. A broad conclusion to be drawn from these data is that, while both XD and XO can use either NADH or hypoxanthine to generate $O_2^{\bullet-}$, XD favours the utilisation of NADH, whereas XO favours hypoxanthine. Although the present data offer no comparison of the rates of $O_2^{\bullet-}$ production by XD and XO via the latter two routes, some indications can be derived from the results of Sanders *et al.*[31] who studied the kinetics of purified human milk XD and XO spectrophotometrically and found that the maximal rate of $O_2^{\bullet-}$ production from XD, in the presence of NADH, was approximately four times faster than that from XO in the presence of xanthine. Bearing in mind that, *in vivo*, the cellular content of the dehydrogenase enzyme could be as high as 95%[8], the concept of $O_2^{\bullet-}$-generation via XD-catalysed oxidation of NADH, rather than by XO-catalysed oxidation of hypoxanthine, is attractive, in at least some human tissues. Moreover, the kinetics of $O_2^{\bullet-}$ production catalysed by human XD are such that increases in NADH levels that are likely to occur during normoxic-ischemic transitions[32–34] will have major effects on $O_2^{\bullet-}$ production.[31]

We have used our gel assay system to examine the activity and the forms of XD/XO in two types of human cells. HUVECs are endothelial cells which have been shown to contain XO.[35] We also used CEMs, a human lymphoblastic leukaemic cell line. We found that conversion of XD to XO occurs in both cell types under hypoxia and the level of conversion varies. However, regardless of the extent of conversion, our data show that, for both cell types and for both XD and XO, $O_2^{\bullet-}$ generation in the presence of NADH is very much greater than in the presence of hypoxanthine. SDS PAGE and Western blotting confirmed the identity of XD and XO in these cells and allowed us to compare molecular weights with those of the purified enzyme, although extra bands were found in hypoxia-treated cell samples. These extra bands were different from the bands described in Figures 2 and 3, since the latter cannot be detected by the monoclonal antibody. Therefore, these extra bands could be due to non-specific cross-reactivity of the antibody, or, alternatively, they may represent additional enzyme fragments formed by unidentified proteases.

The originally proposed mechanism of conversion of XD to XO involved the action of Ca^{++}-dependent proteases.[6] We have used various types of proteases to test their ability to effect this conversion, including serine proteases (trypsin, elastase, thrombin), matrix metalloproteinases (stromelysin-1, gelatinase-A) and calpain. Our results, with purified human milk enzyme, suggest that the matrix metalloproteinases and calpain do not effect XD to XO conversion. We also found that both human neutrophil elastase and trypsin can convert XD to XO. The migration rate of enzyme treated by neutrophil elastase is slower than that of the trypsin-treated enzyme.

The inhibition profiles of XD/XO confirm the different substrate binding sites for NADH and hypoxanthine. Both allopurinol and (–)-BOF-4272 inhibit XD activity by binding at the molybdenum centre, blocking the binding of hypoxanthine by mechanisms that have been studied previously.[36,37] These inhibitors were relatively ineffective against NADH oxidation. Diphenylene iodonium (DPI) is a well described inhibitor of the FAD active sites of a wide spectrum of different flavoprotein enzymes including NADPH oxidoreductase,[38] NADH oxidoreductase[39] and nitric oxide synthase.[40] Therefore, in our study we have used DPI to define the active site of the NADH oxidising activity on XO/XD and to distinguish this from the site of xanthine-based activity. Although our results demonstrate the potent inhibitory capacity of DPI in our *in vitro* system, the biological relevance of this inhibitory action of a non-specific agent remains unclear.

The present demonstration that DPI strongly inhibits XD-associated NADH oxidase activity further complicates the conclusions that can be drawn from the use of this agent in biological

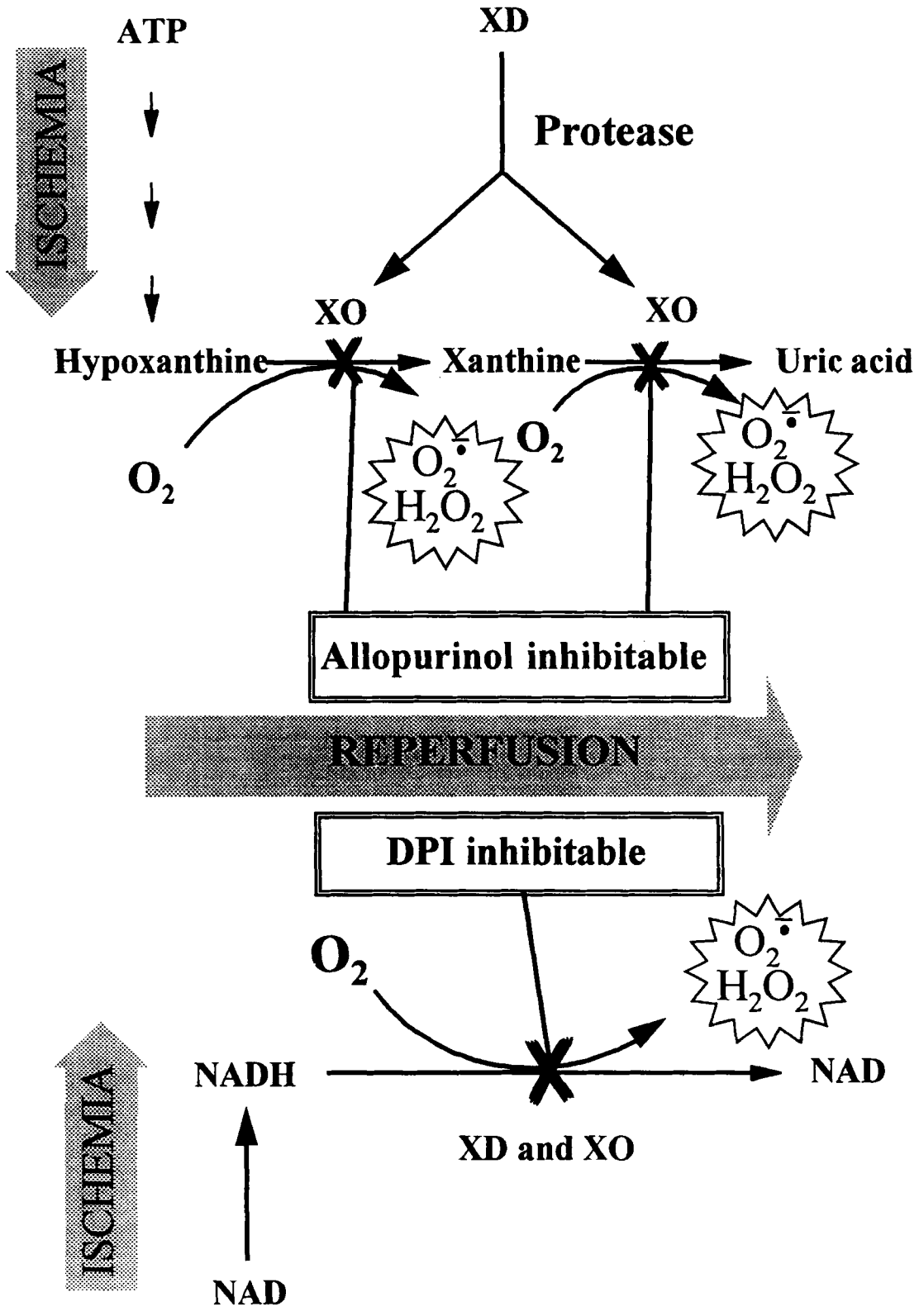

FIGURE 7 Schematic diagram of an extended mechanism for ROS generation by either xanthine dehydrogenase (XD) and xanthine oxidase (XO) during hypoxic reperfusion injury. DPI represents diphenyleneiodonium.

systems, where it was originally assumed to be a specific inhibitor of NADPH oxidase. Another corollary of these inhibition data concerns allopurinol which has been extensively used to detect XO-generated ROS. XD/XO-associated NADH oxidase activity would not be detectable by these means and, as a source of reactive oxygen, may well be more widespread than has been hitherto realised. When both DPI and allopurinol were used in conjunction, the activity of XD/XO towards either NADH or hypoxanthine was completely blocked. This result provides a basis for a new approach to inhibition of XD/XO which may prove to have a greater therapeutic potential in organ transplantation and many hypoxic reperfusion injury related diseases,[2] including rheumatoid arthritis.[41]

In conclusion, our procedures involving the combination of native gels and enzyme activity assays allow the detection and semi-quantitation of both XD and XO in either purified enzyme or cell homogenates. Their use has shown that, at least for the human enzyme forms studied, $O_2^{\cdot-}$ production resulting from NADH oxidation is likely to be a significant factor in hypoxic-reperfusion pathology. Generation of ROS by way of hypoxanthine oxidation according to the commonly-cited mechanism[7] need not be ruled out, especially for non-human species, in which the activity of XO to hypoxanthine or xanthine is much higher.[31] It is, however, worth noting that, according to our present data, calpain is unlikely to be the protease involved in conversion of XD to XO. A modified scheme, showing generation of ROS in hypoxia-reperfusion by two, possibly complementary pathways is shown in Figure 7.

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