Vol. 160, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1989 Pages 140-147

MITOCHONDRIA AND XANTHINE OXIDASE BOTH GENERATE REACTIVE OXYGEN SPECIES IN ISOLATED PERFUSED RAT LIVER AFTER HYPOXIC INJURY

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Received January 2, 1989

Hypoxia caused severe damage in isolated perfused livers from fasted male Fischer rats without evidence of the formation of reactive oxygen species during hypoxia. Reoxygenation caused a significant increase in intracellular oxygen species in the injured liver, as indicated by increases in sinusoidal GSSG efflux and tissue GSSG levels. Both parameters were elevated further by addition of KCN (100 μM) or antimycin A (8 μM). Sinusoidal GSSG efflux was suppressed in part by addition of allopurinol (500 μM) and enhanced by hypoxanthine (250 μM). Xanthine oxidase appears to be a partial source, and damaged mitochondria a continuous and quantitatively greater source, of reactive oxygen as a result of liver injury following hypoxia.

The high susceptibility of the liver to temporary oxygen deprivation and reoxygenation resulting in tissue necrosis and subsequent organ failure is a major clinical problem for liver transplantation, hepatic surgery, and management of shock. The mechanisms of hypoxic or ischemic injury are poorly understood. Reactive oxygen species, generated during reoxygenation of the tissue, have been reviewed as one possible contributor to damage (1). Pharmacological evidence, such as the beneficial effects of the xanthine oxidase inhibitor allopurinol (2-5) or of enzymes that metabolize reactive oxygen species, e.g., superoxide dismutase and catalase (3,4), support the oxygen radical hypothesis.

Our own investigation of the role of reactive oxygen in ischemic or hypoxic injury in the liver has capitalized on the fact that hydrogen peroxide, the dismutation product of the primary metabolite superoxide, is reduced to water by glutathione peroxidase. Glutathione (GSH) provides the reducing equivalents for this reaction through oxidation to its disulfide (GSSG). We and others have found an enhanced efflux of GSSG into bile, blood, or organ perfusate to be a highly sensitive and specific quantitative index of intracellular oxidative stress in vivo (6-8) and in vitro (9-11). Two different models of hepatic injury, i.e., no-flow ischemia and normal-flow hypoxia, have been recently investigated in the isolated, blood-free perfused rat liver to detect and quantify any oxidative stress during reflow or reoxygenation. In the global no-flow ischemia followed by reflow model no evidence was found for formation of GSSG quantitatively sufficient to exceed endogenous defense

0006-291X/89 $1.50

systems at any time during ischemia and reflow (11). In the biologically different model of normal-flow hypoxia, however, an increased sinusoidal efflux and elevated tissue concentration of GSSG indicated a moderately enhanced intracellular formation of reactive oxygen species during reoxygenation (12). The intracellular source of the oxygen radicals was not examined. The aim of the present study was to identify the sources of reactive oxygen formation and elucidate their relative contribution to the oxidative stress during reoxygenation. The data presented here indicate that xanthine oxidase is a temporary source of reactive oxygen formation during the initial reoxygenation period and damaged mitochondria are a persistent and quantitatively greater source of reactive oxygen formation resulting from liver injury following hypoxia.

MATERIALS AND METHODS

Male Fischer rats (Harlan Sprague-Dawley, Inc., Houston, TX) were fasted overnight and livers were isolated and perfused with hemoglobin- and albumin-free Krebs-Henseleit bicarbonate buffer (pH 7.4; 37°C) gassed with carbogen (95% O_2, 5% CO_2). The bile duct had been cannulated with PE-10 tubing. The validity of this model and viability criteria of the perfused liver have been described and discussed in detail (13-15). After a preperfusion phase of 30 min, starting with cannulation of the portal vein, carbogen was replaced by a nitrogen/carbon dioxide mixture (95% N_2, 5% CO_2) for 35 min and then switched back to carbogen for another 30 min (reoxygenation period). The perfusate flow rate was kept constant at ≥ 3 ml/min/g liver weight at any time. In some of the experiments the perfusate contained 10 mM fructose as glycolysis substrate (16), 200 μM hypoxanthine as substrate for xanthine oxidase or 500 μM allopurinol as inhibitor of xanthine oxidase, respectively. Those animals whose livers were perfused with allopurinol were pretreated additionally with the drug (50 mg/kg body weight; p.o.) 18 h prior to the experiment. In other experiments animals were pretreated with the catalase inhibitor 3-amino-1,2,4-triazole (1 g/kg body weight; i.p.) (17) and the liver isolated and perfused 2 h later. To test the superoxide generation of mitochondria, various chemicals known to affect mitochondrial respiration and superoxide formation (18) were perfused during the reoxygenation period after hypoxic damage: 100 μM KCN or 8 μM antimycin A. In these experiments, when the mitochondrial respiration was blocked by KCN or antimycin A, 10 mM fructose was added to the perfusate to provide a substrate for the anaerobic generation of ATP during reoxygenation. To test mitochondrial function in the intact organ, glucose efflux from the liver was determined during infusion of 5 mM lactate (gluconeogenesis capacity) (19). All chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Total glutathione and GSSG were determined in perfusate, bile, and in freeze-clamped tissue with the Tietze assay (20). Glucose, lactate, and lactate dehydrogenase activities in perfusate and tissue ATP levels were determined according to standard procedures (21).

Statistics: All parameters are expressed as mean ± SEM. Comparison of data sets were performed with the unpaired Student's t-test.

RESULTS

Livers from fasted male Fischer rats were highly susceptible to hypoxic injury. As shown in Figure 1, severe cell damage started between 20 and 30 min after initiation of hypoxic perfusion and progressed exponentially as indicated by the dramatic release of lactate dehydrogenase (LDH). After reoxygenation, however, LDH efflux returned rapidly to near initial levels. Tissue ATP levels declined rapidly from 20.3 ± 1.3 nmol ATP/mg protein before hypoxia to 1.2 ± 0.3 after 40 min of hypoxic perfusion and recovered in part

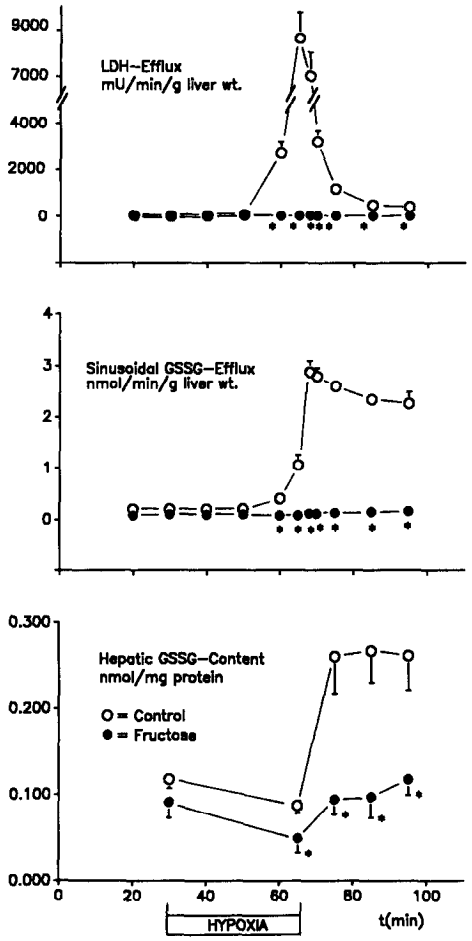

Figure 1. Effect of hypoxia and reoxygenation on the sinusoidal efflux of LDH (upper graph) and GSSG (given as GSH-equivalents) and the tissue GSSG content (lower graph; given as GSH-equivalents) in isolated perfused livers from fasted animals. Livers were perfused with plain perfusate (controls) or perfusate containing 10 mM fructose. After a preperfusion period of 30 min, carbogen was replaced by a nitrogen/carbon dioxide mixture for 35 min (hypoxia) and then switched back to carbogen (reoxygenation) for 30 min. Given are the mean ± SEM of n = 4 experiments per group. If no standard error is shown, the value is smaller than the symbol. *$p < 0.05$ fructose versus controls.

to 7.5 ± 1.0 after 15 min of reoxygenation. Fructose (10 mM) in the perfusate protected the livers effectively from irreversible hypoxic damage. During perfusion with fructose efflux rates of LDH were determined as 1.3 ± 0.5 mU/min/g prior to hypoxia, 13.2 ± 2.0 at 65 min and 12.3 ± 1.4 after 30 min of reoxygenation (Figure 1). Tissue ATP levels in fructose-perfused livers (11.5 ± 3.4 nmol/mg protein at 30 min) declined by 24% ($p < 0.05$) during hypoxia and fully recovered during reoxygenation. The sinusoidal efflux of GSSG as an index of intracellular formation of reactive oxygen species was < 0.2 nmol GSH-eq./min/g liver weight for fructose-perfused livers at all times during hypoxia and reoxygenation (Figure 1). In control livers GSSG efflux increased minimally during the hypoxic period but increased several-fold during reoxygenation at a time when GSH and LDH efflux declined. Measurement of the hepatic GSSG content revealed no enhancement before and during

hypoxia (Figure 1). During reoxygenation, however, the tissue GSSG levels also increased significantly in controls compared to fructose-perfused livers. The total soluble glutathione content (GSH + GSSG) of these livers at 95 min was determined as 14.6 ± 1.2 nmol GSH-eq./mg protein (controls) and 20.6 ± 1.5 (fructose) ($p < 0.05$).

Bile flow and biliary secretion of GSH and GSSG declined in both sets of livers during hypoxia. Whereas there was a total irreversible cholestasis in controls, biliary parameters of fructose-perfused livers recovered to values that represented about 60% (GSH and GSSG secretion rates) and 90% (bile flow) of those measured in livers perfused without hypoxia for 60 to 90 min. However, no increase in biliary GSSG in the fructose-perfused livers was observed at any time during hypoxia and reoxygenation (data not shown).

A potential source of superoxide formation in isolated perfused rat liver may be xanthine oxidase. To test this possibility, the xanthine oxidase inhibitor allopurinol was given in vivo and then added to the liver perfusate. As shown in Figure 2 allopurinol did not affect GSSG efflux into the perfusate before and during hypoxia, but suppressed significantly the sinusoidal GSSG efflux during the first 10 min of reoxygenation. Afterwards, at later time intervals, no significant difference was seen between controls and allopurinol-perfused livers. On the other hand, when the xanthine oxidase substrate hypoxanthine was present in the perfusate, GSSG efflux increased to about twice the control values during the early phase of reoxygenation (Figure 2). There was a decline of the GSSG efflux afterwards, but all values remained significantly above controls. Neither allopurinol nor hypoxanthine affected hepatic GSSG levels differently than controls at any time (Figure 2). Neither compound showed an effect on hepatic LDH efflux during hypoxia or reoxygenation (data not shown).

To test whether mitochondria might be another relevant source of reactive oxygen formation, livers were perfused with inhibitors of the mitochondrial respiratory chain (KCN or antimycin A). These compounds are known to enhance superoxide formation in isolated mitochondria (18) and perfused livers (17). The experiments were performed with livers from animals pretreated with the catalase-inhibitor aminotriazole (AT), since KCN also inhibits catalase. As shown in Figure 3, the sinusoidal efflux of GSSG (AT: 1.2 ± 0.1 nmol GSH-eq./min x g; C: < 0.2) and the tissue GSSG content (AT: 0.28 ± 0.03 nmol GSH-eq./mg protein; C: 0.12 ± 0.02) were significantly higher in AT-pretreated rats under basal conditions as well as after hypoxic damage. These results support the hypothesis that catalase detoxifies a considerable part of reactive oxygen species formed outside the peroxisomes (17). When antimycin A or KCN were present in the perfusate during reoxygenation intracellular GSSG formation was dramatically enhanced. The hepatic GSSG content was doubled and the sinusoidal efflux of GSSG was 3- to 4-fold higher than the already increased values measured in livers without mitochondrial respiratory blockers, indicating a considerable further stimulation of the intracellular formation of reactive oxygen under these conditions. The time-course of the hypoxic damage (LDH-efflux) was similar to the untreated animals in Figure 1 (data not shown).

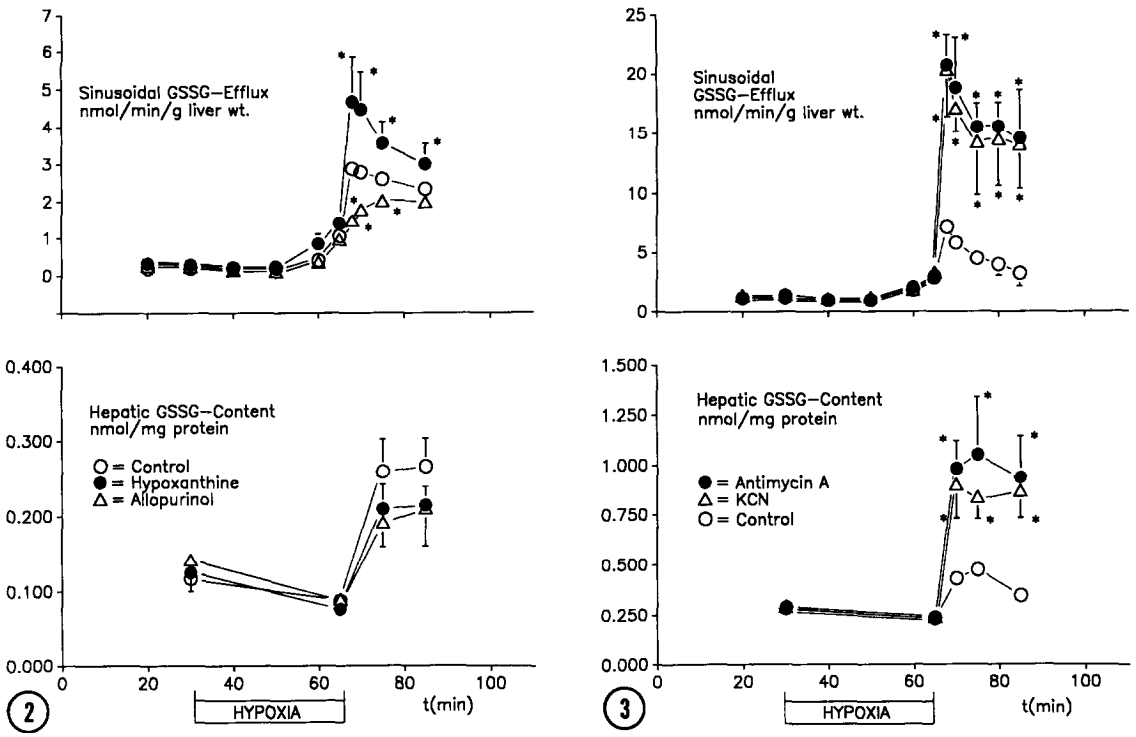

Figure 2. Effect of hypoxia and reoxygenation on the sinusoidal efflux of GSSG (upper graph; given as GSH-equivalents) and the tissue GSSG content (lower graph; given as GSH-equivalents) in livers from fasted animals. Livers were perfused with plain perfusate (controls) or perfusate containing either 250 μM hypoxanthine or 500 μM allopurinol. In the latter case, animals were additionally pretreated with allopurinol (50 mg/kg body weight; p.o.) in vivo. After a preperfusion period of 30 min, carbogen was replaced by a nitrogen/carbon dioxide mixture for 35 min (hypoxia) and then switched back to carbogen (reoxygenation) for 30 min. Given are the mean ± SEM of n = 4 experiments per group. If no standard error is shown, the value is smaller than the symbol. *p < 0.05 controls versus treated group.

Figure 3. Effect of hypoxia and reoxygenation on the sinusoidal efflux of GSSG (upper graph; given as GSH-equivalents) and the tissue GSSG content (lower graph; given as GSH-equivalents) in livers from fasted and aminotriazole-pretreated animals (1 g/kg body weight, i.p.). All livers were perfused with plain perfusate during the 30 min preperfusion period and during 35 min of hypoxia when carbogen was replaced by a nitrogen/carbon dioxide mixture. During reoxygenation livers were perfused with perfusate containing 10 mM fructose (controls), 100 μM KCN and 10 mM fructose or 8 μM antimycin A and 10 mM fructose. Given are the mean ± SEM of n = 4 experiments per group. If no standard error is shown the value is smaller than the symbol. *p < 0.05 controls versus treated group.

To determine the extent that mitochondrial respiratory chain inhibitors affect reactive oxygen formation in the intact organ, livers from AT-pretreated rats were perfused with fructose to prevent hypoxic damage (as shown in Figure 1) and subsequently perfused with KCN or antimycin A during reoxygenation. Under these conditions only the sinusoidal efflux of GSSG increased slightly from 1.09 ± 0.10 to 1.36 ± 0.08 nmol GSH-eq./min/g liver weight. Tissue levels of GSSG and the biliary GSSG excretion were not significantly different from livers reoxygenated without respiratory chain inhibitors.

To verify mitochondrial damage in the intact organ, lactate was infused into the liver to determine the capacity for gluconeogenesis. Non-damaged livers removed 2800 ± 250

nmol lactate/min/g liver weight from the perfusate and released 560 ± 46 nmol glucose/min/g, whereas livers with hypoxic damage removed only 1550 ± 230 nmol lactate/min/g ($p < 0.05$) and released 310 nmol glucose/min/g ($p < 0.05$), when determined after 10 min of reoxygenation.

DISCUSSION

The data presented here indicate that severe cell damage occurred in livers from fasted Fischer rats after 20 to 30 min of hypoxia. The onset of damage and the loss of tissue ATP occurred in these livers about 15 to 20 min earlier than in partially glycogen-depleted livers used in a previous study (12). Livers from fed animals were even more resistant to hypoxic injury (22). On the other hand, addition of the glycolysis substrate fructose in the perfusate totally prevented cell damage, confirming recent papers by Anundi et al. (16,23). Fructose also preserved organ functions such as bile flow and biliary secretion of GSH and GSSG during hypoxia and reoxygenation. These observations indicate that the capability to maintain adequate intracellular ATP levels through the availability of glycolysis substrates is of critical importance for the initiation of hypoxic cell damage in these livers (24).

The purpose of this study was to identify the intracellular sources of reactive oxygen formation in the intact organ. The fact that the xanthine oxidase inhibitor allopurinol significantly attenuated GSSG formation and release during the first 10 min of reoxygenation, and that hypoxanthine continuously enhanced GSSG release, indicates that at least a part of the xanthine dehydrogenase activity was converted to an oxidase and that this enzyme generated reactive oxygen during reoxygenation. However, the amount of reactive oxygen formed seemed to be limited by the availability of enzyme substrates. Hypoxanthine and xanthine are water-soluble and easily washed out from the liver (19). Furthermore, hypoxanthine can also be used in the salvage pathway for the resynthesis of adenine nucleotides. The available intracellular substrate concentration for xanthine oxidase should therefore decline rapidly after onset of reoxygenation and thus restrict the formation of reactive oxygen by this enzyme.

The allopurinol experiments provide evidence as well for another intracellular source of reactive oxygen formation that is not sensitive to allopurinol inhibition. Damaged mitochondria have been proposed previously as a potential source of reactive oxygen (25), since mitochondria release considerable amounts of superoxide even under physiological conditions (17,18,26) and mitochondria are damaged during hypoxia and reoxygenation (19,27). Two different blockers of the mitochondrial respiratory chain, i.e., antimycin A (a selective inhibitor of cytochrome b) and KCN (an inhibitor of cytochrome a/a_3 but also of catalase) were used. Both compounds are known to increase superoxide release from mitochondria (17,18,26). Infusion of KCN or antimycin A into non-damaged livers enhanced GSSG formation minimally during reoxygenation whereas the same respiratory chain blockers increased GSSG formation several-fold in damaged livers, thus providing evidence that damaged mitochondria are the major source of reactive oxygen generation after hypoxic injury. KCN and antimycin A are equally effective when used in livers with

inhibition of catalase (Figure 3). In untreated livers the stimulation of GSSG formation was more pronounced with KCN than with the specific inhibitor antimycin A (H. Jaeschke and J.R. Mitchell, unpublished). These different results are explained by the fact that KCN, in addition to the inhibition of cytochrome a/a_3, also inhibits catalase and thus provides more substrate for glutathione peroxidase in part by stimulating reactive oxygen formation in mitochondria and in part by inhibition of the peroxide detoxifying enzyme. Our data support earlier reports by Oshino and Chance, who estimated that the peroxisomal enzyme catalase can degrade essentially all of the hydrogen peroxide generated inside the peroxisomes and up to 50% of the hydrogen peroxide formed outside these organelles (17,26).

The data presented here support our earlier findings (12) recently confirmed by others (28) that oxyradicals are not involved in the initiation of hypoxic damage. However, formation of reactive oxygen during the early reoxygenation period was proposed to be involved in the generation of a chemotactic signal leading to neutrophil activation, capillary plugging, and vasculature oxidative stress (29,30,31). On the other hand, a continuous release of oxyradicals from mitochondria may damage these organelles directly, causing a further impairment of the oxidative phosphorylation capacity (32).

In summary, this paper presents evidence for the enhanced formation of reactive oxygen during reoxygenation after hypoxic liver injury. Xanthine oxidase was identified as a temporary source at the beginning of the reoxygenation period whereas damaged mitochondria represent the continuous and quantitatively dominating source of reactive oxygen formation in the injured liver.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant GM-34120 and USPHS Grant RR-05425. The authors thank Mr. Michael Fisher and Mr. Bradley Black for skillful technical assistance.

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