Tissue- and cell-specific expression of mouse xanthine oxidoreductase gene *in vivo*: regulation by bacterial lipopolysaccharide

Mami KUROSAKI,* Marco LI CALZI,* Eugenio SCANZIANI,† Enrico GARATTINI* and Mineko TERA0*‡

*Molecular Biology Unit, Centro Daniela e Catullo Borgomainerio, Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea 62, 20157 Milano, Italy,

and †Istituto di Anatomia Patologica Veterinaria e Patologia Aviare, Facoltà di Medicina Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

The expression of the xanthine oxidoreductase gene was studied in various mouse organs and tissues, under basal conditions and on treatment with bacterial lipopolysaccharide. Levels of xanthine oxidoreductase protein and mRNA were compared in order to understand the molecular mechanisms regulating the expression of this enzyme system. The highest amounts of xanthine oxidoreductase and the respective mRNA are observed in the duodenum and jejunum, where the protein is present in an unusual form because of a specific proteolytic cleavage of the primary translation product present in all locations. Under basal conditions, multiple tissue-specific mechanisms of xanthine

oxidoreductase regulation are evident. Lipopolysaccharide increases enzyme activity in some, but not all tissues, mainly via modulation of the respective transcript, although translational and post-translational mechanisms are also active. *In situ* hybridization studies on tissue sections obtained from mice under control conditions or with lipopolysaccharide treatment demonstrate that xanthine oxidoreductase is present in hepatocytes, predominantly in the proximal tubules of the kidney, epithelial layer of the gastrointestinal mucosa, the alveolar compartment of the lung, the pulpar region of the spleen and the vascular component of the heart.

INTRODUCTION

Xanthine oxidoreductase (XOR) is a molybdoflavoprotein which catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid [1]. It is a single gene product [2], a homodimer of two identical 150 kDa subunits [3], and it exists in two interconvertible forms, xanthine dehydrogenase (EC 1.1.1.204; XD) and xanthine oxidase (EC 1.1.3.22, formerly EC 1.2.3.2; XO). The former uses NAD⁺ as the acceptor of reducing equivalents whereas the latter transfers them to molecular oxygen. XD can be converted into XO, either irreversibly by proteolysis or reversibly through oxidation of crucial cysteine residues [4,5].

On the basis of its substrate specificity, XOR is believed to play a key role in the catabolism of purines, although its physiological function has not yet been clarified in detail. It may also be important in the protection of the organism from local oxidative stress by virtue of its ability to produce uric acid, a strong physiological antioxidant [6]. On the other hand, in certain situations, it may become a pathogenetic mediator. In fact, XO (unlike XD) is capable of producing toxic superoxide radicals, thus it is implicated as a possible cause of tissue damage in various clinical and experimental conditions [7–12].

Acute intraperitoneal administration of bacterial lipopolysaccharide (LPS) to mice mimics bolus release from Gram-negative bacteria after antibiotic therapy or perforation of the gastrointestinal tract. Oxygen-radical production, which is associated with LPS-induced cytotoxicity, is implicated in injury to endothelial and other types of cells [13]. XO represents a source of superoxide anions and the enzyme may thus mediate some of the toxic effects induced by LPS. The endotoxin is known to influence XOR activity in the liver and in a few other tissues [14]. Recently we demonstrated that XOR mRNA is

augmented in the liver after challenge of mice with sublethal doses of LPS [15]. Furthermore, XOR mRNA is modulated in fibroblasts and endothelial cells by type-I and type-II interferons [16,17], which are known to be released into the circulation during infection.

In this study we report on the expression of XOR apo- and holo-enzyme in various mouse tissues and compare this with the relative expression of the respective mRNA under basal conditions and on treatment with an acute dose of LPS. We also establish the cell types responsible for the synthesis of XOR in the various tissues by means of *in situ* hybridization using a specific mouse XOR complementary RNA as a probe.

MATERIALS AND METHODS

Animal treatments

CD1 male mice obtained from Charles River Italia (Calco, Como, Italy), weighing 25–30 g, were used for all the experiments. Bacterial LPS (Sigma, St. Louis, MO, U.S.A.) was dissolved in saline and administered intraperitoneally to the mice (four animals in each experimental group) at a concentration of 2 mg/kg body weight. Unless otherwise specified, mice were killed by cervical dislocation 16 h after treatment.

Enzyme assay

The organs were homogenized in 2–10 vol. of 50 mM Tris/HCl buffer (pH 8.0) containing 0.5 mM EDTA, 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 2 mM leupeptin and 0.15 µM aprotinin unless otherwise specified. After centrifugation

Abbreviations used: XOR, xanthine oxidoreductase; XD, xanthine dehydrogenase; XO, xanthine oxidase; LPS, lipopolysaccharide; poly(A)⁺, polyadenylated; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; G6PDH, glucose-6-phosphate dehydrogenase.

‡ To whom correspondence should be addressed.

at 105000 *g* for 45 min, cytosolic supernatants were used for XOR assay [18]. Briefly, total XOR activity, corresponding to the sum of XD and XO, was measured in the presence of NAD⁺ in the reaction mixture, whereas total XO activity was measured in the absence of the nicotinamide cofactor. The irreversible form of XO was measured in the absence of NAD⁺, after reduction of the enzyme in the presence of dithiothreitol (DTT) at 4 °C for 30 min. One unit of activity is defined as the amount of enzyme capable of transforming 1 nmol of hypoxanthine into xanthine and uric acid in 1 min at 37 °C. Proteins were determined using the Bio-Rad kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with BSA (Sigma) as standard.

Western-blot analysis

The protein homogenates prepared for XOR assay were pooled and used for Western-blot analysis after electrophoresis on 8% polyacrylamide gels. Conditions for gel electrophoresis, blotting and detection of the specific XOR bands were as described previously [19]. Immunoreactive protein bands were revealed by a chemiluminescence-based procedure using the ECL detection kit (Amersham) according to the instructions of the manufacturer. XOR protein was quantified by densitometric analysis with an RAS 3000 videoimaging system (Amersham).

In vitro translation

Total and polyadenylated [poly(A)⁺]RNA were extracted by standard procedures [20]. Poly(A)⁺RNA was translated *in vitro* into protein with commercially available rabbit reticulocyte lysates (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. The final reaction mixture contained 14 µg of poly(A)⁺ RNA, 140 µCi of [³⁵S]methionine (> 1000 Ci/mmol; Amersham), 120 units of RNasin (Promega), 20 mM amino acid mixture, 123 µl of rabbit reticulocyte lysate in a total volume of 175 µl. The reaction mixture was diluted to 2 ml by addition of the following buffer: 0.05 M Tris/HCl (pH 7.5), 0.15 M NaCl, 0.1% (v/v) Nonidet P-40, 0.001 M EDTA, 0.25% gelatin and 0.02% (w/v) NaN₃. One-half was incubated in the presence of the specific XOR immunoglobulin fraction, and the other half was challenged with a non-immune immunoglobulin fraction, at 4 °C, overnight. Immunoprecipitates were adsorbed to Protein G-Sepharose (Pharmacia) before electrophoresis in 8% polyacrylamide gel as described above.

Northern-blot analysis

Total RNA was prepared from tissues of four mice, 8 or 16 h after treatment with vehicle alone or LPS [20]. The RNA (30 µg) was fractionated on a 1% agarose/6% formaldehyde gel and blotted to a nylon membrane (GeneScreen Plus; New England Nuclear, Boston, MA, U.S.A.). Membranes were hybridized to the radiolabelled 2 kb fragment of the mouse XD cDNA, ZAP64 [15]. The conditions for labelling and hybridization were as previously described [15,19].

Preparation of riboprobes and *in situ* hybridization

The 2 kb mouse XD cDNA fragment, ZAP64 [15], was subcloned into the plasmid vector Bluescript KS (Stratagene, LaJolla, CA, U.S.A.) and used as a template for the synthesis of sense and antisense riboprobes, employing T3 and T7 RNA polymerases (Stratagene) in the presence of [³⁵S]thio]UTP (specific radioactivity 1200 Ci/mmol, Amersham). Template DNA was degraded by DNAase I (Pharmacia), and the average length of the

riboprobes was adjusted to approx. 150 nucleotides by alkaline treatment [21].

In situ hybridization was performed by standard methodology [21] using paraffin-embedded mouse tissue sectioned to 5 µm thickness and mounted on chromalum (Sigma)-containing gelatin-coated slides. Hybridization was performed in a solution containing 50% formamide, 0.3 M NaCl, 0.02 M Tris/HCl (pH 8), 0.005 M EDTA, 0.001 M NaPO₄, 10% dextran sulphate, 1 × Denhardt's [0.02% sodium citrate, 0.02% poly(vinyl pyrrolidone), 0.02% BSA], 0.04 M DTT, 0.5 mg/ml yeast tRNA and 6 µg/ml riboprobe (specific radioactivity approx. 5 × 10⁸ c.p.m./µg of RNA) at 50 °C overnight. Slides were washed in 50% formamide/2 × SSC/0.1 M DTT (where 1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65 °C for 10 min, and then treated with 20 µg/ml RNAase A (Boehringer) in 0.01 M Tris/HCl (pH 7.5)/0.001 M EDTA at 37 °C for 30 min. Slides were further washed sequentially at 50 °C in 50% formamide/5 × SSC/0.01 M DTT for 1 h, in 2 × SSC/0.01 M DTT for 15 min twice, and in 0.1 × SSC/0.01 M DTT for 15 min twice. Slides were subsequently dehydrated by immersion in graded ethanol series containing 0.3 M ammonium acetate, air-dried, and dipped in Ilford nuclear track emulsion (diluted 1:1 with water) (Ilford, Moberley, Cheshire, U.K.). After 10 days of exposure, the slides were developed in D19 developer (Kodak). The sections were stained with haematoxylin/eosin according to standard cytochemical methodology.

RESULTS AND DISCUSSION

Modulation of XOR activity by LPS

Absolute amounts of XOR activity and the proportion of this enzyme in the XO and XD forms were determined under basal conditions and after treatment with LPS. The results of an experiment representative of two are shown in Table 1. Under basal conditions, by far the highest levels of XOR activity are observed in the small intestine, followed by the lung and liver. Low levels are found in the kidney, spleen and heart. In the intestine, a steep gradient of XOR is evident from the most proximal to the most distal portion. The duodenum contains approx. 2–2.5-fold more XOR activity than the jejunum and the ileum, and 24-fold more than the colon and the rectum. Our results are consistent with data recently reported by Mohamedali et al. [22] from a study on the distribution of XO and adenosine deaminase, the two rate-limiting enzymes in the catabolism of purines. In most tissues, a variable amount of total XOR activity is present in the XO form and the rest is represented by the XD form, which is obtained by subtraction of XO from XOR. The ratio of XO/XOR is high in the intestinal tract whereas it is lower in the lung, liver, kidney, spleen, stomach and heart. In all the tissues, a substantial amount of XO is present in the reversible form, as *in vitro* treatment of the extracts with DTT converts XO into XD. In fact, whereas the amount of total XOR does not change after DTT treatment (results not shown), a considerable decrease in XO activity is observed. The proportion of irreversible XO is approx. 25% of the total activity in all the organs examined, except for the duodenum and jejunum where this value is higher (around 40%).

Treatment of animals with LPS for 16 h results in a statistically significant induction of XOR activity in various tissues and organs. The highest increase (3-fold) is observed in the kidney, followed by spleen, liver, rectum and colon. XOR is refractory to induction by LPS in oesophagus, stomach, jejunum, ileum, lung and heart. By contrast, a consistent, but statistically not significant, decrease in XOR activity is observed in the duodenum. Thus LPS increases XOR activity in a tissue-specific

Table 1 XOR activity in various mouse tissues

Tissues were isolated from animals 16 h after administration of a single intraperitoneal dose of LPS (2 mg/kg) in saline (LPS) or saline alone (control). They were then homogenized and assayed for content of total XO, irreversible XO and total XOR (XD + XO). Each value is the mean $\pm$ S.D. of four separate animals. * $P < 0.05$, ** $P < 0.01$ compared with control after one way analysis of variance (Tukey's test).

Tissue	XO (munits/mg of protein)		XD + XO (munits/mg of protein)		Irreversible XO (munits/mg of protein)		XO/(XD + XO) (%)		Irreversible XO/(XD + XO) (%)		Fold induction
	Control	LPS	Control	LPS	Control	LPS	Control	LPS	Control	LPS	
Oesophagus	2.0 $\pm$ 0.1	2.5 $\pm$ 0.7	3.3 $\pm$ 0.6	4.2 $\pm$ 1.3	0.8 $\pm$ 0.3	1.1 $\pm$ 0.1	61 $\pm$ 15	60 $\pm$ 3	24 $\pm$ 4	26 $\pm$ 11	1.3
Stomach	1.5 $\pm$ 0.1	2.2 $\pm$ 0.2*	3.7 $\pm$ 1.5	4.3 $\pm$ 1.0	0.8 $\pm$ 0.6	1.2 $\pm$ 0.3	41 $\pm$ 2	51 $\pm$ 6	22 $\pm$ 16	28 $\pm$ 3	1.2
Duodenum	54.5 $\pm$ 10.7	41.1 $\pm$ 18.1	72.2 $\pm$ 18.6	56.9 $\pm$ 25.5	29.4 $\pm$ 9.2	24.3 $\pm$ 8.9	75 $\pm$ 12	72 $\pm$ 15	41 $\pm$ 3	43 $\pm$ 4	0.8
Jejunum	29.0 $\pm$ 10.6	30.7 $\pm$ 12.3	38.0 $\pm$ 18.7	40.3 $\pm$ 17.8	14.7 $\pm$ 11.8	14.3 $\pm$ 5.8	76 $\pm$ 4	76 $\pm$ 4	39 $\pm$ 10	35 $\pm$ 7	1.1
Ileum	23.3 $\pm$ 1.3	20.7 $\pm$ 1.8	26.2 $\pm$ 7.9	25.9 $\pm$ 5.7	6.3 $\pm$ 1.3	7.1 $\pm$ 1.5	89 $\pm$ 5	80 $\pm$ 7	24 $\pm$ 5	27 $\pm$ 1	1.0
Colon	2.2 $\pm$ 0.2	4.4 $\pm$ 1.4*	3.0 $\pm$ 0.3	5.3 $\pm$ 0.6**	0.9 $\pm$ 0.2	1.2 $\pm$ 0.4	73 $\pm$ 7	83 $\pm$ 14	30 $\pm$ 3	23 $\pm$ 6	1.8
Rectum	2.1 $\pm$ 0.3	6.4 $\pm$ 2.4*	3.1 $\pm$ 0.5	7.3 $\pm$ 1.5**	0.7 $\pm$ 0.1	1.3 $\pm$ 0.3**	68 $\pm$ 15	58 $\pm$ 11	22 $\pm$ 3	18 $\pm$ 6	2.4
Liver	2.6 $\pm$ 0.8	8.4 $\pm$ 2.5**	5.1 $\pm$ 1.1	12.7 $\pm$ 1.2**	1.2 $\pm$ 0.4	3.6 $\pm$ 0.7**	51 $\pm$ 13	66 $\pm$ 15	23 $\pm$ 3	28 $\pm$ 3	2.5
Lung	3.7 $\pm$ 0.4	6.5 $\pm$ 0.5**	6.1 $\pm$ 1.2	8.9 $\pm$ 1.3*	1.4 $\pm$ 0.4	2.3 $\pm$ 0.6	67 $\pm$ 7	73 $\pm$ 6	23 $\pm$ 4	26 $\pm$ 4	1.5
Kidney	0.7 $\pm$ 0.1	2.8 $\pm$ 0.2**	2.1 $\pm$ 0.7	6.2 $\pm$ 0.6**	0.8 $\pm$ 0.4	1.7 $\pm$ 0.4	33 $\pm$ 6	45 $\pm$ 4*	38 $\pm$ 12	27 $\pm$ 6	3.0
Heart	1.2 $\pm$ 0.2	1.3 $\pm$ 0.7	2.9 $\pm$ 0.7	2.8 $\pm$ 0.6	0.6 $\pm$ 0.1	0.7 $\pm$ 0.2	41 $\pm$ 10	46 $\pm$ 13	20 $\pm$ 5	25 $\pm$ 6	1.0
Spleen	1.0 $\pm$ 0.1	2.5 $\pm$ 0.2**	2.0 $\pm$ 0.9	5.1 $\pm$ 0.8**	0.4 $\pm$ 0.1	1.3 $\pm$ 0.4**	50 $\pm$ 7	49 $\pm$ 4	20 $\pm$ 6	25 $\pm$ 8	2.6

manner. Whether this phenomenon reflects cell-specific regulation of the enzyme, natural resistance of the duodenum and jejunum to LPS or differences in the distribution, uptake and catabolism of the bacterial wall component in the various tissues remains to be established. Except for kidney, where LPS induces XO more effectively than XD, in all tissues, the bacterial endotoxin does not significantly alter the ratio between the XO and XD forms. In addition, LPS treatment has no effect on the amount of XO that can be converted into XD by DTT.

Effect of LPS on immunoreactive XOR protein and correlation with enzyme activity

XOR is a complex enzyme system that requires molybdenum, FAD and NAD as cofactors [23–25]. Thus steady-state levels of XOR are potentially modulated at the post-translational level through control of the balance between apo- and holo-enzyme. We investigated whether the amounts of XOR apo- plus holo-protein correlate with the levels of enzyme activity under basal conditions and after treatment with LPS. For this purpose, the same homogenates as used for the determination of enzyme activity shown in Table 1 were pooled and used for Western-blot analysis, using a specific polyclonal antibody [19]. An experiment representative of two highly reproducible ones is shown in Figure 1(a). In kidney, the XOR band is not detectable under basal conditions. After SDS/PAGE, a single band of approx. 150 kDa is visible for most of the tissues obtained from control animals. This is consistent with the size of the primary translation product in its monomeric form, as predicted from nucleotide sequencing of the respective cDNA [15]. The minor bands at lower molecular mass observed in the liver and ileum on LPS induction represent non-specific degradation of the protein and are not a consistent finding.

In the duodenum and jejunum, a major immunoreactive band of approx. 90 kDa is observed under basal conditions and after LPS treatment. To clarify the nature of this band, we performed an *in vitro* translation experiment using rabbit reticulocyte lysates and poly(A)⁺ RNA extracted from the duodenum and liver of animals treated with vehicle or LPS respectively. As shown in Figure 2(a), specific XOR protein bands with an apparent molecular mass 150 kDa were immunoprecipitated after translation of both duodenum and liver poly(A)⁺ RNA. The identity

of the immunoprecipitation products with XOR was verified by the absence of any specific band after challenge with preimmune immunoglobulins. Furthermore, as expected, concentrations of the immunoprecipitation product were highest in lysates containing RNA extracted from the duodenum followed by the liver of LPS-treated and control animals respectively. These results, along with the presence in the duodenum of an XOR mRNA of a size indistinguishable from that observed in all the other tissues (see Figure 4), suggest that the primary XOR translation product detectable in the first portion of the small intestine is not unusual. It is worth noting that primer-extension analysis supports this conclusion [2]. In addition, as shown in Figure 2(b), high concentrations of soya-bean trypsin inhibitor in the homogenization buffer shifted the mobility of most duodenal and jejunal XOR on SDS/PAGE to the same position as that observed in the liver. These data demonstrate that the 90 kDa band observed in the first part of the intestine is the result of *in vitro* proteolysis during homogenization of the 150 kDa XOR subunit by an as yet unrecognized protease, as suggested by Duke et al. [26]. Although this phenomenon is clearly not significant under physiological conditions, it may acquire importance in pathological states involving cell damage or necrosis, where the protease may gain access to the XOR substrate and act on it.

Quantitative results from densitometric analysis of the autoradiogram presented in Figure 1(a) are illustrated in Figure 1(b). In good agreement with the levels of enzyme activity under basal conditions, the duodenum contains the highest amounts of XOR immunoreactive band, followed by jejunum, ileum, colon, lung, stomach, rectum and liver. Oesophagus, heart and spleen contain very small amounts of the protein. In kidney, XOR immunoreactive protein becomes detectable on LPS induction. The bacterial endotoxin significantly increases the intensity of the XOR band observed in the rectum, liver, spleen, stomach and oesophagus. In the small intestine, colon, heart and lung, treatment with LPS does not result in increased XOR protein. In contrast, the duodenum is the only tissue in which a fall in XOR protein concentration is evident as a consequence of the treatment. This is consistent with the decrease in XOR activity reported in Table 1.

The steady-state levels of XOR activity and immunoreactive protein in the various tissues before and after treatment with LPS

Figure 1 Western-blot analysis of XOR protein in tissue homogenates obtained from control and LPS-treated mice

(a) Animals were treated with 2 mg/kg LPS in saline (LPS) or with vehicle alone (C). At 16 h after injection, animals were killed, tissues were homogenized and subjected to Western-blot analysis using an XOR-specific polyclonal antibody. The immunoreactive band was revealed by use of a chemiluminescent substrate after incubation with the secondary antibody linked to horseradish peroxidase. Western-blot analysis was carried out with 100 μ g of protein extracted from the indicated tissues. The XOR band in the lane corresponding to the LPS-treated kidney is visible in the original autoradiography although it does not appear clearly in the picture. The positions of the molecular-mass markers are indicated (myosin, 200 kDa; phosphorylase *b*, 97 kDa; BSA, 68 kDa). (b) Densitometric analysis of the autoradiogram shown in (a); the results are normalized relative to the absorbance for lung under control conditions which was taken as 1. The numbers represent the fold increase in level of XOR immunoreactive protein after LPS treatment relative to control conditions. □, Control; ■, LPS.

are compared in Table 2. If the basal levels of total XOR activity are divided by the corresponding concentration of immunoreactive protein in the various tissues, an index of the proportions of the protein present in the holo- and apo-forms is obtained. The higher the number, the greater the amount of XOR in the holo-form. Under basal conditions most of the tissues have a high proportion of XOR protein in the holoenzyme form, with the oesophagus showing the highest value. In contrast, in the stomach, colon and rectum, there seems to be a relative deficiency in the post-translational mechanisms controlling the assembly of the active enzyme, as the proportion of XOR protein in the holo-form is low. Treatment of animals with LPS does not lead to major changes in the proportion of XOR protein in the holo-form.

Effects of LPS on the levels of the XOR transcript in mouse tissues

In a previous report, we demonstrated that XOR mRNA is induced in the liver of LPS-sensitive strains of mice after systemic treatment with the bacterial toxin [15]. To correlate the steady-state levels of protein with the corresponding mRNA, we assessed the time course of induction after LPS treatment of the XOR

transcript, immunoreactivity and enzyme activity in the liver, by Northern-blot, Western-blot and enzyme analysis respectively. Densitometric analysis of the Northern blot reveals a rapid transient increase in XOR mRNA at 8 h (Figure 3a). At 16 h, the levels of transcript have already returned to baseline. The data are normalized for the amount of RNA loaded in each lane as assessed by determination of mRNA coding for the L32 ribosomal protein [27]. This is a good indicator of the amount of RNA loaded, as the level of expression of this transcript is relatively constant regardless of the tissue and treatment (less than 1.5-fold difference in the various organs; see also Figure 4a). As shown in Figure 3(b), induction of XOR immunoreactivity follows the increase in the respective mRNA, and maximal levels of the protein are observed at 16 h with a subsequent return to baseline levels at 24 h. Similarly, induction of enzyme activity is highest at 16 h, as illustrated in Figure 3(c). These results indicate that there is a lag of at least 8 h between up-regulation of XOR mRNA and increase in the respective translation product, whereas assembly of the active enzyme from the primary translation product is almost immediate.

On the basis of the kinetics of XOR transcript induction observed in liver after LPS treatment, 8 and 16 h were the time points selected for the determination by Northern-blot analysis

Figure 2 *In vitro* translation of XOR mRNA extracted from duodenum and liver: effect of high concentrations of soya-bean trypsin inhibitor on the mobility of duodenal XOR protein on PAGE in reducing and non-reducing conditions

(a) *In vitro* translation was performed using rabbit reticulocyte lysates enriched with poly(A)⁺ RNA (14 µg) extracted from control duodenum (lanes 1, 2 and 7) and from control liver (lanes 5, 6 and 9) or liver treated with LPS for 16 h (lanes 3, 4 and 8). The products of *in vitro* translation before (lanes 7, 8 and 9; 2% of the total translation product) and after immunoprecipitation with the specific XOR polyclonal antibody (lanes 1, 3 and 5) or preimmune immunoglobulin fraction (lanes 2, 4 and 6) are shown. (b) Liver, jejunum and duodenum were homogenized in the presence of leupeptin (2 mM), aprotinin (0.15 µM), PMSF (0.1 mM), EDTA (0.5 mM) and soya-bean trypsin inhibitor (12.5 µM) and subjected to Western-blot analysis using an XOR-specific polyclonal antibody. The immunoreactive band was revealed by use of a chemiluminescent substrate after incubation with the secondary antibody linked to horseradish peroxidase. Western-blot analysis was carried out with 100 µg of protein extracted from each of the indicated tissues. Prestained molecular-mass markers, indicated on the right, are myosin (200 kDa), phosphorylase *b* (97 kDa) and BSA (68 kDa).

Table 2 Correlation of XOR activity with immunoreactive protein in various tissues of mice under basal conditions and on treatment with LPS

Results were obtained by dividing total XOR activity (from Table 1) by relative XOR protein (from densitometric analysis in Figure 1b). As explained in the text, the resulting value is an index of the amount of XOR present in the apo- and holo-protein form. N.D., not determined; the amounts of XOR protein were too low to be quantified by densitometric analysis of the autoradiograms.

Tissue	Ratio of XOR activity/protein	
	Control	LPS
Oesophagus	33.0	21.0
Stomach	4.6	2.7
Duodenum	14.2	20.8
Jejunum	10.0	13.0
Ileum	9.4	10.4
Colon	2.3	3.8
Rectum	3.9	2.5
Liver	12.8	7.9
Kidney	N.D.	68.9
Heart	17.0	15.6
Lung	6.1	8.1
Spleen	11.8	15.9

of steady-state mRNA levels in all the other tissues. To check the specificity of the effects observed on XOR mRNA, the blots were always rehybridized with cDNA probes coding for the L32 ribosomal protein and the product of glucose-6-phosphate dehydrogenase (G6PDH) respectively. Representative results after

Figure 3 Kinetics of the induction of XOR mRNA, immunoreactive protein and enzyme activity in mouse liver after LPS administration

Four animals per experimental group were killed at the indicated time after LPS administration; livers were homogenized and used for the preparation of RNA or protein. For the samples used for Northern- and Western-blot analysis, individual extracts were pooled. For the samples used for determination of enzyme activity, individual extracts were assayed separately. (a) RNA samples were subjected to Northern-blot analysis and the resulting filter was sequentially hybridized with radiolabelled mouse XOR and L32 ribosomal cDNAs. The results of the densitometric analysis conducted on these autoradiograms are shown and are expressed as the ratio of absorbance values determined for XOR and L32 ribosomal RNAs respectively. The amount of L32 ribosomal RNA was constant in all the samples (less than 20% variation). (b) Equivalent amounts of protein extract (100 µg) were electrophoresed on SDS/8% polyacrylamide gels, blotted onto nitrocellulose and subjected to Western-blot analysis. The quantitative results obtained after densitometric scanning of the corresponding autoradiogram are shown. Expression of XOR in lung under basal conditions was used to standardize the data as in Figure 2. The absorbance obtained for the lung under control conditions is taken as 1. (c) Total XOR (XD + XO) activity was measured on the same liver extracts as used for Western-blot analysis before pooling of the samples, thus the results are means $\pm$ S.D. for four separate animals. Asterisks indicate that the values are significantly higher than those observed before treatment with LPS (time 0) after one way analysis of variance ($P < 0.01$ according to Turkey's test).

8 h of treatment with saline or LPS are shown in Figure 4(a). Both under basal conditions and after treatment with bacterial endotoxin, an XOR transcript of similar length (approx. 4500 bases) is observed in all the tissues albeit at different concentrations. The length of XOR mRNA is consistent with that determined by molecular cloning of the respective cDNA and gene [2,15]. To obtain quantitative data on the expression of XOR mRNA, densitometric analysis of the autoradiogram presented in Figure 4(a) and of a similar autoradiogram (not shown) obtained in a separate experiment, in which animals were

Figure 4 Effects of LPS on XOR mRNA levels in various mouse tissues

(a) Total RNA was obtained from the indicated tissues of four animals treated for 8 h with 2 mg/kg LPS in saline (LPS) or saline alone (C). RNA (30 µg/lane) was loaded on a 1% formaldehyde/agarose gel and subjected to Northern-blot analysis. After transfer, nylon membranes were sequentially hybridized with mouse XOR, mouse L32 ribosomal and human G6PD cDNAs. The positions of 28 S and 18 S rRNA are shown on the right. (b) Densitometric analysis of the autoradiogram shown (a) (□, control; ■, LPS for 8 h) and of an equivalent autoradiogram obtained from Northern-blot analysis of RNA extracted from the indicated tissues of four animals, 16 h after intraperitoneal injection of LPS in saline (▨). As the values obtained for the latter group were no different from those obtained after 8 h of treatment with saline, they are not included. Values are expressed as the ratio of the absorbance determined for XOR and L32 RNAs respectively. These values are normalized for the ratio of XOR/L32 observed for the lung in control conditions, and taken as 1. The amount of L32 rRNA was constant in all the samples (less than 20% variation), demonstrating that the same amount of RNA was loaded in each lane.

killed 16 h after treatment with LPS, was performed and is shown in Figure 4(b). Under basal conditions, the XOR mRNA is extensively expressed in the duodenum, lung, jejunum, ileum, stomach and heart. Relatively low levels of the transcript are found in all the other tissues. Consistent with measurements of immunoreactive protein and enzyme activity, a steep gradient in the expression of XOR mRNA is observed in the small intestine from the most proximal to the most distal region. If the results presented in Figures 1(b) and 4(b) are compared, it is evident that, under basal conditions, there is a general correlation between the steady-state levels of XOR transcript and the respective protein in the various tissues. This suggests that the rate of mRNA accumulation is generally the limiting factor in the expression of XOR activity in a given organ or tissue. Notable exceptions are stomach and heart, where relatively high levels of XOR mRNA are associated with very low levels of the respective protein. In these two tissues, under basal conditions,

the rate of translation of the XOR transcript may play a more important role than in other organs.

After 8 h of LPS treatment, the amounts of transcript are markedly decreased in the duodenum, relatively unaffected in the stomach, colon, heart and lung, and augmented in all the other tissues examined. The highest increase is observed in the kidney followed by rectum, ileum, liver, spleen and jejunum. In most tissues, the effect of LPS on the XOR transcript is reduced or completely abrogated after 16 h of treatment with LPS. Exceptions are the colon, where a dramatic rise in the levels of XOR transcript is observed between 8 and 16 h, and the stomach, heart and spleen, where the level of induction remains constant at 8 and 16 h. Notice that, although differences in the basal expression of G6PDH mRNA in the various tissues are evident, LPS does not affect concentrations of the transcript. This demonstrates that the bacterial endotoxin does not have a general effect on the accumulation of RNA polymerase II-

Figure 5 *In situ* hybridization of XOR mRNA in liver, lung, kidney, spleen and heart

Liver, lung, kidney, spleen and heart were obtained from mice 16 h after treatment with LPS in saline or saline alone as indicated. Tissues were fixed in paraformaldehyde, embedded in paraffin, cut to 5 μ m thickness and processed for *in situ* hybridization with mouse XOR sense and antisense riboprobes. (a) LPS-treated liver hybridized with sense XOR riboprobe; (b) LPS-treated liver hybridized with antisense XOR riboprobe; (c) control liver hybridized with antisense XOR riboprobe; (d) and (e) LPS-treated lung hybridized with antisense XOR riboprobe; (f) LPS-treated kidney hybridized with antisense XOR riboprobe; (g) LPS-treated spleen hybridized with antisense XOR riboprobe; (h) and (i) LPS-treated heart hybridized with antisense XOR riboprobe. The magnification for (a), (b), (c), (e) and (g) is $1.25 \times 6.3 \times 25$, for (d) $1 \times 5 \times 16$, for (f) $1.6 \times 8 \times 25$, for (h) $1.6 \times 8 \times 40$ and for (i) $2.5 \times 12.5 \times 100$. Arrowheads in (g) indicate the position of a group of XOR-positive cells.

dependent transcripts. It is difficult to correlate the levels of XOR mRNA and protein on treatment with LPS, as the respective kinetics of synthesis and degradation are different. However, in this and various other experiments, we observed that XOR mRNA up- and down-regulation by LPS is generally more relevant from a quantitative point of view than the modulation of the respective apo- and holo-protein.

Determination of XOR-positive cells by *in situ* hybridization

To determine which cell types are responsible for the expression and regulation of the XOR gene in the various mouse tissues

before and after treatment with LPS, *in situ* hybridization was performed on paraffin-embedded sections with the use of a riboprobe synthesized from the recently isolated mouse XOR cDNA [15].

As shown in Figures 5 and 6 for the liver and the duodenum respectively, the silver grains observed on the sections are the result of specific hybridization of the antisense riboprobe to the XOR transcript, as no accumulation of grains (compare Figures 5a and 5b and 6d and 6e) is observed on vicinal sections hybridized with an equally labelled sense riboprobe. Specificity of the XOR signal was verified in the same way for all the other tissues, although the results with sense XOR riboprobe are not

Figure 6 *In situ* hybridization of XOR mRNA in the gastrointestinal tract

Stomach, duodenum, jejunum, colon and rectum were obtained from mice 16 h after treatment with LPS in saline or saline alone as indicated. Tissues were fixed in paraformaldehyde, embedded in paraffin, cut to 5 μ m thickness and processed for *in situ* hybridization with mouse XOR sense and antisense riboprobes. (a), (b) and (c) Mucosa of the fundus, corpus and pylorus from LPS-treated animals respectively hybridized with antisense XOR riboprobe; (d) and (e) two consecutive sections from control duodenum, hybridized with sense and antisense XOR riboprobes respectively; (f) LPS-treated duodenum hybridized with antisense XOR riboprobe; (g) control jejunum hybridized with antisense XOR riboprobe; (h) LPS-treated colon hybridized with antisense XOR riboprobe; (i) LPS-treated rectum hybridized with antisense XOR riboprobe. The magnification for all the panels is $1.25 \times 6.3 \times 25$. Arrowheads in (h) indicate the position of a group of XOR-positive cells.

shown. In the liver (Figures 5a–5c), XOR mRNA is expressed predominantly in hepatocytes and the distribution of the grains throughout the organ is patchy, with areas of high density interrupted by areas of low density. This suggests that the synthesis of XOR is by a heterogeneous population of hepatocytes with no obvious relation to the general architecture of the organ. In the lung (Figures 5d and 5e), no XOR message is observed in cells lining bronchi and bronchioli, but a uniform distribution of the granules is evident at the level of the alveolar wall. However, it is impossible to establish whether XOR mRNA is associated with the epithelial or the endothelial component of the alveolus. In the heart (Figures 5h and 5i), it is clear that

cardiocytes express very low levels of the XOR message, whereas we observed greater accumulation of the transcript in the endothelial component of the ventriculi. The fact that the vascular component of the heart is the richest in XOR is of extreme importance given the involvement of the enzyme in the tissue damage observed during the reperfusion phase of acute ischaemia [7].

In line with the results obtained by Northern-blot analysis, under basal conditions (results not shown), the kidney is the only tissue that does not contain XOR-positive cells. After LPS treatment, the number of XOR-positive cells observed in liver (compare Figures 5b and 5c), heart (Figures 5h and 5i) and

spleen (Figure 5g) is increased, whereas, in lung, LPS does not markedly affect their number or distribution (Figures 5d–5e). In the liver, although the number of grains associated with cells expressing XOR is increased by LPS treatment, the regional distribution is not altered. In the kidney (Figure 5f), after bacterial endotoxin treatment, XOR-expressing cells are predominantly at the level of the proximal tubuli, although positive cells are sometimes observed in a few glomeruli. In the spleen, the XOR transcript is associated with the lymphocytes present in the pulpar region of the organ (Figure 5g). In liver and kidney, where XOR mRNA up-regulation by LPS is accompanied by an increase in enzyme activity, the specific cellular localization of the enzyme may have a pathological significance, as the two organs are targets for the toxic effects of the bacterial endotoxin. The latter organ is particularly interesting. In fact, LPS administration is characterized by signs of hepatocellular degeneration and necrosis which are multifocal in nature and become apparent within 24 h. All this correlates with the regional and cellular distribution of XOR as well as with the kinetics of induction of the enzyme. It would be tempting to speculate that superoxides generated by XOR inside the hepatocyte may have a role in mediating the observed toxicity.

A more extensive characterization of the cells synthesizing XOR mRNA in the gastrointestinal tract was carried out because of the high level of expression of the transcript in this tissue (Figure 6). Under basal conditions, cells from the most proximal two-thirds of the stomach (cardia and corpus ventriculi) do not express detectable amounts of the transcript (results not shown). On LPS induction, the mucosal layer of these two segments of the organ become XOR-positive with a diffuse and patchy pattern of staining (Figures 6a and 6b for cardia and corpus ventriculi respectively). In contrast, the pylorus expresses low but detectable amounts of XOR message, which is significantly increased on treatment with LPS (Figure 6c). In the duodenum, the epithelium that covers the villi uniformly expresses considerable amounts of the XOR transcript under basal conditions and these levels are unaffected by treatment with LPS for 16 h (Figures 6e and 6f). The underlying stromal layer with corresponding blood vessels as well as the tunica muscularis do not express appreciable levels of XOR message. In the epithelial layer, differentiated enterocytes covering the villus are strongly positive, whereas less differentiated cells at the base of the villus or inside the cryptae are generally negative. As we proceed along the small intestine through the jejunum (Figure 6g) to the ileum (results not shown), the number of epithelial XOR-positive cells decreases dramatically. Consistent with the quantitative results obtained by Northern-blot analysis, at the level of the colon and the rectum, very few epithelial cells in the mucosa stain positive for the XOR transcript under basal conditions (results not shown). LPS consistently increases the number of XOR-positive cells in the mucosa of these two regions (Figures 6h and 6i respectively). As to the possible physiopathological implications of our findings, the high level of XOR expression in the mucosal layer in the first part of the small intestine suggests a role for the enzyme in the defence mechanisms active at the level of the epithelia lining of the intestinal wall. High local concentrations of uric acid may be a component of the cytoprotective mucous layer lining the abluminal side of the enterocyte along with prostaglandins and other components. It is also possible that the superoxides produced by XO may represent a barrier against the proliferation of the potentially pathogenic micro-organisms contained in the intestinal lumen. In contrast, during endotoxaemia, up-regulation of the enzyme in the enterocytes of the colon and rectum and its down-regulation in the duodenal epithelium may be compatible with a role in the intestinal

toxicity observed in severe endotoxaemia which includes detachment of epithelial cells from the mucosal basement membrane [28].

In the past, localization of XOR at the single-cell level has not been the object of a systematic study, although scattered observations in the intestine, liver and mammary gland have been reported in a few animal species [29–32]. These data were, however, generated by cytochemical techniques which generally lack specificity and, moreover, gave contrasting results. The only study in which an immunocytochemical approach was used is that of Jarasch et al [33], which was conducted on a bovine model. The authors reported the expression of XOR in the vascular component of the intestinal wall and suggested that the enzyme may be a specific marker for the endothelium. The data reported here are substantially at variance with the observations in the bovine model [32], which may be explained by the different technology used (immunocytochemistry compared with *in situ* hybridization) or, more likely, species-specific differences. Nevertheless, except in heart and possibly lung, our results clearly do not support the idea that XOR is specifically localized in the vascular component of the various tissues and organs.

This work was supported in part by grants from the Consiglio Nazionale delle Ricerche, Progetto Finalizzato 'Biotecnologie e Biostrumentazione', 'Ingegneria Genetica', 'Chimica Fine' and the Associazione per la Ricerca contro il Cancro (AIRC). We are grateful to Dr. Takeshi Nishino (Nippon Medical School, Tokyo, Japan) for the gift of XOR polyclonal antibodies and to Ms. Daniela Cavallotti for technical assistance in the preparation of tissue sections. We are also grateful to Professor S. Garattini, Dr. M. Salmona and Dr. E. Fadlon for critical reading of the manuscript. M.L.C. is the recipient of a fellowship from 'La Via di Natale', Pordenone, Italy.

REFERENCES

- Dixon, M. and Webb, E. C. (1964) in *Enzymes*, 2nd edn., pp. 203–204, Academic Press, New York
- Cazzaniga, G., Seldin, M. F., Terao, M., Lo Schiavo, P., Galbiati, F., Segalla, F. and Garattini, E. (1994) *Genomics* **23**, 390–402
- Carpani, G., Racchi, M., Ghezzi, P., Terao, M. and Garattini, E. (1990) *Arch. Biochem. Biophys.* **279**, 237–241
- Della Corte, E. and Stirpe, F. (1972) *Biochem. J.* **126**, 739–745
- Amaya, Y., Yamazaki, K., Sato, M., Noda, K., Nishino, T. and Nishino, T. (1990) *J. Biol. Chem.* **265**, 14170–14175
- Terao, J. and Matsushita, S. (1988) *Free Rad. Biol. Med.* **4**, 205–208
- Parks, D. A. and Granger, D. N. (1983) *Am. J. Physiol.* **245**, G285–G289
- McCord, J. M. (1985) *N. Engl. J. Med.* **312**, 159–163
- Fridovich, I. (1970) *J. Biol. Chem.* **245**, 4053–4057
- de Groot, H. and Littauer, A. (1988) *Biochem. Biophys. Res. Commun.* **155**, 278–282
- Suzuki, M., Grisham, M. B. and Granger, N. (1991) *J. Leukocyte Biol.* **50**, 488–494
- Akaike, T., Ando, M., Oda, T., Doi, T., Ijiri, S., Araki, S. and Maeda, H. (1990) *J. Clin. Invest.* **85**, 739–745
- Brigham, K. L., Meyrick, B., Berry, L. C., Jr. and Repine, J. E. (1987) *J. Appl. Physiol.* **63**, 840–850
- Ghezzi, P., Bianchi, M., Mantovani, A., Spreafico, F. and Salmona, M. (1984) *Biochem. Biophys. Res. Commun.* **119**, 144–149
- Terao, M., Cazzaniga, G., Ghezzi, P., Bianchi, M., Falciani, F., Perani, P. and Garattini, E. (1992) *Biochem. J.* **283**, 863–870
- Falciani, F., Ghezzi, P., Terao, M., Cazzaniga, G. and Garattini, E. (1992) *Biochem. J.* **285**, 1001–1008
- Dupont, G. P., Huecksteadt, T. P., Marshall, B. C., Ryan, U. S., Michael, J. R. and Hoidal, J. R. (1992) *J. Clin. Invest.* **89**, 197–202
- Reiners, J. J., Jr., Pence, B. C., Barcus, M. C. S. and Cantu', A. R. (1987) *Cancer Res.* **47**, 1775–1779
- Falciani, F., Terao, M., Goldwurm, S., Ronchi, A., Gatti, A., Minoia, C., Li Calzi, M., Salmona, M., Cazzaniga, G. and Garattini, E. (1994) *Biochem. J.* **298**, 69–77
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Wilkinson, D. G. (ed.) (1992) *In Situ Hybridization: A Practical Approach*, IRL Press, Oxford
- Mohamedali, K. A., Guicherit, O. M., Kellems, R. E. and Rudolph, F. B. (1993) *J. Biol. Chem.* **268**, 23728–23733
- Rajagopalan, K. V. and Handler, P. (1967) *J. Biol. Chem.* **242**, 4097–4107

- 24 Bray, R. C. (1975) *Enzymes* 3rd Ed. **12**, 299–419
- 25 Johnson, J. L. (1980) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M. P., ed.), pp. 345–383, Pergamon Press, Oxford
- 26 Duke, E. J., Joyce, P. and Ryan, J. P. (1973) *Biochem J.* **131**, 187–190
- 27 Meyuhas, O. and Perry, R. P. (1980) *Gene* **10**, 113–129
- 28 Wallace, J. L., Steel, G. L., Whittle, B. J. R., Lagente, V. and Vargaftig, B. (1987) *Gastroenterology* **93**, 765–773
- 29 Kooij, A., Frederiks, W. M., Gossrau, R. and Van Noorden, C. J. F. (1991) *J. Histochem. Cytochem.* **39**, 87–93
- 30 Frederiks, W. M. and Marx, F. (1991) *J. Histochem. Cytochem.* **41**, 667–670
- 31 Ibrahim, B. and Stoward, P. J. (1978) *Histochem. J.* **10**, 615–617
- 32 Wohlrab, F. and Seidler, E. (1978) *Acta Histochem.* **62**, 120–132
- 33 Jarasch, E. D., Grund, C., Bruder, G., Heid, H. W., Keenan, T. W. and Franke, W. W. (1981) *Cell* **25**, 67–82

Received 18 July 1994/19 September 1994; accepted 5 October 1994