(1) Propagating synovial inflammation

In rheumatoid joint disease, conditions of ischaemia and reperfusion prevail in the synovium. This is as a consequence of an inadequate perfusion of the highly metabolically active synovial tissue and the facility for intra-articular pressure-induced fluctuations in blood supply [1]. A detailed morphometric study of rheumatoid and normal synovium [2] has confirmed the former and accounted for the low levels of $O_2$ detected directly from rheumatoid knee joints (Figure 1, top), in addition to explaining earlier paradoxical perfusion studies [3]. The morphometry showed that, although rheumatoid synovium is subjected to aggressive angiogenesis, the distribution of new vasculature is not conducive to the complete perfusion of the joint through to the often effused joint space (Figure 1, bottom).

Increased pressure-induced anomalies arise because the joint, including the synovium, is contained rigidly in a ligamentous capsule offering resistance to pressure in any direction. Thus, in joints containing an inflammatory effusion, exercise results in intra-articular pressure rises, exceeding the capillary perfusion pressure [11]. The capillary perfusion pressure in inflammation is of the order of 30–60 mmHg and there is, accordingly, the potential for parts of the synovial capillary bed to be occluded, so inducing acute ischaemia in an already hypoxic environment. In mobile patients, who may be subjecting their joints to pressure-induced ischaemia, subsequent

Abbreviations used: ROS, reactive oxygen species; TNF, tumour necrosis factor; XOR, xanthine oxidoreductase.
rest allows reperfusion and reoxygenation of the synovium. The resulting repeated ischaemia-reperfusion cycles provide conditions for the generation of reactive oxygen species (ROS) from xanthine oxidoreductase (XOR), which has been detected in endothelial cells of the synovium [4,5]. In comparison with normal synovial tissue, rheumatoid synovium showed up to 60 times higher levels of XOR, possibly because of up-regulation of the enzyme by the characteristically high levels of cytokines and the hypoxic nature of rheumatoid synovium [6-8]. Synovial ROS generation by rheumatoid and osteoarthritic human tissue subjected to simulated ischaemia-reperfusion conditions has been assessed by ESR spectroscopy [9,10]. Levels of oxidizing species correlated with the degree of inflammation present and the density of capillaries in the specimens, and were partially and variably reduced by both competitive and non-competitive inhibitors of the Mo centre of XOR. This supports the partial involvement of this redox-site enzyme in the process. No studies have defined the involvement of the FAD or FeS redox centres which are capable of generating ROS and possibly reactive nitrogen species (see below).

(2) Causing bone erosion
Recent studies have identified a role for XOR in cytokine-induced bone resorption, another facet of the pathology of inflamed joints in rheumatoid arthritis. It is well established that both local and hormonal factors influence osteoblast and osteoclast function in complex ways to balance precisely the formation and resorption of bone. The communication between these two cell types is often established by direct stimulation of the osteoblasts which then signal to the osteoclasts for the induction of resorptive activity. Although the nature of this signalling has not been definitively identified, it does seem to be achieved by characteristically small and labile factors. ROS, particularly H$_2$O$_2$, having such characteristics are often considered as intracellular messengers and are involved in cell to cell communication. On
this basis, we have shown that osteoblasts produce H₂O₂ and that stimulation by tumour necrosis factor α (TNFα) (a potent resorption stimulant) increases this production. The use of TNF as an appropriate stimulant was based on the knowledge of its release during hypoxic conditions, abundance in rheumatoid synovia and its potent XOR-inducing activity in various cell types, as well as being a potent inducer of resorption in vitro and in vivo. Furthermore, we reported that H₂O₂ can enhance in vitro bone resorption by freshly isolated rat osteoclasts in the absence of parathyroid hormone-responsive osteoblasts [11]. These observations are supported by a wealth of literature indicating the redox sensitivity of bone resorption [12,13]. Our subsequent investigations involved the elucidation of the source of such species. We established the presence of the enzyme in freshly isolated rat calvarial osteoblasts and showed that the potent bone-resorption-stimulating cytokine, TNFα, could dramatically increase levels of XOR mRNA and activity in these cells. Moreover, the XOR inhibitor allopurinol was found to reduce Ca²⁺ release as a measure of resorption in mouse calvaria in vitro.

(3) Promoting vasculitis and spreading disease?

The proposed pathogenic mechanism of ischaemia–reperfusion injury involves the generation of ROS, after oxidation of hypoxanthine by XOR, and depends upon the well-known properties of bovine milk or rat liver enzymes. Human XOR has been purified from breast milk and characterized with a remarkably low activity towards hypoxanthine, xanthine and other conventional reducing substrates [14], which donate their electrons directly to the Mo centre. However, NADH-oxidizing activity involving the FAD centre rather than Mo exists in human XOR as well as that of other species. The substrate-specificity of human XOR in different organs is not clearly understood. The enzyme in the liver and intestine [15,16] has relatively high activity towards xanthine, like that from non-human sources. In contrast, the enzyme in the heart, synovium [17] and probably most human tissues has activity and substrate specificity profiles similar to that of the breast milk enzyme [18], which is believed to have a largely inactive Mo centre. This may be the basis for the apparent lack of XOR activity, particularly in the heart, where xanthine-based compounds have been utilized as substrates. Similarly, XOR activity in human plasma has been reported to be very low or absent, as measured by conventional assays. In view of the clearly elevated levels of synovial XOR in rheumatoid arthritis and the higher activity of the human enzyme towards NADH rather than towards xanthine [18], we have used a sensitive chemiluminescence assay to detect NADH-oxidizing activity in the plasma of patients with rheumatoid arthritis and normal controls [19]. Such activity was found to be significantly higher in the patients and to correlate strongly with biochemical markers of disease activity including the hepatic acute-phase proteins, possibly indicating a hepatic source of the plasma enzyme. Moreover, immunoprecipitation experiments identified XOR as the source of the plasma NADH oxidase activity. These findings suggest that XOR may be an important source of ROS in hypoxic joints by virtue of its NADH-based activity, therefore not depending on xanthine-based activity or conversion of the dehydrogenase to the oxidase form. The lower pH in rheumatoid joints also predisposes to a higher activity towards NADH oxidation which increases with lower pH. Xanthine-based activity, on the other hand, is inhibited by lowered pH. The presence of a circulating form of NADH oxidase activity, inactive within the plasma but activatable by appropriate substrate availability, suggests a novel mechanism for propagating disease and developing complications such as vasculitis. Recent evidence showing the specific high-affinity binding of XOR to certain glycosaminoglycans [20] supplies one potential mechanism for the redistribution of plasma XOR to tissue-specific endothelium-associated glycosaminoglycan.

(4) Generating NO under conditions of hypoxia?

Another attribute of XOR related to pH is its ability to convert NO₃⁻ to NO₂⁻ [21]. As has been described, the human enzyme favours its ability to oxidize NADH, rather than xanthine, with resultant superoxide formation. In ischaemia, however, NADH or xanthine can donate electrons which, rather than forming superoxide, reduce NO₃⁻ to NO₂⁻. In our continuing studies [22] we have found that XOR converts NO₃⁻ into NO₂⁻ using the Mo centre of the enzyme when O₂ is restricted (Figure 2). This activity is detectable at physiological pH but increases with
Proposed mechanism for nitrate reductase activity of XOR

Electron donors such as xanthine or NADH pass an electron to the enzyme, which can shuttle around the molecule and ultimately be transferred to NO⁻ at the Mo centre or O₂ at the FAD centre (also to NAD⁺ when available as a dehydrogenase). The extent of transfer to NO⁻ is dependent on the concentration of the preferred electron acceptor O₂. When transfer to O₂ at the FAD centre is blocked [by diphenyleneiodonium (DPI) for example] nitrate reductase activity is optimal.

Nitrite reductase activity can utilize either xanthine or NADH as electron donors, and it appears that either the Mo or the FAD centre can receive the electrons which may be passed to NO⁻ at the FeS centre (Figure 3). One product of this reaction is NO as detected by an isolated NO probe and an ozone chemiluminescence system [22,23].

We believe these findings to have important consequences; for example, under specified low-O₂ conditions, XOR will metabolize dietary NO⁻ to NO₂ and then may form NO, which cannot be efficiently formed under hypoxic conditions by nitric oxide synthase, as this enzyme requires O₂. The effect of NO in inflammatory conditions and its possible interaction with ROS to form peroxynitrite suggest that XOR may play a pivotal role in diseases where hypoxic episodes are a factor.

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