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RESPECTIVE ROLES OF FREE RADICALS AND ENERGY SUPPLY IN HYPOXIC RAT LIVER INJURY AFTER REOXYGENATION

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Livers from fasted rats subjected to 60 min of hypoxia followed by 25 min of reflow exhibited a significant release of lactate dehydrogenase (LDH) and protein into the perfusate, together with high rates of oxygen consumption, depletion of hepatic glutathione (GSH) and accumulation of thiobarbituric acid reactants (TBAR) in the liver. These changes were observed in the presence and absence of added xanthine (25 μM) and were significantly diminished when experiments were carried out in the presence of either 1 mM allopurinol or 100 μM Trolox. Allopurinol inhibited by 79% the production of uric acid by the liver, which was not altered by Trolox. Hypoxia-reflow studies performed in the presence of 25 μM 2,4-dinitrophenol (DNP) showed a drastic enhancement in LDH (244%) and protein (104%) efflux from the liver, compared with the effects found in its absence, with a moderate increase (35%) in tissue TBAR levels. Liver perfusion in the presence of both allopurinol and DNP exhibited a normalization of the tissue content of GSH and TBAR, while the net increase in LDH and protein release elicited by DNP alone was inhibited by only 20 and 25%, respectively. Similar results were obtained in experiments in which allopurinol was replaced by Trolox. These studies indicate that production of oxygen free-radicals are involved in hypoxic liver injury upon reflow, but its relative importance is significantly diminished when energy stores are severely diminished.

KEY WORDS: Free radicals, energy supply, hypoxia, reoxygenation, liver injury, antioxidants, mitochondrial uncoupling.

INTRODUCTION

Hypoxia-reperfusion injury to tissues implies that deprivation of O_2 and substrates supply imposes a reductive stress condition, which might give rise to oxidative injury upon reoxygenation.[1-3] The phenomenon is understood in terms of impairment of mitochondrial oxidative phosphorylation, with the onset of two major events, namely, (a) ATP depletion,[4] with loss of cellular transport ATPase functions[3] and altered cation homeostatis (i.e., increased intracellular Ca^{2+}),[5] and (b) accumulation of reducing equivalents and AMP,[4,6] with the concomitant enhanced production of O_2-derived free radicals upon reflow.[1,3] Membrane lipid peroxidation induced by oxygen free radicals has been found associated with hypoxia-reperfusion injury in several tissues,[3-7] however, the occurence of such mechanism in the liver is controvertial.[8] The relative importance of free radicals and energy supply in determining this phenomenon was assessed in the isolated perfused rat liver. For this purpose, the low perfusion flow followed by reflow hypoxic model[9] was used, under the influence of either the xanthine oxidase inhibitor allopurinol or the antioxidant Trolox, in the absence and presence of the mitochondrial uncoupler 2,4-dinitrophenol (DNP).

MATERIAL AND METHODS

Male Wistar rates weighing 182 ± 4 g (mean ± S.E.M.) ($n = 33$) with a liver/body weight ratio of 2.96 ± 0.05 g of liver/100 g body weight ($n = 33$) were fasted 24 h prior to experiments.

Before surgical preparation, the animals were anesthetized with 50 mg of nembutal/kg, intraperitoneally. Livers were perfused with hemoglobin-free Krebs-Heinseleit bicarbonate beffer (pH 7.4; 37°C) saturated with 95% O_2–5% CO_2, in an non-recirculating system, continuously monitoring O_2 concentration.[10] Following 25 min of perfusion, the initial flow rate (4.50 ± 0.18 ml/g liver/min ($n = 33$)) was reduced to 0.59 ± 0.02 ($n = 33$) for 60 min.[9] At the end of the hypoxic period, the flow rate was returned to the initial value, controlled by the use of a flow meter placed in the tube leading to the portal vein, and perfusion was continued for an additional 25 min period. Experiments using 25 μM xanthine in the influent perfusate were carried out in the absence of additives (A), or in the presence of either 1 mM allopurinol during all the perfusion period (B), 25 μM DNP infused after 55 min of perfusion (C), or both (D) (Figures 1 and 2). Similar experimental designs were performed in the absence of added xanthine, using 100 μM Trolox instead of allopurinol (Table I). Studies on the effect of DNP on hypoxic liver injury were carried out by infusing 25 μM DNP during hypoxia, 30 min before reflow (Figure 1C).

Samples of the effluent perfusate were collected during the whole perfusion period at 5 min intervals, for the determination of lactate dehydrogenase (LDH) activity,[11] and protein[12] and uric acid[13] contents. Rates of efflux were calculated from the effluent

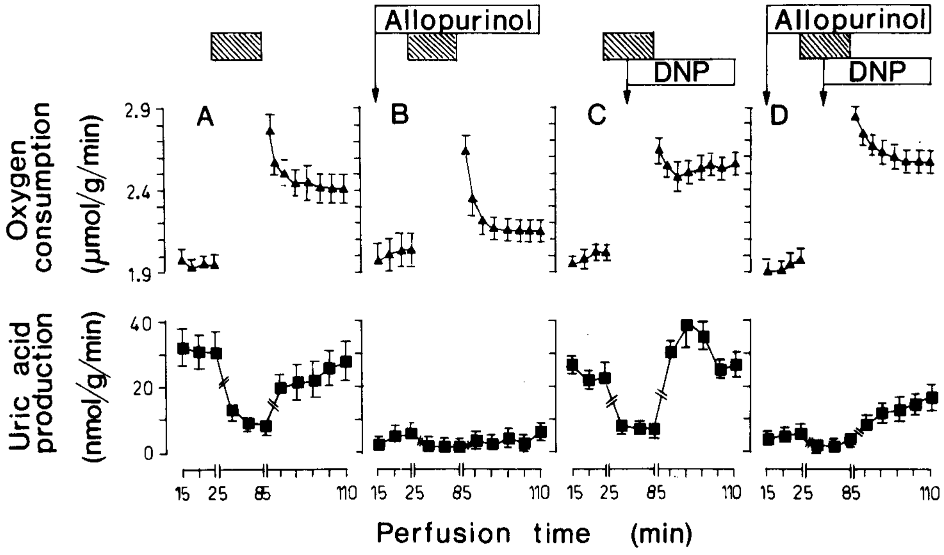

FIGURE 1 Effects of allopurinol (1 mM), 2,4-dinitrophenol (DNP) (25 μM) and allopurinol plus DNP on rates of oxygen consumption and uric acid production by perfused rat livers during hypoxia and reoxygenation. Experiments were carried out with a perfusion fluid containing 25 μM xanthine through all the perfusion period. The means ± S.E.M. are shown (n = 5–6 livers per experimental group, as indicated in Table I). Striped bars indicate periods of low flow perfusion, as described in Methods.

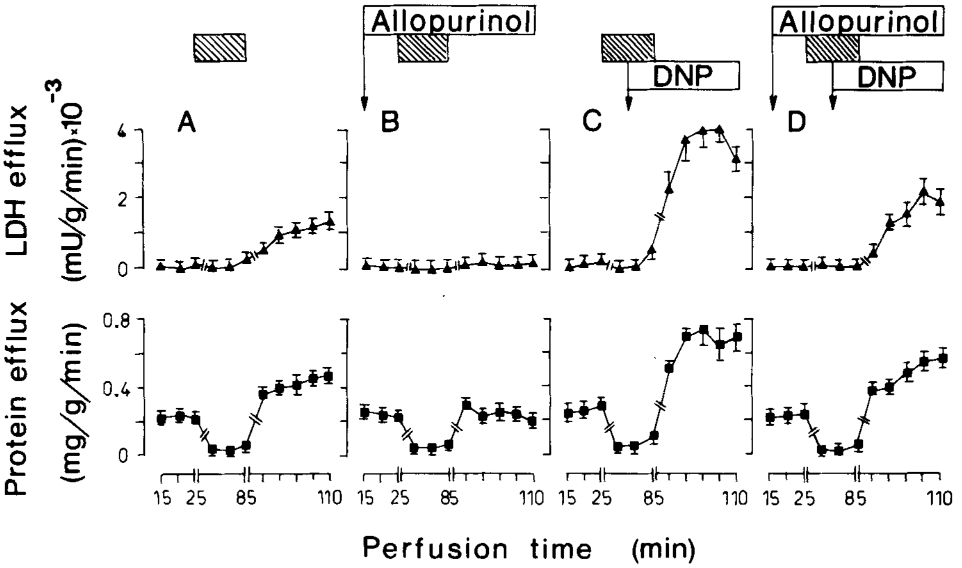

FIGURE 2 Effects of allopurinol (1 mM), 2,4-dinitrophenol (DNP) (25 μM) and allopurinol plus DNP on rates of lactate dehydrogenase (LDH) and protein efflux from perfused rat livers during hypoxia and reoxygenation. Other conditions as in Figure 1.

TABLE I

Hepatic release of uric acid, lactate dehydrogenase (LDH) and protein during reoxygenation of perfused rat livers subjected to hypoxia, under the influence of allopurinol, Trolox, 2,4-dinitrophenol (DNP) and combinations of allopurinol or Trolox with DNP

	Parameters*		
Experimental conditions	Uric acid production (nmol/g of liver)	LDH efflux (U/g of liver)	Protein efflux (mg/g of liver)
A. - Perfusion with 25 μM xanthine			
a) No additions	497.5 ± 34.7 (6)⁺	21.0 ± 1.9 (6)	3.73 ± 0.28 (6)
b) Allopurinol (1 mM)	99.5 ± 10.5 (5)	4.1 ± 0.5 (5)	0.42 ± 0.05 (5)
c) DNP (25 μM)	727.1 ± 65.4 (5)	72.4 ± 4.6 (5)	7.71 ± 0.60 (5)
d) Allopurinol (1 mM) + DNP (25 μM)	313.1 ± 35.0 (5)	30.8 ± 4.0 (5)	4.79 ± 0.54 (5)
B. - Perfusion without added xanthine			
e) No additions	149.2 ± 13.1 (3)	9.6 ± 0.6 (3)	1.92 ± 0.20 (3)
f) Trolox (100 μM)	145.0 ± 14.3 (3)	0.7 ± 0.1 (3)	0.50 ± 0.06 (3)
g) DNP (25 μM)	160.0 ± 11.3 (3)	20.6 ± 1.5 (3)	2.66 ± 0.28 (3)
h) Trolox (100 μM) + DNP (25 μM)	179.1 ± 13.5 (3)	13.7 ± 1.4 (3)	2.00 ± 0.22 (3)

*Total uric acid, LDH and protein effluxes were calculated by integration of the area under the respective time curves of sinusoidal releases upon reoxygenation (85–110 min perfusion), as shown in Figures 1 and 2.

⁺Values shown represent the means ± S.E.M. for the number of perfusions indicated in parentheses. Statistical studies: LDH and protein efflux, a versus b and c, e vs f and g, d vs b and c, h vs f and g ($P < 0.001$). Uric acid production, a vs b, c vs d (P (0.01); a vs c ($P < 0.05$); a vs e ($P < 0.001$); e vs f, g and h, not significant.

activities or concentrations, referred to the corresponding flow rates and wet liver weights. At the end of the perfusion period (110 min), samples of liver tissue were taken to determine the content of reduced glutathione (GSH),[14] thiobarbituric acid reactants (TBAR)[15] and protein,[12] as well as tissue LDH activity.[11] One unit of LDH activity is equivalent to 1 μmol/min.

All chemicals used were obtained from Sigma (St. Louis, MO, USA), except for Trolox (Aldrich Chemical Co. Inc., Milwaukee, W, USA). Values shown represent the mean ± S.E.M. for the number of perfusions indicated. Statistical differences between the different perfusion conditions were assessed by the Student's t-test for unpaired data.

RESULTS

Reoxygenation of perfused livers after 60 min of hypoxia produced a 22% increase in O_2 consumption, with no changes in uric acid production, compared to pre-hypoxia values (Figure 1A). Concomitantly, LDH and protein release at the end of the experiments were 14-fold and 100% higher than pre-hypoxia values, respectively (Figure 2A). The addition of 1 mM allopurinol to the perfusion fluid elicited a 79% inhibition of uric acid production by perfused livers (Table I). In this situation, O_2 consumption (Figure 1B) and LDH and protein efflux (Figure 2B) were comparable during the pre-hypoxia and reflow periods. However, allopurinol inhibited by 80 and 89% the total LDH and protein released during reflow, respectively, when compared to experiments performed in its absence (Table I). The presence of DNP during reflow increased the hepatic O_2 uptake by 26% over pre-hypoxia values (Figure 1C). Reoxygenation in the presence of DNP increased by 46% the total uric acid production by the liver compared to control values (Table I). In this condition, LDH and protein release were enhanced by 22-fold and 140% over pre-hypoxia values, respectively (Figure 2C), while increases of 244% and 104% were found when values in the presence of DNP are compared to the respective efflux rates obtained in the absence of the uncoupler (Table I). Control experiments similar to those of Figures 1C and 2C, but without hypoxia, revealed that DNP did not significantly modify LDH and protein efflux, as well as liver GSH and TBAR contents (data not shown). DNP also increased liver O_2 uptake in the presence of allopurinol (Figure 1D). Allopurinol in the presence of DNP inhibited uric acid production by 37% and 57%, compared to control values and to those found with DNP alone, respectively (Table I). During the reflow period, LDH and protein efflux from perfused livers increased by 13-fold and 82% with allopurinol plus DNP over pre-hypoxia values, respectively (Figure 2D). Increases of 47% and 27% in total LDH and protein efflux are observed upon reflow, when values obtained with allopurinol plus DNP are compared with those found under no-addition conditions (Table I). LDH and protein efflux rates from perfused livers after reperfusion were significantly correlated, in the different conditions studied ($r = 0.96$; $p < 0.001$).

Contents of liver GSH and TBAR were determined at the end of the perfusion period in all the conditions shown in Figures 1 and 2, as well as in comparable normoxic conditions. Hypoxia elicited a significant diminution in liver GSH in the absence or presence of DNP, together with enhanced TBAR levels (Table II), when compared to normoxic conditions. These changes were reverted to control values when hypoxia was induced in the presence of allopurinol (Table II).

TABLE II

Hepatic content of reduced glutathione (GSH) and thiobarbituric acid reactants (TBAR) after reoxygenation of rat livers subjected to hypoxia, under the influence of allopurinol, 2,4-dinitrophenol (DNP) and allopurinol plus DNP

Experimental conditions	GSH (μmol/g of liver)	TBAR (pmol/mg of protein)
A. - Normoxic conditions*	5.32 ± 0.46 (4)	27.2 ± 5.1 (4)
B. - Hypoxic conditions§		
a) No additions	3.40 ± 0.18 (6)$^+$	60.8 ± 5.2 (6)$^+$
b) Allopurinol (1 mM)	5.05 ± 0.03 (5)	29.3 ± 2.6 (5)
c) DNP (25 μM)	3.23 ± 0.13 (5)$^+$	82.2 ± 8.9 (5)$^{+,\ddagger}$
d) Allopurinol (1 mM) + DNP (25 μM)	4.74 ± 0.24 (5)	28.8 ± 2.3 (5)

*Liver perfusions carried out in the absence of hypoxia or additions for 110 min.

§Liver perfusions carried out as described in Figures 1 and 2.

$^+P < 0.001$, compared to normoxic conditions.

$^\ddagger P < 0.05$, compared to (a).

Liver perfusion experiments, comparable to those presented in Figures 1 and 2, were performed in the absence of added xanthine and revealed similar changes in hepatic O_2 consumption and in GSH or TBAR contents (data not shown). As expected, the production of uric acid by perfused livers during reflow dropped by 70% in this condition, compared to that found in the presence of 25 μM xanthine, and was not modified by Trolox, DNP, or Trolox plus DNP (Table I). Accordingly, total LDH and protein efflux from perfused livers during reflow corresponded to 45% and 50% of those observed in experiments with added xanthine, in the absence of additives, respectively (Table I). These parameters were diminished by 93% and 74%, when livers perfusion are carried out in the presence of 100 μM Trolox (Table I). As in the case of experiments with added xanthine, LDH and protein release upon reflow were enhanced by 114% and 39% by DNP, over values obtained in the absence of the uncoupler (Table I). These efflux rates were decreased by 33% and 25%, when the effect of DNP is assessed in the presence of Trolox (Table I).

DISCUSSION

Hypoxia produced by low flow perfusion of livers from fasted rats elicited significant liver injury upon reoxygenation, whose magnitude seems to be related to purine metabolites availability. Reperfusion liver damage coincided with an elevation of TBAR and diminution of GSH levels in the tissue, which, together with the enhanced content and sinusoidal efflux of hepatic GSSG previously reported,[16] are indicative of oxidative stress. In addition to a high free-radical mediated lipid peroxidative activity that might be coupled to the operation of the xanthine oxidase system, TBAR formation may derive from alternative processes such as prostaglandin biosynthesis,[17] that could increase in response to injury. An enhanced O_2 utilization in purine catabolism and lipid peroxidation, and in mitochondrial oxidative phosphorylation triggered by the low energy charge that prevails during hypoxia,[18] could contribute to set the hepatic O_2 uptake at a higher steady state rate during reflow, compared to that of the pre-hypoxia period. When reoxygenation took place in the presence of allopurinol or Trolox, liver injury was drastically reduced, with normalization of tissue

TBAR and GSH levels and of the O_2 uptake rate. These data inidcate that production of oxygen free radicals plays a major role in hypoxic liver injury upon reflow, and that protection would require effective antioxidant interventions and sufficient energy supply, to restore cellular functions altered by hypoxia.[3-6]

The role of energy supply in determining hypoxia liver injury upon reflow was assessed by inducing uncoupling of mitochondrial oxidative phosphorylation, in conditions which do not alter the prooxidant-antioxidant balance of the liver cell. Reoxygenation of hypoxic livers in the presence of DNP produced a drastic enhancement of liver injury, in relation to that found in the absence of the uncoupler, when perfused with or without added xanthine. This finding was seen concomitantly with a moderate increase in hepatic lipid peroxidation and was completely abolished by allopurinol. Although these results point out to the involvment of xanthine oxidase-mediated processes in hypoxic liver damage by mitochondrial uncoupling, its relative contribution to the overall phenomenon induced by DPN seems to be rather small. In fact, allopurinol was found to decrease by only 20 to 25% the net increments in total LDH and protein release elicited by DNP. This effect may be related to the lower inhibition (57%) or uric acid production when reflow of hypoxic livers occurred in the presence of allopurinol and DNP, compared to that seen with allopurinol alone (79%). It is possible that the high rate of electron flow through the mitochondrial respiratory chain imposed by the uncoupler would increase NAD^+ availability for the NAD^+-dependent form of liver xanthine dehydrogenase. This could partially overcome the inhibition of xanthine dehydrogenase by allopurinol, with uric acid being produced without free radical generation. The contention that free radical processes seem to play a minor role in hypoxic liver injury when ATP availability is severe reduced, is strongly supported by data obtained with Trolox. This chain-breaking antioxidant dimished by 37% and 11% the net enhancement in hepatic LDH and protein efflux by DNP, respectively, without altering xanthine oxidase function. Thus, exacerbation of hypoxic liver injury upon reflow by mitochondrial uncoupling might be mainly ascribed to insufficient replenishment of cellular energy stores, condition which could accentuate the accumulation of cytosolic Ca^{2+} set in by hypoxia, as uncouplers rapidly release Ca^{2+} from mitochondrial pools.[19] In this respect, it is interesting to note that mitochondrial uncoupling by DNP has been reported to markedly inhibit enzyme release seen upon reoxygenation of the isolated perfused hypoxic heart.[20] The different DNP effect on hypoxic injury observed in liver and heart could be due to the different structural and functional properties of both tissues. In fact, recovery of ATP levels upon reflow would produce heart injury by excessive contracture of myocardial elements, effect that would be avoided by ATP depletion upon mitochondrial uncoupling.[20]

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