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In situ analysis of ischaemia/reperfusion injury in rat liver studied in three different models

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Summary. Animal models of liver ischaemia and reperfusion are frequently used to study the consequences on liver cells of transient oxygen deprivation. In 3 different rat models we studied ischaemia/reperfusion effects on liver cell membrane integrity, cytoplasmic enzyme proteins and enzyme activities by *in situ* histochemical techniques. *In vivo* ischaemia, as well as no-flow hypoxia, or N_2-induced hypoxia in isolated perfused livers, reduced the activity of 5′-nucleotidase, a sensitive marker for plasma membrane damage in hepatocytes. As little as 2 minutes of reoxygenation in each model resulted in leakage of soluble enzymes from parenchymal and non-parenchymal liver cells, as shown by decreased protein level and activity of cytoplasmic enzymes. Whereas a multifocal decrease was observed after *in vivo* reperfusion, a decrease was found in all periportal and midzonal cells after blood-free reoxygenation. As judged by alkaline phosphatase activity and immunohistochemistry, an influx of inflammatory cells was not found in the *in vivo* model. Our findings indicate that reoxygenation itself, rather than restoration of flow, accounts for the loss of soluble enzymes from liver cells after a period of hypoxia. *In situ* detection of enzyme protein and activity proved useful for the examination of very early ischaemia/reperfusion effects on rat liver cells.

Keywords: hypoxia, isolated liver perfusion, histochemistry, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, xanthine oxidoreductase, 5′-nucleotidase

Ischaemia/reperfusion injury has been studied extensively in liver (for reviews, see Kehrer *et al.* 1990; Lemasters *et al.* 1993; Das 1994; Jaeschke 1995) and various models have been developed to study different aspects of ischaemia/reperfusion injury (Bradford *et al.* 1986; Kehrer & Starnes 1989; Jaeschke & Mitchell 1990). One of the major findings with the use of these model studies is that reperfusion aggravates damage induced by ischaemia. Three frequently applied models of ischaemia/reperfusion in liver are directly compared in the present study. The first is the ischaemia/reperfusion model *in vivo*. In this model, tissue damage is caused particularly by interactions between damaged endothelium and adhering cells, reactive oxygen species and inflammatory mediators (for review, see Jaeschke 1995). The second is the no-flow/reflow model in an isolated liver perfusion system. The liver endures oxygen

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deprivation and subsequent reoxygenation by buffer only, thus avoiding blood-endothelium interactions. In this model, damage is caused mainly be resumption of flow leading to mechanical shear stress on endothelial cells (Caldwell-Kenkel *et al.* 1991). The third model is based on continuous flow and N_2-induced hypoxia in the isolated liver perfusion system, thus avoiding flow interruption and mechanical stress when oxygenation is restored.

We studied the localization of rat liver enzymes and their activities *in situ* in a search for early effects of ischaemia/reperfusion in the 3 experimental models. The activity of the plasma membrane-bound enzyme 5′-nucleotidase (5′-Nase) was selected as a sensitive marker for ischaemic damage to hepatocytes (Farber *et al.* 1981; Frederiks *et al.* 1988). Histochemical detection of the activities of 3 cytoplasmic enzymes (lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PDH) and xanthine oxidoreductase (XOR)) as well as immunohistochemical localization of the proteins of LDH and G6PDH were used to reveal enzyme leakage from hepatocytes (Lemasters *et al.* 1983). G6PDH activity was also used to observe changes in monocytes and macrophages, because this marker is particularly high in these cells (Hosemann *et al.* 1979). Monocytes and macrophages were identified in serial sections by specific antibodies and immunohistochemistry. The comparatively high activity of XOR in Kupffer cells and sinusoidal endothelial cells (Kooij *et al.* 1991) enabled the study of ischaemia/reperfusion effects on these non-parenchymal cells. Influx of polymorphonuclear leucocytes (PMNs) has been shown to contribute to ischaemia/reperfusion injury (Jaeschke 1995). PMNs were therefore identified on the basis of their high alkaline phosphatase (AlP) activity (Wachstein 1946).

Materials and methods

Animals

Mature male Wistar rats ($n = 3$ in each experimental group; 200–225 g, Harlan CPB, Zeist, The Netherlands) were handled according to the official guidelines of the University of Amsterdam. Rats were kept for one week in a constant-climate environment with respect to temperature, humidity and daylight cycle. Animals were fasted overnight with free access to water before operation. Operation procedures were performed between 1000 h and 1200 h to avoid chronobiological variations.

In vivo *ischaemia/reperfusion*

In vivo liver ischaemia was induced in rats under ether anaesthesia by clamping branches of both portal vein and hepatic artery to the median and left lateral lobes for 60 minutes. Livers were reperfused by removal of the clamp. Liver tissue was obtained after 0, 2 or 30 minutes reperfusion. It is known from a previous study, that 60 minutes of liver ischaemia will lead to focal necrosis after 24 hours of reperfusion (Frederiks *et al.* 1982).

Isolated perfused liver system

Rats were anaesthetized by administration of sodium pentobarbital (60 mg/kg body wt i.p.). Livers were removed surgically and perfused in a non-recirculating fashion (Scholz *et al.* 1973) at room temperature via a cannula inserted into the portal vein with Tris-buffered physiological salt solution (TBS, pH 7.45), equilibrated with 100% O_2 at a rate of 4 ml/min/g liver to provide an adequate supply of oxygen (Sies 1978). The composition of TBS was: NaCl (143 mM), KCl (5 mM), $MgCl_2$ (1.2 mM), $CaCl_2$ (2 mM), D-glucose (11 mM), tris(hydroxymethyl)-aminomethane (4 mM). This medium was designed to allow parallel studies with cerium chloride added to determine *in situ* production of hydrogen peroxide (Briggs *et al.* 1975) which required a phosphate and carbonate-free perfusion medium (I.H. Straatsburg and W.M. Frederiks, unpublished). The pH of the perfusate during the experiments was maintained within the physiological range. Since preliminary studies indicated that in isolated perfused livers a much shorter period of hypoxia leads to extensive cellular damage after 2 hours of reoxygenation in comparison with the ischaemia/reperfusion model *in vivo* (Straatsburg and Frederiks, unpublished results), we chose to investigate early effects of only 20 minutes of hypoxia and subsequent reoxygenation in the isolated perfused livers. Control livers were perfused continuously with oxygenated TBS for 50 minutes, including an initial equilibrium period of 30 minutes oxygenation.

No-flow/reflow in the isolated perfused rat liver

Isolated livers were equilibrated with oxygenated TBS for 30 minutes and the flow was then stopped for 20 minutes. Following this period of no-flow hypoxia, livers were reperfused with oxygenated TBS for 0, 2 or 60 minutes.

Hypoxia/reoxygenation in the isolated perfused rat liver

After an initial oxygenation period of 30 minutes, livers were perfused with hypoxic TBS (saturated with 100% N_2) for 20 minutes. After this period of hypoxia, livers

were perfused with oxygenated TBS for 0, 2 or 60 minutes.

At the end of all experiments, the left lateral and median lobes were obtained, cut into small tissue blocks up to 0.5 cm^3, snap-frozen in liquid nitrogen and stored at −80°C.

Enzyme histochemistry

Cryostat sections (8 μm) were cut at −25°C on a motor-driven cryostat (Bright, Huntingdon, UK), picked up onto glass slides and stored at −20°C until use. Sections were allowed to dry and warm up before incubations at 37°C for at least 10 minutes. Incubation procedures were designed to demonstrate the specific and maximal activity of each enzyme.

5′-Nase activity (EC 3.1.3.5) was demonstrated (incubation time (t_{inc}) 10 minutes) using a lead salt method (Frederiks & Marx 1988). The incubation medium contained 18% (w/v) polyvinyl alcohol (PVA; weight average M_r 70 000–100 000; Sigma, St Louis, MO) in 100 mM Tris-maleate buffer (pH 7.2), 5 mM AMP (Boehringer, Mannheim, FRG) as substrate, 10 mM $MgCl_2$ and 7.2 mM $Pb(NO_3)_2$. Lead phosphate was visualized using 1% (w/v) ammonium sulphide.

LDH activity (EC 3.2.1.23) was demonstrated (t_{inc} 5 minutes) using a medium containing 18% PVA in 100 mM phosphate buffer (pH 7.45), 150 mM sodium lactate (Serva, Heidelberg, FRG) as substrate, 3 mM NAD^+ (Boehringer) as coenzyme, 5 mM sodium azide, 0.32 mM 1-methoxyphenazinemethosulphate (1-mPMS; Serva) as electron carrier and 5 mM tetranitro-blue tetrazolium salt (tetranitro BT; Serva) as final electron acceptor (Van Noorden & Vogels 1989).

XOR activity (xanthine : NAD^+ oxidoreductase, EC 1.1.1.204 (dehydrogenase or D-form, XDH) plus superoxide anion producing xanthine : O_2 oxidoreductase, EC 1.2.3.22 (oxidase or O-form, XO)) was demonstrated (t_{inc} 30 minutes) using a tetrazolium salt method (Frederiks *et al.* 1996). The medium contained 18% PVA in 100 mM phosphate buffer (pH 8.0), 0.5 mM hypoxanthine (Sigma) as substrate, 0.32 mM 1-mPMS and 5 mM tetranitro BT. The medium was applied to sections immediately after cutting to prevent loss of enzyme activity (Kooij *et al.* 1991).

G6PDH activity (EC 1.1.1.49) was demonstrated (t_{inc} 10 minutes) using the tetrazolium salt method for dehydrogenases with 10 mM glucose-6-phosphate (Serva) as substrate and 0.8 mM $NADP^+$ (Boehringer) as coenzyme (Butcher & Van Noorden 1985).

AIP activity (EC 3.1.3.1) was demonstrated (t_{inc} 15 minutes) using an indoxyltetrazolium salt method with 18% PVA in 100 mM Tris-HCl buffer (pH 9), 0.7 mM 5-bromo-4-chloro-3-indolyl phosphate (Boehringer) as substrate and 5 mM $MgCl_2$ as activator of the enzyme (Van Noorden & Jonges 1987).

Control incubations were performed in the absence of substrate (and coenzyme). After incubations, sections were rinsed in hot water to stop the reaction immediately and to remove the viscous incubation medium. Sections were mounted in glycerol jelly.

Immunohistochemistry

Two cytoplasmic enzyme proteins were demonstrated using the polyclonal antibodies rabbit-anti-yeast G6PDH (Sigma; dilution 1 : 1000) and rabbit-anti-porcine LDH-5 (M4 subunit; Biogenesis, Poole, UK; 1 : 250). Cryostat sections (8 μm) were air-dried (1 hour), fixed in cold acetone (10 minutes, −20°C) and dried again (10 minutes). Endogeneous peroxidase activity was blocked with 88 mM H_2O_2 and 15 mM NaN_3 in 0.01 M phosphate buffered saline (PBS, pH 7.4) (10 minutes). Antigens were exposed for 30 minutes using a solution of Tris (10 mM), EDTA (5 mM), NaCl (150 mM), 0.25% gelatine and 0.05% Tween-20 (Sigma) at pH 8.0. Between all further incubation steps, sections were thoroughly rinsed in PBS. Antibodies were diluted in PBS containing 0.2% (w/v) bovine serum albumin (BSA) and 1% (v/v) normal rat serum to block non-specific background staining. Sections were incubated (60 minutes, room temperature) with either specific antibodies or, for the controls, with an irrelevant rabbit IgG (1 : 200) or PBS/BSA. Sections were then incubated with horse-radish peroxidase labelled goat-anti-rabbit IgG (DAKO A/S, Glostrup, Denmark; 1 : 100; 90 minutes). Peroxidase activity was detected by incubating the sections for 10 minutes in a medium containing 0.5 mg/ml diaminobenzidine (Fluka Chemie, Buchs, Switzerland), 3 mM H_2O_2 and 50 mM Tris-HCl buffer (pH 7.6). Monocytes and resident macrophages were identified using the monoclonal antibodies ED1 and ED2 (Serotec, Hilversum, The Netherlands) (Dijkstra *et al.* 1985; Barbé *et al.* 1992). Serial sections were first incubated with ED1 or ED2 (1 : 500; 60 minutes) and, after rinsing, with horse-radish peroxidase labelled rabbit-anti-mouse (DAKO A/S; 1 : 200; 45 minutes). Peroxidase activity was detected as described above.

Results

Control livers

5′-Nase activity was found at plasma membranes of vascular endothelial cells and at bile canalicular and sinusoidal plasma membranes of hepatocytes in control

livers (Figure 1a). LDH protein and activity (Figure 1b) were distributed more or less homogeneously over the liver lobules. LDH activity was hardly present in sinusoidal cells. XOR activity was found in hepatocytes, Kupffer cells and sinusoidal endothelial cells (Figure 1c). Activity was highest in Kupffer cells and lowest in periportal hepatocytes. G6PDH activity was distributed more or less evenly over liver lobules (Figure 1d). G6PDH protein was found in similar amounts in hepatocytes and non-parenchymal cells, but G6PDH activity was distinctly higher in non-parenchymal cells (Figure 1d).

Ischaemia/reperfusion in vivo

After *in vivo* ischaemia alone, 5′-Nase activity was decreased at both bile canalicular and sinusoidal plasma membranes of hepatocytes. XOR activity was decreased in all liver cells. Activities of LDH and G6PDH were decreased in pericentral hepatocytes only. Changes in protein levels were not detected. Non-parenchymal cells showed prominent activity of G6PDH.

After 2 and 30 minutes of reperfusion, 5′-Nase activity (Figure 2a) and cytoplasmic enzyme activities (Figure 2b–d) were very low in cell clusters forming confined 'foci'. These 'depletions' were particularly obvious after 30 minutes of reperfusion. G6PDH activity was high in numerous non-parenchymal cells surrounding the foci (Figure 2d). In serial sections, some of these cells could be identified as monocytes and resident macrophages by immunohistochemistry (Figure 3). However, the number of non-parenchymal cells with high G6PDH activity was always larger than the number of positively identified monocytes and macrophages. In foci with decreased activity, protein levels of both LDH (Figure 4) and G6PDH (data not shown) were clearly diminished. XOR activity was absent in non-parenchymal cells with foci after 2 rather than after 30 minutes of reperfusion. There were no signs of PMN accumulation within or around the foci (Figure 5).

Isolated rat liver perfusion

Besides a slight decrease of periportal XOR activity, 50 minutes of normoxic perfusion of rat liver did not induce significant changes (Figure 6a). After no-flow or N_2-induced hypoxia only, activity of 5′-Nase at the sinusoidal and lateral plasma membrane domains of hepatocytes was low in pericentral areas. In periportal areas, 5′-Nase activity was redistributed from the bile canalicular plasma membrane domain over sinusoidal and lateral domains. After no-flow cytoplasmic enzyme activities were slightly lowered (Figure 6b) N_2-induced hypoxia affected cytoplasmic enzyme activities to a larger extent, especially in periportal areas (Figure 6c). Distribution patterns of proteins of LDH and G6PDH were similar to activity patterns.

After 2 minutes of reoxygenation following N_2-induced hypoxia, and to a lesser extent following no-flow, protein levels and activities of all enzymes were strongly decreased in periportal and midzonal cells (Figures 6d, 7). In both models, some XOR-derived final reaction product was observed in or at the luminal face of endothelial cells of central veins and sinusoids (Figure 6d). After 30 minutes of reoxygenation similar distribution patterns of all enzymes were found as after 2 minutes, with the following exceptions: 5′-Nase activity appeared to be restored in bile canalicular plasma membrane domains of periportal and midzonal hepatocytes. Sinusoids were no longer enlarged and did not contain any final reaction product of dehydrogenase activity.

Discussion

We compared early ischaemia/reperfusion injury as expressed by changes in enzyme activities in rat liver cells in 3 frequently applied experimental models. After *in vivo* liver ischaemia, or no-flow or N_2-induced hypoxia in isolated perfused livers, decreased plasma membrane-bound enzyme activity was observed in hepatocytes. Cytoplasmic enzymes also showed decreased activity. In all models, reoxygenation aggravated liver cell damage very rapidly. Leakage of cytoplasmic enzymes occurred after as little as 2 minutes of reperfusion. Focal depletion of enzyme activity was observed after *in vivo* ischaemia/reperfusion. In contrast, in isolated perfused livers changes occurred especially in periportal and midzonal areas after no-flow/reflow or N_2-induced hypoxia/reoxygenation.

In vivo *ischaemia/reperfusion*

One hour of *in vivo* ischaemia caused a general drop in activity of 5′-Nase, which may be related to disturbance of membrane integrity (Farber *et al.* 1981; Frederiks *et al.* 1988). During reperfusion, 5′-Nase activity decreased in foci, possibly due to shedding of membrane blebs containing the enzyme (Frederiks *et al.* 1983), redistribution of 5′-Nase to other plasma membrane domains or as a result of inactivation by reactive oxygen species (Kitakaze *et al.* 1992; Zhai *et al.* 1995). A rapid fall in activity of soluble enzymes was noted in these foci accompanied by loss of the proteins LDH and G6PDH, which is indicative for enzyme leakage (Figures 2–4).

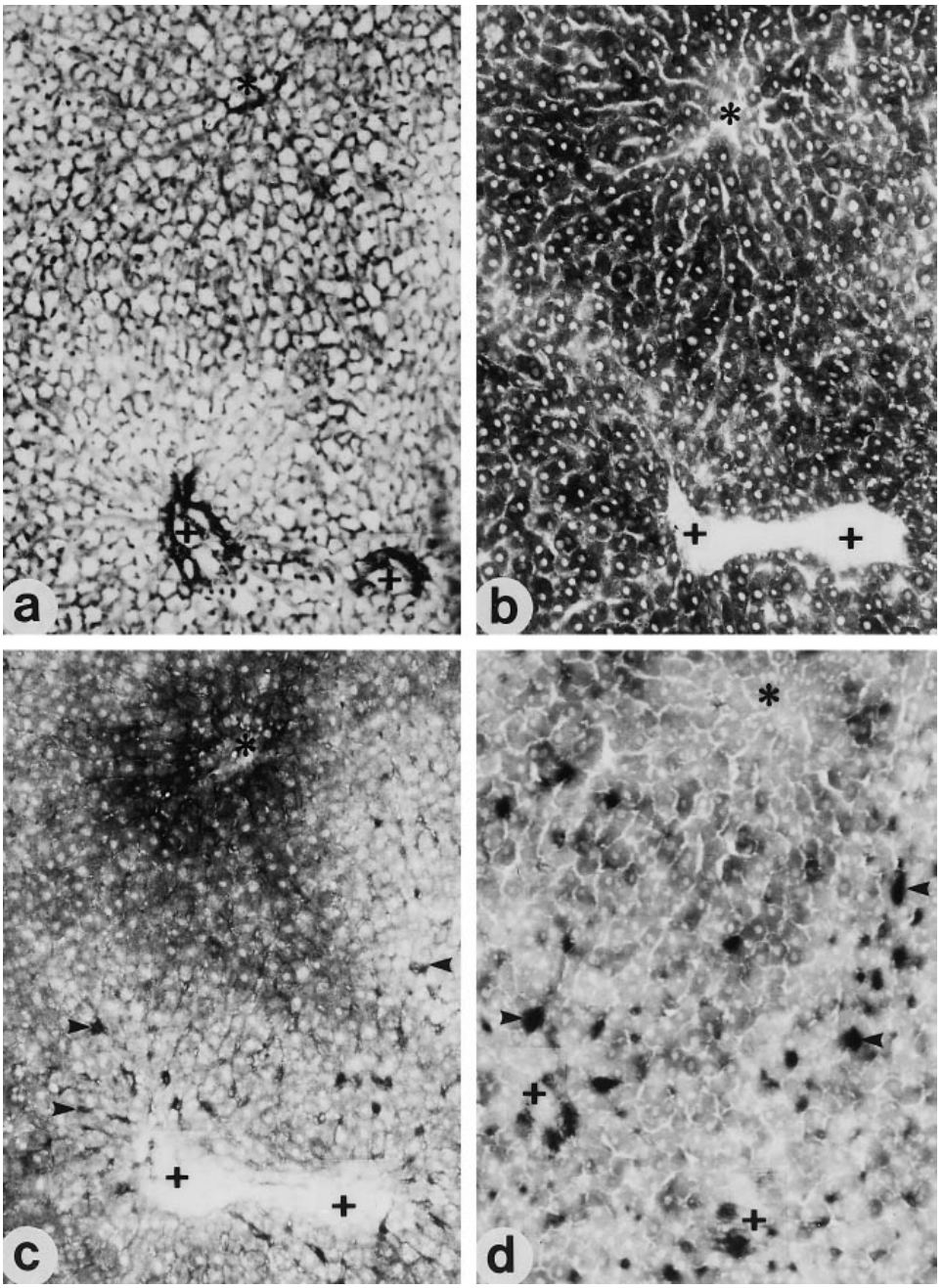

Figure 1. Consecutive sections of control rat liver incubated for the demonstration of activity of a, 5′-nucleotidase; b, lactate dehydrogenase; c, xanthine oxidoreductase and d, glucose-6-phosphate dehydrogenase. 5′-Nucleotidase activity is present at plasma membranes of vascular endothelial cells and at bile canalicular and sinusoidal plasma membranes of hepatocytes. Lactate dehydrogenase activity is distributed more or less homogeneously over liver lobules. Xanthine oxidoreductase activity is found in hepatocytes, especially in pericentral zones, and non-parenchymal cells (arrowheads). Glucose-6-phosphate dehydrogenase activity is homogeneously distributed over liver lobules, but distinctly higher in non-parenchymal cells (arrowheads) than in hepatocytes. *Central vein; +, portal vein. ×120.

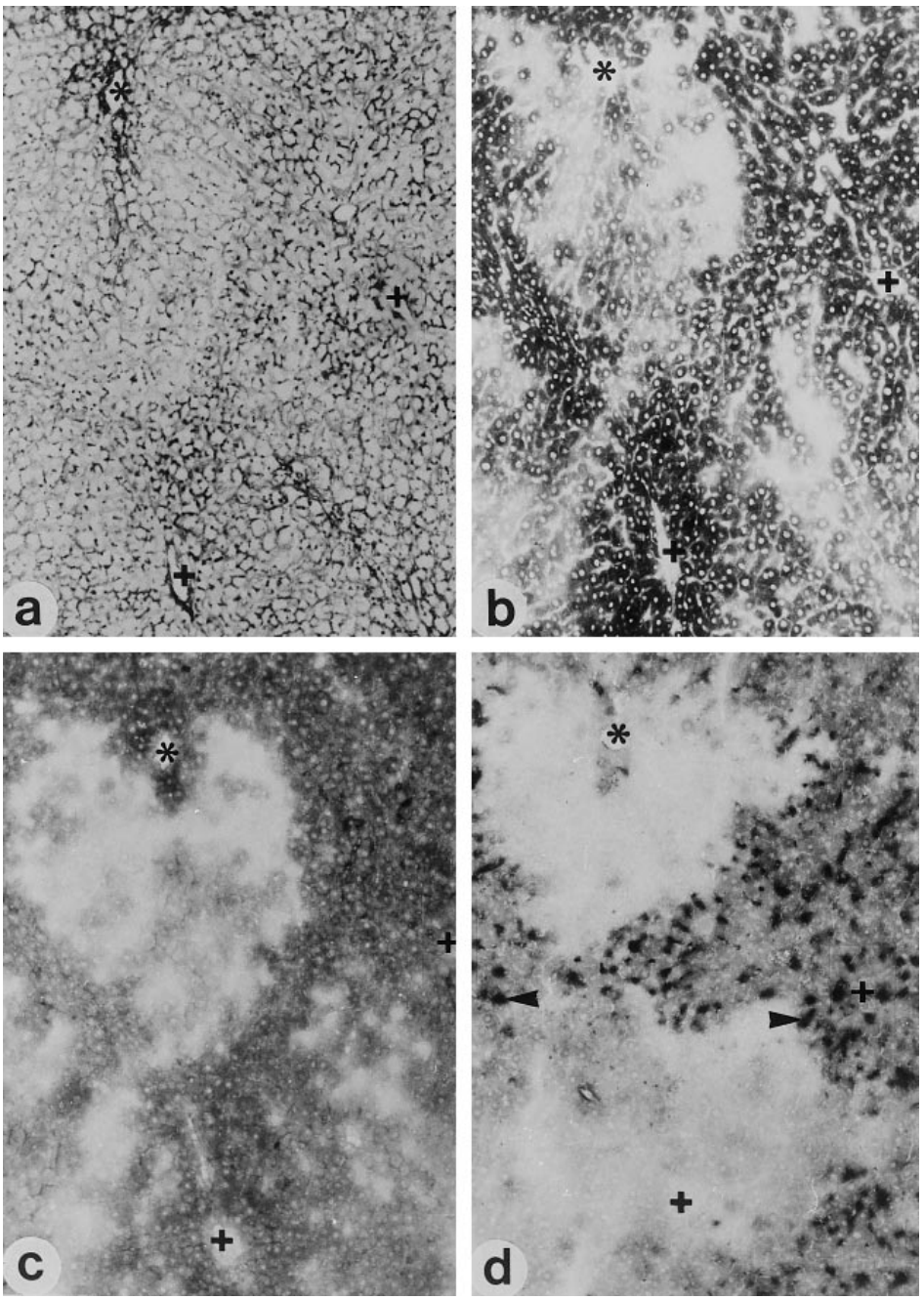

Figure 2. Consecutive sections of rat liver after 60 minutes of *in vivo* ischaemia and 2 minutes of reperfusion incubated for the demonstration of activity of a, 5′-nucleotidase; b, lactate dehydrogenase; c, xanthine oxidoreductase and d, glucose-6-phosphate dehydrogenase. 5′-Nucleotidase activity in hepatocytes is strongly decreased, indicating compromised plasma membrane integrity (a). Cytoplasmic enzyme activities are depleted from parenchymal and non-parenchymal cells within confined foci (B,C,D). An increased number of non-parenchymal cells show high-glucose-6-phosphate dehydrogenase activity around these foci (d, arrowheads). *Central vein, + portal vein. ×80.

A large number of non-parenchymal cells with high G6PDH activity, partly identified as monocytes and resident macrophages by immunohistochemistry, surrounded these depleted foci (Figures 2d and 3). It is likely that activation of Kupffer cells occurred during reperfusion after ischaemia, because infiltration of monocytes into the liver, that is, an increase in the number of ED1-positive cells, was not observed. In all cases, the number of non-parenchymal cells containing high G6PDH activity was larger than the number of cells that were positively identified as monocytes or macrophages (Figure 3). This suggests the existence of other cells which are able to express high levels of G6PDH activity after ischaemia/reperfusion. These cells were not PMNs as is shown in Figure 5.

Enzyme leakage was not observed after *in vivo* ischaemia alone, but directly initiated by reperfusion. However, the reason for multifocal enzyme leakage after *in vivo* ischaemia/reperfusion remains unclear. In isolated perfused livers, enzyme leakage after reoxygenation was observed throughout liver sections, and not just in a few areas. This suggests that blood–endothelium interactions determine locally decreased membrane integrity and loss of soluble enzymes. Although in our study reperfusion was crucial for enzyme leakage, we can not exclude that *insufficient*

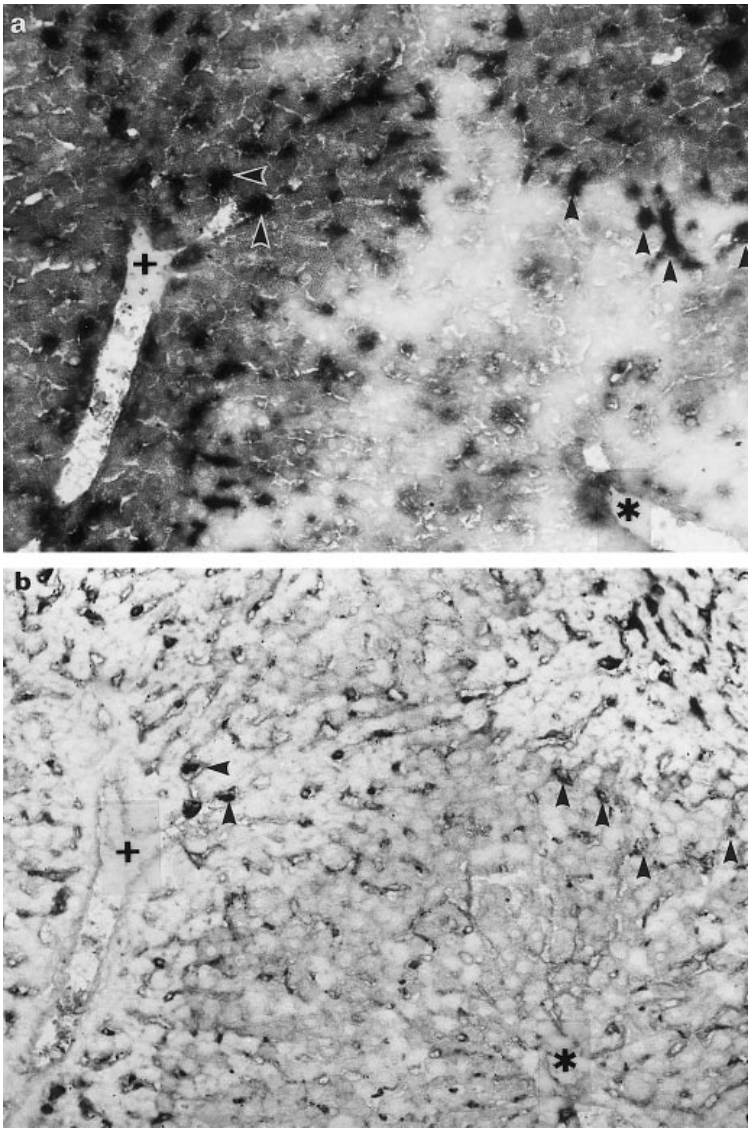

Figure 3. Consecutive sections of rat liver after 60 minutes of *in vivo* ischaemia and 30 minutes of reperfusion incubated for the demonstration of a, glucose-6-phosphate dehydrogenase activity and b, ED2-positive resident macrophages. Non-parenchymal cells with high G6PDH activity around focal lesions are identified partly as Kupffer cells (arrowheads). *Central vein, + portal vein. ×140.

reperfusion and reoxygenation due to an 'immediate or delayed no-reflow phenomenon' (Koo *et al*. 1992; Minor & Isselhard, 1993; Vollmar *et al*. 1994) caused the development of focal lesions. This hypothesis presumes early reoxygenation failure, which can not be caused by PMN accumulation (Figure 5), but possibly by endothelial cell swelling. It has indeed been suggested that leukostasis *per se* does not determine perfusion failure (Vollmar *et al*. 1996).

Hypoxia/reoxygenation or no-flow/reflow in isolated perfused livers

The general decrease of 5′-Nase activity after no-flow or N_2-induced hypoxia indicates that 5′-Nase activity was sensitive to oxygen depletion alone and that flow interruption was not obligatory to cause a decrease in activity. This observation is consistent with the view that 5′-Nase is a very sensitive marker for ischaemic damage to plasma membranes in rat liver (Frederiks *et al*. 1988). Brief reoxygenation, like *in vivo* reperfusion, further decreased 5′-Nase activity. The recovery of activity in pericentral bile canalicular membranes after 30 minutes of reoxygenation or reflow may be related to restoration of microvilli (Frederiks *et al*. 1988).

Brief reflow or reoxygenation (2 minutes) caused a large reduction of both enzyme protein and activity, especially in periportal areas (Figures 3 and 7), indicating

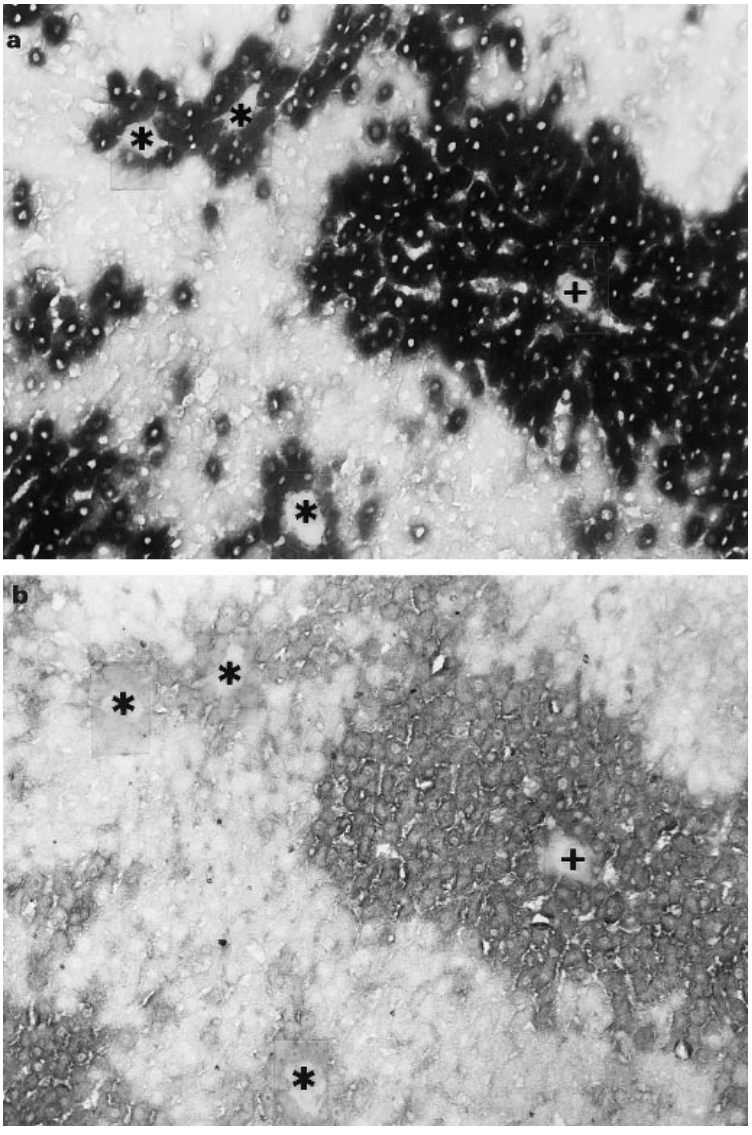

Figure 4. Consecutive sections of rat liver after 60 minutes of *in vivo* ischaemia and 30 minutes of reperfusion incubated for the demonstration of a, lactate dehydrogenase activity and b, protein. Both activity and protein have disappeared from hepatocytes in confined foci. *Central vein, + portal vein, ×140.

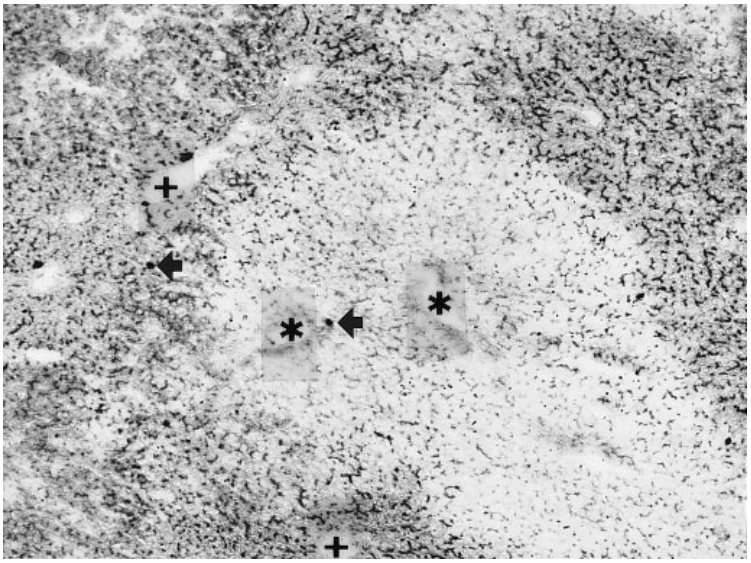

Figure 5. Section of rat liver after 60 minutes of *in vivo* ischaemia and 30 minutes of reperfusion incubated for the demonstration of alkaline phosphatase activity in polymorphonuclear leucocytes. Only a few cells are present (arrow). *Central vein, + portal vein. ×70.

leakage (Lemasters *et al*. 1983). The appearance of cytoplasmic dehydrogenase activities within enlarged periportal sinusoids substantiates that leakage occurs extremely rapid during reperfusion. It was most prominent for XOR (Figures 6d and 7c) and this confirms previous reports on XOR leakage during reperfusion from hepatocytes and sinusoidal endothelial cells (Kooij *et al*. 1994; Federiks & Bosch 1995) and XOR adhesion to plasma membranes of vascular endothelial cells (Tan *et al*. 1993). The conversion of circulating XOR from dehydrogenase (XDH) to oxidase (XO) (Kooij *et al*. 1994) may lead to extracellular superoxide anion production and aggravation of reperfusion injury. Although the production of reactive species during reoxygenation (McCord 1985; Jaeschke *et al*. 1988), possibly by XO (Engerson *et al*. 1987; Brass *et al*. 1992; Wiezorek *et al*. 1994; Kooij *et al*. 1994), has been widely investigated, a role for reactive oxygen species in the development of *early* reperfusion injury in liver is still under debate (Metzger *et al*. 1988; Brass 1995). In conclusion, our study shows that resumption of oxygen delivery itself, rather than reflow, is the major cause of rapid loss of cytoplasmic enzyme activity during reperfusion.

Comparison of the results in models of ischaemia/reperfusion

Hepatocyte plasma membranes were disturbed during experimental hypoxia in all 3 models, as shown by a decrease and/or redistribution of 5′-Nase activity. After reperfusion *in vivo*, we observed mainly pericentral focal membrane changes and enzyme leakage (Figures 2–4), whereas in the isolated perfused livers the most severe activity loss was always observed in periportal and midzonal cells. This discrepancy between the zonation of leakage after ischaemia/reperfusion *in vivo* versus hypoxia/reoxygenation in isolated perfused livers deserves attention. First, reductive stress, i.e. an increased NADH/NAD^+ ratio, is an early panlobular phenomenon during low-flow hypoxia (Suzuki *et al*. 1994) and may be even more important for susceptibility to hypoxic injury than ATP depletion (Khan & O'Brien 1995). After fasting, as applied in our models, the normal distribution gradient of glutathione, with highest concentration in the periportal region, disappears (Bradford *et al*. 1986; Shimizu & Morita 1992). This comparatively large loss of glutathione, both redox-buffer (Khan & O'Brien 1995) and anti-oxidant (Halliwell *et al*. 1992), from periportal and midzonal cells may increase their susceptibility to reductive and oxidant stress. Especially in isolated perfused livers, periportal and midzonal cells may respond more strongly than pericentral cells to reoxygenation stress because the perfusion medium lacks extracellular redox buffers and anti-oxidants.

Because the sinusoidal endothelial lining is responsive to changes in flow conditions (for review see Resnick & Gimbrone 1995), we aimed to study the effect of flow interruption on top of hypoxia on liver enzymes in comparison with continuous flow and hypoxia. The latter condition was achieved by perfusing isolated livers with N_2-enriched medium. Our results show that the most severe enzyme leakage and decrease in activity occurred after N_2-induced hypoxia followed by reoxygenation. Whereas no-flow hypoxia may allow moderate

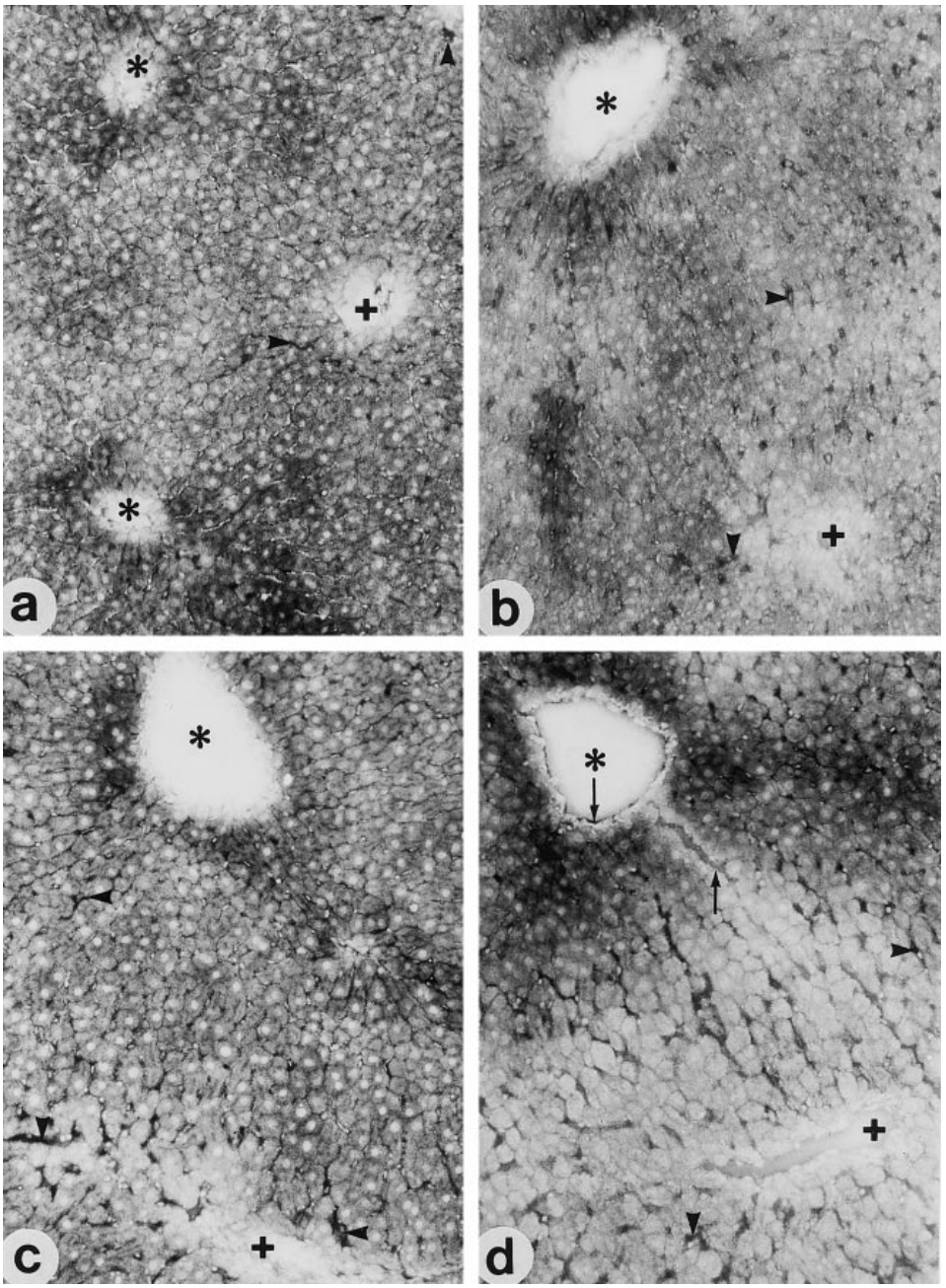

Figure 6. Sections of isolated perfused rat liver after a, 50 minutes of normoxic perfusion; b, 20 minutes of no-flow hypoxia; c, 20 minutes of N_2-induced hypoxic perfusion and d, 20 minutes of N_2-induced hypoxic perfusion plus 2 minutes of reoxygenation incubated for the demonstration of xanthine oxidoreductase activity. After normoxic perfusion or hypoxia, enzyme activity is somewhat decreased in all periportal cells (a–c). After reoxygenation, enzyme activity is strongly decreased in periportal cells and appears extracellularly in dilated sinusoids and at the luminal face of vascular endothelium (d, arrows). *Central vein, + portal vein, arrowheads: non-parenchymal cells. ×120.

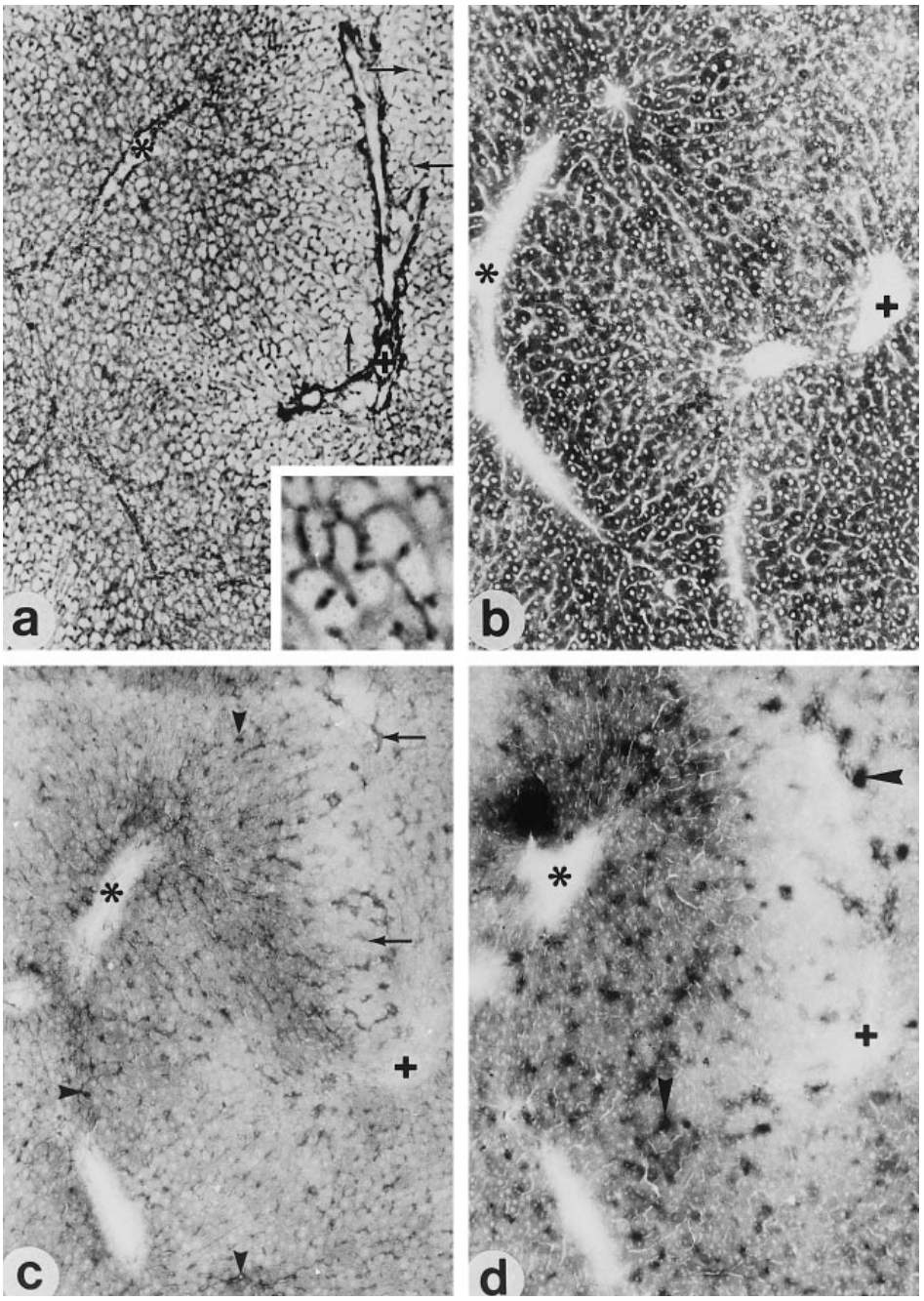

Figure 7. Consecutive sections of isolated perfused rat liver after 20 minutes of no-flow hypoxia and 2 minutes of reflow incubated for the demonstration of activity of a, 5′-nucleotidase; b, lactate dehydrogenase; c, xanthine oxidoreductase and d, glucose-6-phosphate dehydrogenase. 5′-Nucleotidase activity, most obvious at bile canalicular membranes (a, arrows, see insert), as well as cytoplasmic enzyme activities (b–d) are mainly decreased in periportal and midzonal hepatocytes. Xanthine oxidoreductase activity is present in dilated sinusoids (arrows) and in non-parenchymal cells (c, arrowheads). Glucose-6-phosphate dehydrogenase activity remains highest in non-parenchymal cells (d, arrowheads). *Central vein, + portal vein. ×80 (insert ×317).

ATP generation and a protective acidosis, continuous diffusion of substrates for glycolysis and oxidative phosphorylation during N_2-induced hypoxia will cause a rapid decline of hepatic ATP levels to critical values. Also, perfusion with N_2, unlike no-flow hypoxia, renders all liver zones hypoxic and induces severe zone-independent liver damage (Lemasters *et al.* 1981; Bradford *et al.* 1986).

The present study shows for the first time that the decrease in enzyme activities *in situ* in rat livers is mainly an effect of a few minutes of reoxygenation after hypoxia. A simultaneous demonstration of enzyme proteins revealed the leakage of cytoplasmic enzymes from hepatocytes within confined foci. The rapid drop in plasma membrane enzyme activity, suggesting loss of membrane integrity, is likely to initiate other events in reperfusion injury which may eventually lead to cell death. The presence of blood should be held responsible for the focal, mainly pericentral damage, whereas in the blood-free system periportal and midzonal injury predominates. Finally, *in situ* detection of enzyme activities and localization of enzyme proteins prove useful tools for the examination of very early changes in liver cells after different types of experimental ischaemia/reperfusion.

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