Xanthine oxidoreductase is asymmetrically localised on the outer surface of human endothelial and epithelial cells in culture

Magali Rouquette[a], Susanna Page[a], Richard Bryant[a], Mustapha Benboubetra[a], Cliff R. Stevens[b], David R. Blake[b], William D. Whish[a], Roger Harrison[a,*], David Tosh[a]

[a] *Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK*
[b] *Department of Postgraduate Medicine, University of Bath, Bath BA2 7AY, UK*

Received 19 March 1998

Abstract Subcellular localisation of xanthine oxidoreductase (XOR) was determined by indirect immunofluorescence using confocal microscopy in human endothelial and epithelial cell lines and in primary cultures of human umbilical vein endothelial cells. XOR was diffusely distributed throughout the cytoplasm but with higher intensity in the perinuclear region. In non-permeabilised cells, XOR was clearly seen to be asymmetrically located on the outer surfaces, showing, in many cases, a higher intensity on those faces apposed by closely neighbouring cells. Such specific distribution suggests a functional role for the enzyme in cell-cell interactions, possibly involving signalling via reactive oxygen species

Key words: Xanthine oxidoreductase; Immunolocalization; Endothelial; Epithelial; Human

1. Introduction

Xanthine oxidoreductase (XOR) is a molybdenum-containing flavoenzyme that catalyses the hydroxylation of hypoxanthine to xanthine and of xanthine to uric acid in the latter stages of purine catabolism [1]. In mammals, it occurs in two interconvertible forms, xanthine dehydrogenase (EC 1.1.1.204) and xanthine oxidase (EC 1.1.3.22). Both forms of the enzyme can reduce molecular oxygen, although only the dehydrogenase form can reduce NAD, which it prefers as an electron acceptor. Reduction of oxygen leads to superoxide anion and hydrogen peroxide and it is the potential to generate these reactive oxygen species that has led to widespread interest in the enzyme as a pathogenic agent in many forms of ischaemia-reperfusion injury [2]. More recently, reactive oxygen species are being increasingly cited as intermediates in normal signal transduction pathways [3,4].

XOR is widely distributed, being particularly rich in mammary epithelial cells and in capillary endothelium in a range of tissues [5,6]. While the enzyme is generally understood to be cytosolic, there have been very few published investigations of its precise subcellular localisation. Jarasch et al. [5] used both light and electron microscopic immunohistochemical procedures to show that XOR is located throughout the cytoplasm of bovine capillary endothelial cells. This was also found to be the case in rat liver endothelial cells [7,8]. In contrast, using immunoelectron microscopy, Ichikawa et al. [9] concluded that the enzyme was exclusively cytosolic with no significant association with intracellular organelles, including endoplasmic reticulum, Golgi apparatus, lysosomes or peroxisomes.

The apparent confusion over the subcellular location of XOR prompted us to investigate the situation in human cells. The human enzyme is of especial interest, particularly in view of questions regarding its anomalous characteristics and physiological role [10]. We have, accordingly, made use of confocal microscopy in immunolocalisation of the enzyme in human endothelial and epithelial cells in culture. We show here that XOR is present not only in the cytoplasm but also on the outer surface of all three cell types studied. Moreover, the extracellular enzyme shows a strongly polarised distribution, being in many cases concentrated on those surfaces closely apposed by neighbouring cells.

2. Materials and methods

2.1. Materials

RPMI 1640 culture medium was obtained from ICN, Costa Mesa, CA, USA. Penicillin, streptomycin and foetal calf serum (FCS) were from Life Technologies, Paisley, UK. Rabbit anti-TGN 46 antibody was a kind gift from Dr. George Banting (Department of Biochemistry, University of Bristol, UK). Rabbit anti-(bovine milk XOR) was from Chemicon International, Harrow, UK. All other reagents, unless otherwise stated, were from Sigma, Poole, UK.

2.2. Cell culture

EA-hy-926, a permanent endothelial cell line [11], was a gift from Dr. Andrew George, Imperial College School of Medicine, Hammersmith Hospital, London. The cells were routinely grown in an atmosphere of 5% CO_2/95% air in 75-cm^2 flasks at 37°C, as previously described [12]. Growth medium, RPMI 1640, containing 10% (v/v) FCS and penicillin/streptomycin [12], was changed every 3–4 days. The cells grew to form a confluent monolayer after approximately 7 days, exhibiting typical endothelial cell characteristics and were shown, by immunofluorescence (results not shown), to be positive for factor VIII, as reported by Edgell et al. [11].

HB4a is a human mammary epithelial cell line, conditionally immortalised by transfection with SV40 virus [13] and kindly donated to us by Dr T. Kamalati and Professor B. Gusterson of the Institute for Cancer Research, Royal Cancer Hospital, Sutton, UK. HB4a cells were routinely grown at 37°C in 75-cm^2 culture flasks, in an atmosphere of 5% CO_2/95% air as previously described [14]. Growth medium, RPMI 1640 containing 10% (v/v) FCS and other supplements [14], was changed every 3–4 days. Cells grew to confluence, forming a strict monolayer after 9 days, showing a characteristic 'crazy paving' appearance and stained strongly positive (results not shown) for the epithelial cell marker, cytokeratin [14].

Human umbilical vein endothelial cells (HUVECs) were obtained from human umbilical veins (kindly donated by the nursing staff of the Princess Anne Wing, Royal United Hospitals, Bath) and cultured essentially as described by Jaffe et al. [15].

*Corresponding author. Fax: (44) (1225) 826779.
E-mail: bssrh@bath.ac.uk

Abbreviations: XOR, xanthine oxidoreductase; FCS, foetal calf serum; HUVEC, human umbilical vein endothelial cell; DIC, differential interference contrast

PII S0014-5793(98)00385-8

2.3. Immunolabelling of cells and confocal microscopy

Cells were seeded (approx. 2×10^5 cells/ml) in four-chambered glass slides (Nunc Inc., Naperville, IL, USA), incubated for 24 h at 37°C and washed twice with pre-warmed PBS before fixing for 20 min at room temperature with 4% (w/v) formaldehyde in PBS.

Cells were permeabilised by incubation with 0.1% (w/v) saponin in PBS for 45 min, then incubated with rabbit polyclonal anti-(human XOR) antibodies in PBS (0.02 mg/ml), containing 0.1% (w/v) saponin, 3% (v/v) normal goat serum and 1% (w/v) BSA, for 2 h at room temperature. The cells were washed three times with 0.1% (w/v) saponin in PBS before incubation, for 2 h at room temperature, with secondary antibody [FITC-conjugated goat anti-rabbit IgG (0.025 mg/ml, Jackson Immunoresearch Labs. Inc., West Grove, PA, USA)], diluted 1:100 in the same diluant as for the primary antibodies. The cells were then washed three times with PBS containing 0.1% (w/v) saponin, before removing the chambers from the slides prior to confocal microscopy.

Unpermeabilised cells were obtained and treated as above, except that saponin was omitted throughout.

The permeabilised or unpermeabilised nature of the cells was confirmed by immunolabelling with rabbit anti-TGN 46 antibody, which is specific for the trans-Golgi network (results not shown) [16].

Images were collected on a confocal laser-scanning microscope (LSM 510, with either ×40 1.30 NA or ×63 1.40 NA apochromatic objective; Carl Zeiss, Welwyn Garden, UK). The 488 lines of an argon laser were used for excitation of FITC.

2.4. Assay for XOR enzymic activity

Cell extracts were prepared and assayed for total (oxidase plus dehydrogenase) activity as previously described [14], using a sensitive fluorimetric procedure [17]. EA-hy-926 and HB4a cells contained 1–2 pmol isoxanthopterin/min/mg. Activity of HUVECs was below the lower limit of sensitivity of the assay (0.1 pmol isoxanthopterin/min/mg).

2.5. Heparin-Sepharose treatment of growth medium

A column (3.5 cm×1.5 cm) of heparin-Sepharose (Sigma) was washed with appropriate growth medium (30 ml) lacking FCS. Growth medium (100 ml) containing FCS (10%) was then passed through the column and collected in a sterile container. The column was washed with 25 mM sodium phosphate buffer, pH 7.4, until A_{280} reached a baseline level, and then with the same buffer containing 1 M NaCl. Protein-containing fraction (A_{280}) was assayed for XOR enzymic activity as described above.

2.6. Generation and affinity purification of rabbit polyclonal anti-(human XOR) antibodies

Antibodies were generated and affinity-purified as previously described [14]. Their specificity has been previously established by immunoprecipitation of XOR from HB4a cell extracts [14]. Incubation of HB4a cell extracts with the gel-bound specific antibodies removed 100% of XOR enzymic activity, while SDS-PAGE of the immunoprecipitate showed only the characteristic band of XOR, apart from bands attributable to the antibodies themselves [14].

3. Results

Three human cell types, including endothelial (EA-hy-926) and epithelial (HB4a) cell lines and primary endothelial (HUVEC) cells in culture, were studied using affinity-purified rabbit anti-(human XOR) antibodies (see Section 2). In all cases, immunolocalisation of XOR in permeabilised cells showed the enzyme to be diffusely distributed throughout the cytoplasm, although fluorescence in the perinuclear region was more intense (Fig. 1). Immunolocalisation of XOR in unpermeabilised cells clearly showed the presence of the enzyme on the outer cell surface, the distribution being localised to specific areas of the cell (Fig. 2). In several cases, XOR appeared to be concentrated on those parts of the surface that apposed or were extending towards neighbouring cells (Fig. 2C, arrows).

Use of commercially supplied rabbit anti-(bovine milk XOR) antibody gave the same results as those described above (results not shown).

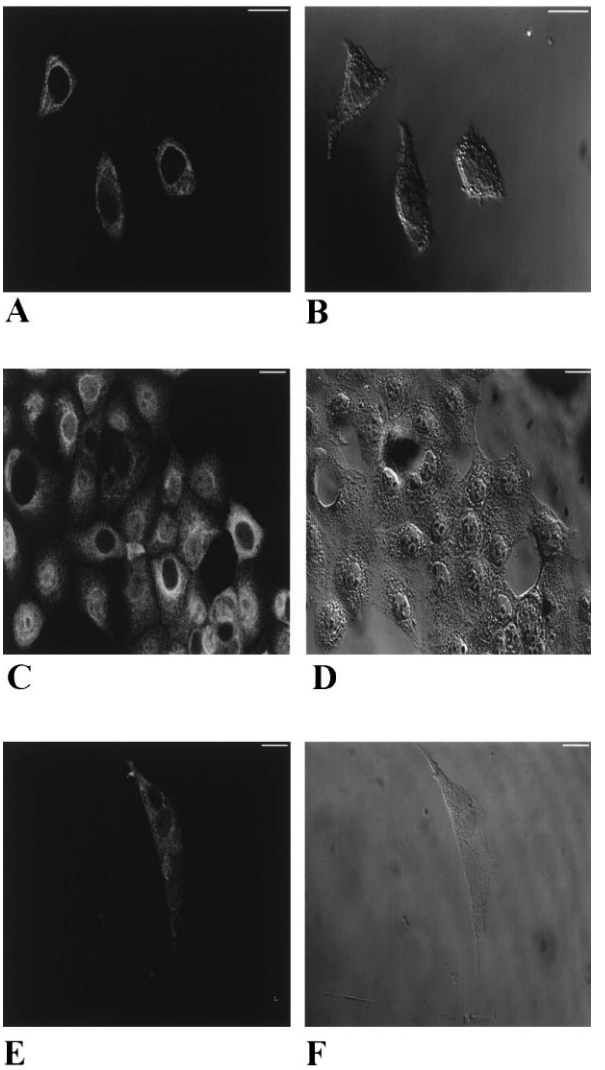

Fig. 1. Distribution of XOR in permeabilised EA-hy-926 cells (A, B), HB4a cells (C, D) and HUVECs (E, F). For experimental details see Section 2. Immunofluorescent (A, C, E) and differential interference contrast (DIC) (B, D, F) images are shown. Magnification ×630 (A, B), ×400 (C–F); bar, 20 μm.

Because of the possibility that XOR on the surface of our cells in culture originated in the growth medium (which contains FCS) the latter was assayed for XOR. Enzymic activity could not be detected by the sensitive fluorimetric procedure (see Section 2). In view of the high affinity of human XOR for heparin [18,19], we sought to concentrate any small amounts of XOR in the growth medium by passage down a column of heparin-Sepharose (see Section 2). In none of six batches of serum was XOR activity detectable by fluorescence assay when the heparin column was subsequently eluted with 1 M NaCl, conditions known to elute the human enzyme [19]. Two further control experiments addressed this issue. In the first of these, parallel cultures of EA-hy-926 or HB4a cells were grown in 75-cm^2 flasks in the corresponding growth medium that had, or had not been passed down a heparin-Sepharose column (see Section 2). In each case, cells were then seeded onto duplicate wells of glass slides as usual and subjected to

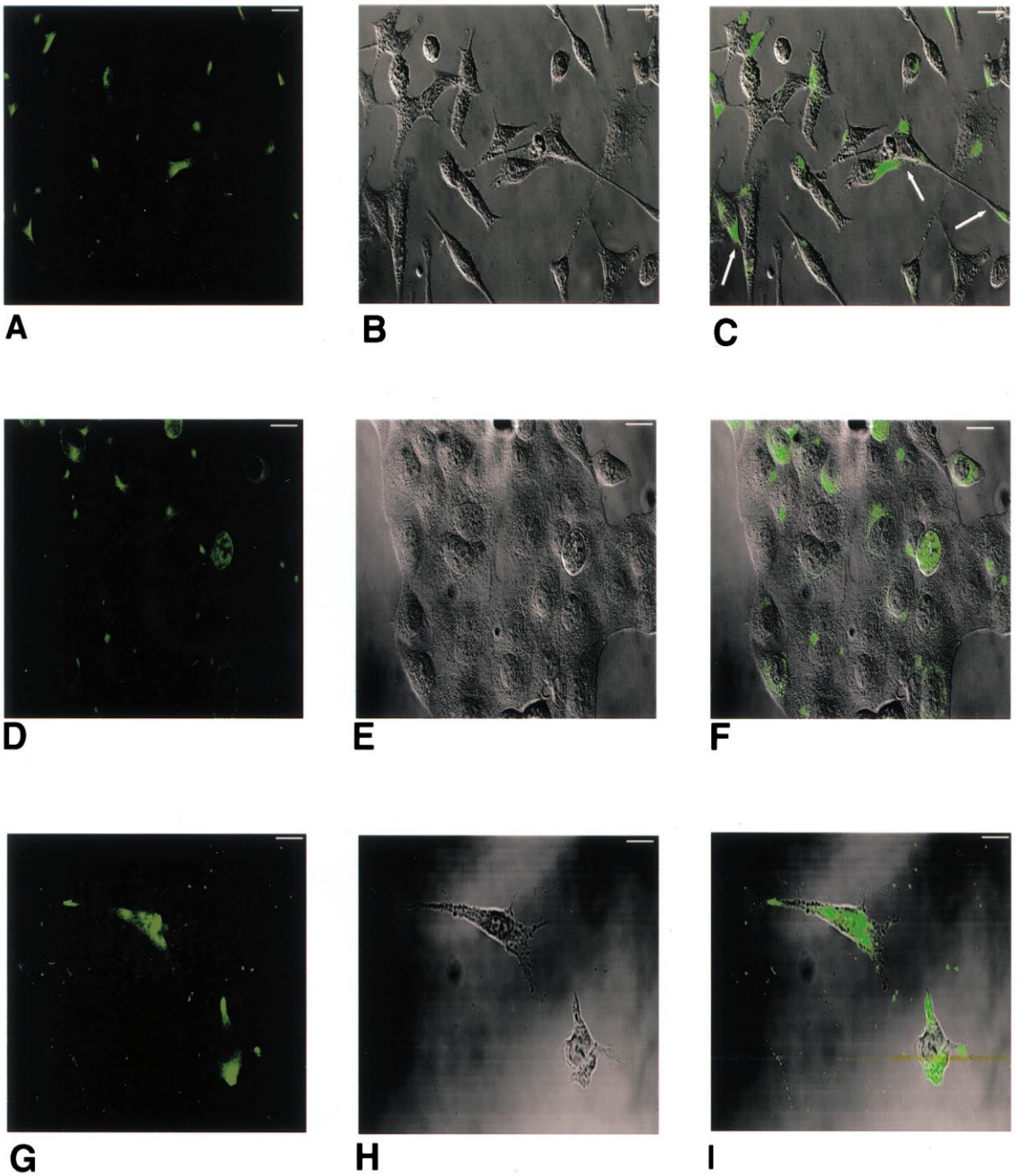

Fig. 2. Distribution of XOR in unpermeabilised EA-hy-926 cells (A–C), HB4a cells (D–F) and HUVECs (G–I). For experimental details see Section 2. Immunofluorescent (A, D, G) and DIC (B, E, H) images are overlaid (C, F, I) to emphasise the polarised distribution. Arrows (C) show examples where fluorescence is concentrated on surfaces that appose those of neighbouring cells. Magnification ×400; bar, 20 μm.

immunolabelling. No difference was apparent in the distribution or intensity of the fluorescence patterns between unpermeabilised cells grown in heparin-treated and untreated medium, nor was there any significant difference in total XOR activity in the cells, as assayed fluorimetrically. Results for EA-hy-926 cells are shown in Fig. 3C–F, in which the polarised distribution of the enzyme is again clearly seen. In the second control experiment, heparin-Sepharose beads (300 μl) were washed twice with PBS before incubation overnight, with continuous gentle agitation, either with growth medium (containing FCS), PBS or with PBS containing bovine XOR (10 μg/ml). Subsequent labelling with anti-XOR antibody showed clear immunofluorescence on the surface of the beads in the latter but not the former case (Fig. 3). Similar incubation of heparin beads with normal goat serum, used as a blocking agent in immunolabelling, also failed to lead to immunofluorescence on the surface of the beads.

4. Discussion

While reactive oxygen species are increasingly being considered as agents of signal transduction [3,4], their source is seldom clear, and XOR, with its capacity for generation of superoxide anion and hydrogen peroxide, is in many cases an attractive candidate [10]. The subcellular localisation of the enzyme is clearly relevant to its function and it is with

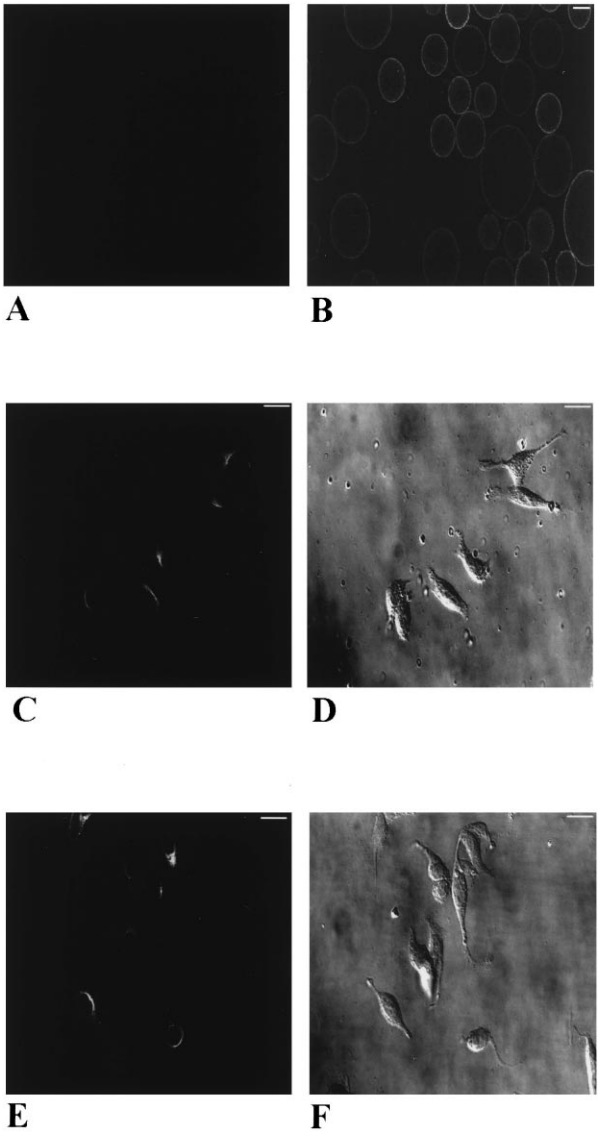

Fig. 3. Control experiments showing that cell surface XOR is not derived from growth medium. Heparin beads were incubated for 24 h with growth medium (A) or with PBS containing bovine XOR (B) (see Section 3). C, D and E, F show EA-hy-926 cells grown in medium (containing FCS), that has (E, F) or has not (C, D) been preabsorbed on a heparin-Sepharose column (see Section 2). Immunofluorescence (A–C, E) and DIC (D, F) images were obtained as described in Section 2. Magnification ×100; bar, 50 μm (A, B); ×400; bar, 20 μm (C–F).

this in mind that we examined the former in cultured human cells.

XOR is generally assumed to be a cytoplasmic enzyme, although its precise localisation is unclear, having been described as being both peroxisomal [7,8] and exclusively cytosolic [9]. In the permeabilised cells of the present study, XOR was seen to be generally distributed throughout the cytoplasm but with more intense staining in the perinuclear region. This latter localisation has not, to our knowledge, been suggested previously and has interesting implications concerning possible functions of the cytoplasmic enzyme. A perinuclear location would, for example, accord with a role for XOR as a source of reactive oxygen species that activate nuclear transcription factors, such as NF-κB [20].

XOR was clearly detected on the outer surface of unpermeabilised cells of all three human cell types studied. While extracellular localisation of XOR has previously been proposed in bovine aortic endothelial cells [21,22], our presently reported findings constitute the first detailed evidence of such a localisation in any cell type. In view of the potential importance of these results, it was necessary to eliminate the possibility that surface enzyme is derived from exogenous sources, such as, for example FCS in the growth media. Growth media did not contain levels of XOR above the limit of sensitivity of the fluorimetric assay. This, in itself, does not necessarily preclude the presence of lower levels of enzyme. However, XOR was not detected in the growth media following attempted concentration of the enzyme by chromatography on heparin-Sepharose, nor were any differences in immunolabelling detected when any of the three cell types was grown in preabsorbed medium. Moreover, growth medium for all three cells failed to show fluorescence labelling of heparin-Sepharose beads when incubated with the latter. Similar results were obtained with goat serum, routinely used as a blocking agent in immunolabelling. Finally, it is highly unlikely that cell surface XOR originated in lysed neighbouring cells, which are at low density early in their growth cycle and are essentially 100% viable.

If we accept that the extracellular XOR is indeed an endogenous enzyme, then the mechanisms of its secretion come into question. The classical secretory pathway of protein biosynthesis involves transfer from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane and depends upon the presence of a cleavable signal peptide [23]. Human XOR has no signal peptide [24,25] and is not known to be glycosylated, a consequence of the classical secretory pathway. However, increasing numbers of polypeptides with these characteristics, that are nevertheless secreted from both prokaryotic and eukaryotic cells, are being discovered [26] and it may well be that XOR is another such protein using a non-classical secretory pathway. In view of the relatively high affinity of XOR for heparin [18,19], it is interesting to note an earlier suggestion [27] that muscle L-14 lectin, exported by a non-classical pathway, would thus be separated from glycoconjugates, with which it interacted, until after its secretion. Similar considerations could apply to XOR, which may be expected to bind to cell surface glycosylaminoglycans following secretion. It is noteworthy that incubation of EA-hy-926 cells with heparin, followed by washing, failed to significantly diminish the intensity of staining (results not shown), suggesting that other glycosylaminoglycans may be involved.

Our results clearly show that XOR is not only present on the outer surface of cultured human endothelial and epithelial cells, but is asymmetrically distributed, in many cases appearing to be localised to surfaces apposed to those of closely neighbouring cells. This extracellular localisation and particularly its polarised nature strongly suggest a role for XOR in cell-cell interactions, possibly involving signalling via reactive oxygen species. We believe this to be an entirely novel concept worthy of detailed further investigations. Such investigations are, however, beyond the scope of the present study.

Acknowledgements: This work was supported in part by grants from the Arthritis and Rheumatism Council and Phytopharm Ltd. The

Biotechnology and Biological Sciences Research Council and the University of Bath are gratefully acknowledged for post-graduate research studentships (to S.P. and R.B. respectively). Mrs Joan Whish is gratefully acknowledged for excellent technical support.

References

[1] Bray, R.C. (1975) in: The Enzymes (Boyer, P.D., Ed.), Vol XII, 3rd edn., pp 299–419, Academic Press, New York.
[2] McCord, J.M. (1985) New Engl. J. Med. 312, 159–163.
[3] Khan, A.U. and Wilson, T. (1995) Chem. Biol. 2, 437–445.
[4] Winyard, P.G. and Blake, D.R. (1997) Adv. Pharmacol. 38, 403–421.
[5] Jarasch, E.-D., Grund, C., Bruder, G., Heid, H.W., Keenan, T.W. and Franke, W.W. (1981) Cell 25, 67–82.
[6] Kooij, A., Bosch, K.S., Fredieriks, W.M. and Van Noorden, C.J.F. (1992) Virchows Arch. B Cell Pathol. 62, 143–150.
[7] Angermuller, S., Bruder, G., Volkl, A., Wesch, H. and Fahimi, H.D. (1987) Eur. J. Cell Biol. 45, 137–144.
[8] Dikov, V.A., Alexandrov, I., Russinova, A. and Boyadjieva-Michailova, A. (1988) Acta Histochem. 83, 107–115.
[9] Ichikawa, M., Nishino, T., Nishino, T. and Ichikawa, A. (1992) J. Histochem. Cytochem. 40, 1097–1103.
[10] Harrison, R. (1997) Biochem. Soc. Trans. 25, 786–791.
[11] Edgell, W.R., McDonald, C.C. and Graham, J.B. (1983) Proc. Natl. Acad. Sci. USA 80, 3734–3737.
[12] Rouquette, M., Stevens, C.R., Blake, D.R., Harrison, R., Whish, J. and Whish, W.D.H. (1997) Biochem. Soc. Trans. 25, 532S.
[13] Stamps, A.C., Davies, S.C., Burman, J. and O'Hare, M.J. (1994) Int. J. Cancer 57, 865–874.
[14] Page, S., Powell, D., Benboubetra, M., Stevens, C.R., Blake, D.R., Selase, F., Wolstenholme, A. and Harrison, R. (1998) Biochim. Biophys. Acta (in press).
[15] Jaffe, E.A., Nachman, R.L., Becker, C.G. and Minick, C.R. (1973) J. Clin. Invest. 52, 2745–2756.
[16] Ponnambalam, S., Girotti, M., Yaspo, M.-L., Owen, C.E., Perry, A.C.F., Suganuma, T., Nilsson, T., Fried, M., Banting, G. and Warren, G. (1996) J. Cell Sci. 109, 675–685.
[17] Beckman, J.S., Parks, D.A., Pearson, J.D., Marshall, P.A. and Freeman, B.A. (1989) Free Radical Biol. Med. 6, 607–615.
[18] Adachi, T., Fukushima, T., Usami, Y. and Hirano, K. (1993) Biochem. J. 289, 523–527.
[19] Sanders, S., Eisenthal, R. and Harrison, R. (1997) Eur. J. Biochem. 245, 541–548.
[20] Pahl, H.L. and Baeuerle, P.A. (1994) BioEssays 16, 497–502.
[21] Bulkley, G.B. (1991) Abstr. Int. Congr. Oxygen Radicals 5th, p. 28.
[22] Schiller, H., Vickers, S., Hildreth, J., Mather, I., Kuhajda, F. and Bulkley, G. (1991) Circ. Shock 34, A435.
[23] Rapoport, T.A. (1992) Science 258, 931–936.
[24] Ichida, K., Amaya, Y., Noda, K., Minoshima, S., Hosoya, T., Sakai, O., Shimizu, N. and Nishino, T. (1993) Gene 133, 279–284.
[25] Xu, P., Hueckstead, T.P., Harrison, R. and Hoidal, J.R. (1994) Biochem. Biophys. Res. Commun. 199, 998–1004.
[26] Kuchler, K. (1993) Trends Cell Biol. 3, 421–426.
[27] Cooper, D.N.W. and Barondes, S.H. (1990) J. Biol. Chem. 110, 1681–1691.