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# SUPEROXIDE GENERATION BY KUPFFER CELLS AND PRIMING OF NEUTROPHILS DURING REPERFUSION AFTER HEPATIC ISCHEMIA

HARTMUT JAESCHKE*, ABRAHAM P. BAUTISTA, ZOLTAN SPOLARICS and JOHN J. SPITZER

**Center for Experimental Therapeutics, Baylor College of Medicine, Houston, Texas, and Department of Physiology, Louisiana State University Medical Center, New Orleans, Louisiana*

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The objective of this study was to identify the cellular source of the vascular oxidant stress in hepatic ischemia-reperfusion injury in male Fischer rats. Nonparenchymal cells (Kupffer cells, endothelial cells) and neutrophils were isolated from postischemic liver lobes by collagenase-pronase digestion followed by centrifugal elutriation. The spontaneous and stimulated generation of superoxide by these cells were subsequently quantified *in vitro*. Large Kupffer cells from the postischemic lobes spontaneously generated 300% more superoxide than similar cells from control animals. No difference in spontaneous superoxide formation was found when the small Kupffer cells were compared. No other cells isolated from the postischemic lobes or control liver including neutrophils released any detectable superoxide spontaneously. In contrast, small Kupffer cells and neutrophils from the postischemic liver generated significantly more superoxide after stimulation with phorbol ester or opsonized zymosan than the controls. The considerably higher response with zymosan stimulation compared to phorbol ester indicates a particular priming for a receptor-mediated signal transduction pathway during reperfusion. These studies demonstrate that Kupffer cells are the principal source of the oxidant stress during the initial reperfusion phase after hepatic ischemia. The priming of neutrophils during this time may be an important factor for the later neutrophil-induced injury phase.

KEY WORDS: Kupffer cells, neutrophils, oxygen radicals, ischemia-reperfusion injury, liver

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## INTRODUCTION

The importance of reactive oxygen species in the pathogenesis of ischemia-reperfusion injury in the liver had been implicated in many pharmacological intervention studies.[1] Since there is no evidence that quantitatively enough reactive oxygen is formed intracellularly during reperfusion to cause parenchymal cell injury through lipid peroxidation[2,3] or thiol oxidation,[4,5] our attention focussed on the role of liver macrophages (Kupffer cells) and neutrophils as potential sources of extracellular reactive oxygen formation.[6] Two phases of reperfusion injury were identified after hepatic no-flow ischemia, an initial Kupffer cell-induced oxidant stress and injury[7] and a later severe neutrophil-induced injury.[8] The conclusion that Kupffer cells and not neutrophils are mainly responsible for the initial injury was based on two experimental findings: 1. The drastic increase of plasma glutathione disulfide (GSSG) concentrations during reperfusion indicating an extracellular oxidant stress.[6] 2. The postischemic increase of plasma GSSG levels could be enhanced or attenuated by respective alterations of the Kupffer cell activity in the liver, while no correlation was





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found between plasma GSSG levels and the initial accumulation of neutrophils in the postischemic liver.[7] Morphological changes of the Kupffer cells during reperfusion after cold ischemia provide further evidence for the postischemic activation of Kupffer cells.[9] However, direct evidence for an increased formation of reactive oxygen by Kupffer cells in ischemia-reperfusion injury is missing. Furthermore, the role of hepatic endothelial cells as potential source of reactive oxygen is unknown. The present study addressed these problems by isolating Kupffer cells, endothelial cells and neutrophils from the postischemic and from the nonischemic liver lobes. A similar experimental approach could identify Kupffer cells as the principal source of reactive oxygen following endotoxin administration.[10,11] Our results indicate an enhanced spontaneous superoxide formation only by large Kupffer cells and a significant priming of the smaller Kupffer cell fraction and particularly of neutrophils during reperfusion.

## MATERIALS AND METHODS

### *Animals*

Fed male Fischer 344 rats (Harlan Sprague Dawley Inc, Houston, Texas) with a body weight of 240–290 g were used in all experiments. After anesthetizing the animal with pentobarbital (60 mg/kg), the blood flow to the medium and left lateral hepatic lobes was occluded with an atraumatic bulldog clamp for 45 min; reflow was initiated by removal of the clamp. After 60 min of reperfusion a blood sample was obtained from the vena cava for the determination of plasma alanine aminotransferase (ALT) activity (Test kit DG 159-UV, Sigma Chemical Co., St Louis, MO) and the different cell types of the liver were isolated from the postischemic and the nonischemic lobes.[10,11]

### *Isolation of Kupffer Cells and Hepatic Granulocytes*

The liver was perfused in a circulating system for 5–10 min with 25 mg collagenase (Sigma) in 100 ml Hank's balanced salt solution supplemented with 0.001% deoxyribonuclease (Sigma) and 1 mmol $CaCl_2$. The organ was surgically removed, the postischemic and nonischemic lobes were separated and cut into small pieces and digested further with 0.2% pronase (Sigma), 0.001% deoxyribonuclease in Gey's balanced salt solution, at room temperature for 20–30 min. The digested tissues were strained through a layer of nylon mesh. The cell suspension was centrifuged at 50 × g for 2 min to separate the parenchymal cells from the nonparenchymal cells. Endothelial and different subpopulations of Kupffer cells (arbitrarily classified as $KC_{1,2,3}$) were separated according to cell density[12,13] by centrifugal elutriation at 875 × g using different flow rates. Endothelial cells were eluted at 23 ml/min flow rate. $KC_1$, the smallest of the Kupffer cell fraction, were collected at 29 ml/min flow rate. This cell fraction was not used in this study because it was usually contaminated with 30–70% endothelial cells. $KC_2$ (small Kupffer cells) were eluted at 45 ml/min flow at 875 × g. The large Kupffer cells ($KC_3$) were collected at 45 ml/min flow rate at 1 × g. Hepatic neutrophils were separated from the Kupffer cells using Ficoll-Hypaque gradient centrifugation as described below. Purity of Kupffer cell preparations were always above 90% and for hepatic neutrophils it was 95–99%, as assessed by morphology (Giemsa-Wrights stain) and peroxidase stain.

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*Isolation of Blood Granulocytes*

Whole blood was anticoagulated with 100 units/ml heparin and diluted 1 : 3.5 with Hank's balanced salt solution. One ml of 6% dextran (500 000 MW, Sigma) was added to 9 ml of diluted whole blood. The red cells were allowed to sediment and the leukocyte-rich supernatant was collected. Blood granulocytes were separated from the mononuclear cell fraction by Nycomed (Accurate Chemicals, Westbury, NY) and Ficoll-Histopaque (Sigma) density gradient centrifugation using two gradients: 1.077 and 1.119. Granulocytes were collected at the interface between these two gradients. Using this method, purity of neutrophils was above 95%, as assessed by morphology (Giemsa-Wrights stain) and peroxidase stain.

*Peroxidase Stain*

Peroxidase positive cells were identified using a modified staining technique described previously.[14] Twenty $\mu$l of cell suspension ($1 \times 10^6$ cells/ml) was added to 180 $\mu$l of Gey's balanced salt solution containing 1 mg/ml of diaminobenzidine (Sigma) and 1% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ). The cells were incubated for 10 min at 37°C. An aliquot (5 $\mu$l) was taken and examined under the microscope. Brown-stained cells were considered peroxidase positive cells.

*Measurement of Superoxide Anions by Isolated Hepatic Cells*

Approximately 0.75 to $1.0 \times 10^6$ cells/well in Hank's balanced salt solution supplemented with 1 mM $CaCl_2$, 2 mM sodium azide (to inhibit cytochrome oxidase), 0.079% bovine serum albumim and 5 mM glucose were plated on six-well culture plates (Costar). Ferricytochrome c (Sigma) was added at a final concentration of 50 $\mu$M. Phorbol myristate acetate (Sigma) or opsonized zymosan (Sigma) was added at final concentrations of 1 $\mu$M and 1.98 mg/well, respectively. Negative control wells contained additionally 500 units/well of bovine liver superoxide dismutase (Sigma S4761). The suspension was incubated at 37°C for 60 min in a humidified environment containing 5% $CO_2$. The amount of reduced ferricytochrome c was measured using a molecular extinction coefficient of 21.1 $mM^{-1} cm^{-1}$ from a change in absorbance at 550 nm.[15] Superoxide formation was calculated from the SOD-inhibitable reduction of ferricytochrome c and expressed as nmol/h/$10^6$ cells.

## RESULTS

Kupffer cells isolated from untreated animals spontaneously generated low amounts of superoxide during incubation *in vitro* while neither endothelial cells (Table I) nor hepatocytes (data not shown) released superoxide under these conditions. Since no demonstrable accumulation of neutrophils was observed in these livers, "control" neutrophils were isolated from the peripheral blood. No spontaneous release of superoxide by these neutrophils was detected (Table I). When the cells were isolated from livers, which were subjected to 45 min of no-flow ischemia and 60 min of reperfusion, only the large Kupffer cells ($KC_3$) showed a more than 300% increase of the spontaneous superoxide formation over basal values; interestingly, the $KC_3$ fraction from the nonischemic lobes also produced more superoxide compared to controls (Table I). Superoxide production by all other cell types isolated from the

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TABLE I
Spontaneous generation of superoxide by nonparenchymal liver cells and neutrophils[1]

| | Control[2] | Lobes[2] | |
|---|---|---|---|
| | | Nonischemic | Postischemic |
| EC | 0.03 ± 0.03 | 0 | 0.02 ± 0.02 |
| $KC_2$ | 1.01 ± 0.29 | 1.64 ± 0.52 | 1.13 ± 0.45 |
| $KC_3$ | 1.00 ± 0.24 | 3.68 ± 0.95* | 4.16 ± 1.03* |
| Neutrophils[3] | 0 | N.T.[4] | 0 |

[1] Data are given as mean ± SE and are expressed as nmol $O_2^-$/h/$10^6$ cells.
[2] Cells were isolated from livers of untreated animals (control) ($n$ = 7) and from animals after 45 min of hepatic ischemia and 60 min of reperfusion ($n$ = 5).
[3] Neutrophils were isolated only from the postischemic lobes or from the peripheral blood in control animals.
[4] Not tested due to insufficient cell yield.
*$p < 0.05$ (compared to controls).

postischemic and nonischemic liver lobes did not significantly differ from those of the same cell type isolated from control livers (Table I). Stimulation of the $KC_3$ fraction with phorbol ester (PMA) or opsonized zymosan enhanced the release of superoxide by about 1 nmol/h/$10^6$ cells over basal values in control cells (Figure 1). A similar or slightly higher increase was observed for PMA and zymosan stimulation in $KC_3$ cells isolated from the postischemic lobes (Figure 1), i.e., the cells showed an increased spontaneous superoxide formation but no evidence for a relevant priming effect. In contrast, the small Kupffer cell fraction from the ischemic liver lobes ($KC_2$) did not produce spontaneously more superoxide than controls, however, generated considerably more superoxide upon stimulation with PMA and particularly with zymosan

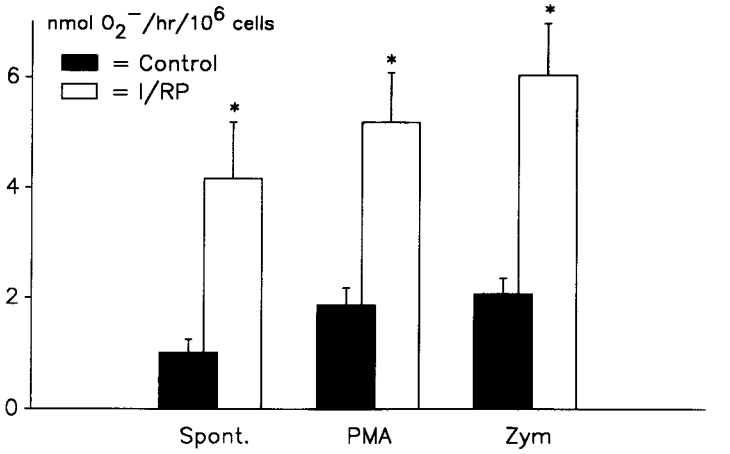


FIGURE 1 Spontaneous, phorbol ester (PMA)- and opsonized zymosan (Zym)-stimulated generation of superoxide by the large Kupffer cell fraction ($KC_3$) isolated from livers of control animals and the postischemic liver lobes of animals subjected to 45 min of hepatic ischemia and 60 min reperfusion (I/RP). Data are given as mean ± SE of n = 7 animals in the control group and $n$ = 5 animals in the I/RP group. *$p < 0.05$ (Control versus I/RP).

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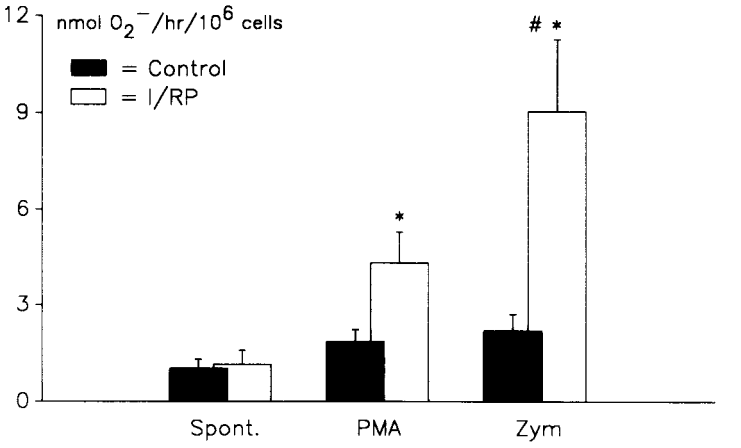


FIGURE 2 Spontaneous and phorbol ester (PMA)- and opsonized zymosan (Zym)-stimulated generation of superoxide by the small Kupffer cell fraction ($KC_2$) isolated from livers of control animals and the postischemic liver lobes of animals subjected to 45 min of hepatic ischemia and 60 min reperfusion (I/RP). Data are given as mean ± SE of $n = 7$ animals in the control group and $n = 5$ animals in the I/RP group. *$p < 0.05$ (Control versus I/RP). #$p < 0.05$ (PMA versus Zymosan).

(Figure 2). No significant difference in the superoxide formation was found between Kupffer cell fractions isolated from the postischemic and nonischemic lobes (data not shown). A similar priming effect as seen in the $KC_2$ fraction was also observed in neutrophils isolated form the postischemic lobes (Figure 3). The priming for stimulation with zymosan was also more than two-fold greater than for PMA. Superoxide

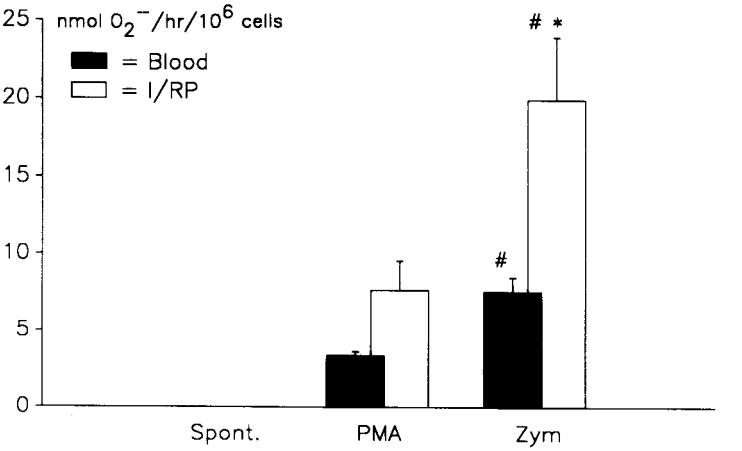


FIGURE 3 Spontaneous, phorbol ester (PMA)- and opsonized zymosan (Zym)-stimulated generation of superoxide by neutrophils isolated from the peripheral blood in controls or the postischemic liver from animals subjected to 45 min of hepatic ischemia and 60 min reperfusion (I/RP). Data are given as mean ± SE of $n = 7$ animals in the control group and $n = 5$ animals in the I/RP group. *$p < 0.05$ (Blood versus I/RP). #$p <$ (PMA versus Zymosan).

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generation by endothelial cells increased from $0.03 \pm 0.03$ nmol/h/$10^6$ cells to $0.25 \pm 0.08$ with PMA and $0.13 \pm 0.07$ with zymosan stimulation in control animals. Hepatocytes did not release any detectable superoxide even with additional stimulation. No difference in superoxide production was measured between endothelial cells or hepatocytes isolated from untreated livers and postischemic and nonischemic lobes (data not shown).

## DISCUSSION

The objective of this paper was to identify the cellular source of the vascular oxidant stress during reperfusion after hepatic ischemia. Indirect evidence, based on the observation that the stimulated GSSG formation in the vasculature could be further enhanced or attenuated by the preischemic activation or inactivation of Kupffer cells *in vivo*, suggested Kupffer cells as the principal source of the postischemic oxidant stress.[7] Our data support this conclusion by demonstrating a 300% increase of the spontaneous superoxide production by Kupffer cells isolated from the postischemic lobes. However, there is a clear difference between the two major Kupffer cell fractions isolated from these lobes. Only the large fraction ($KC_3$), which has an average diameter of 15 $\mu$m[11] and presumably represents Kupffer cells from the periportal region,[16] produced more superoxide spontaneously. The smaller Kupffer cell fraction ($KC_2$), which is supposed to represent cells from the midzonal and periportal area, and the infiltrating neutrophils isolated from the postischemic liver did not generate higher amounts of superoxide without additional stimulation. When exposed to PMA or opsonized zymosan, however, these cells generate 2–4-fold more superoxide than similarly stimulated cells from untreated animals. Our results provide direct evidence that only a particular fraction of Kupffer cells is responsible for the early postischemic oxidant stress. We have also demonstrated that another fraction of these cells together with the accumulating neutrophils are significantly primed for enhanced superoxide production. The infiltration of primed neutrophils provides the basis for the later, severe neutrophil-dependent injury phase in this model.[8]

There are striking similarities between the findings in ischemia-reperfusion injury and various models of endotoxemia. Intravenous injection of a bolus dose of endotoxin causes an immediate increase of plasma GSSG levels, a response that can be potentiated by Kupffer cell activation.[17] When Kupffer cells and neutrophils are isolated from the liver 3 h after endotoxin administration, the $KC_3$ fraction is the main source of superoxide in these animals.[10,11] Similar to hepatic ischemia-reperfusion, the smaller Kupffer cells and the accumulating neutrophils are highly primed after endotoxin.[10,11] These results suggest that the liver injury by inflammatory cells such as neutrophils is a two-step process: it is initiated by a chemotactic activation of neutrophils leading to hepatic neutrophil accumulation and priming of these cells followed by a further activation to release cytotoxic mediators, e.g., reactive oxygen and proteases. In support of this hypothesis we observed massive infiltration of neutrophils into the liver several hours before the onset of the neutrophil-induced oxidant stress and liver injury in the galactosamine/endotoxin shock model of the mouse.[18] An interesting difference between the endotoxin and ischemia-reperfusion models is that endotoxin seems to prime small Kupffer cells ($KC_2$) and neutrophils particularly for stimulation with PMA and considerably less for opsonized zymosan.[10,11] In contrast, ischemia-reperfusion primed the same cell types to release much more superoxide when stimulated with opsonized

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zymosan than by PMA (Figures 2, 3). Phagocytic cells generate superoxide through the membrane-bound enzyme NADPH oxidase, which can be activated through different signal transduction pathways.[19] Superoxide production by PMA is a protein kinase C (PKC) - dependent pathway; PMA directly binds to and activates PKC.[20] On the other hand, opsonized zymosan induces a receptor-mediated activation of a signal transduction pathway that is insensitive to PKC inhibitors.[21,22] It was recently shown in rat bone marrow-derived macrophages, opsonized zymosan but not PMA is able to induce a respiratory burst, providing direct evidence for a PKC-independent signal transduction pathway in these cells.[23] The significantly higher response after zymosan stimulation compared to PMA suggest that hepatic ischemia and subsequent reperfusion primes rat Kupffer cells and accumulating neutrophils for enhanced reactive oxygen production particularly through a receptor-mediated, PKC-independent pathway but also, to a lesser degree, through a PKC-dependent pathway.

Another interesting finding of this study is the fact that Kupffer cells from the postischemic as well as the nonischemic lobes were similarly activated and/or primed. Preliminary data from our laboratory demonstrated that depletion of serum complement attenuated the postischemic increase of plasma GSSG levels and the infiltration of neutrophils into the liver.[24] Since the complement factors $C_{3a}$ and $C_{5a}$ are known to induce superoxide formation by Kupffer cells,[25,26] a general activation of the complement cascade in serum during reperfusion could explain the simultaneous activation and priming of Kupffer cells in the postischemic and nonischemic lobes. Although it was recently shown that cultured Kupffer cells can generate superoxide when subject to hypoxia and reperfusion,[27] the present findings can not be explained only by this observation.

In summary, the data shown in this paper provide direct evidence for the large Kupffer cells as the principal source of the vascular oxidant stress after liver ischemia and also demonstrates a significant priming of the smaller Kupffer cells and neutrophils, particularly for a receptor-mediated superoxide formation. No evidence was found for a contribution of hepatic endothelial cells or hepatocytes to the postischemic oxidant stress.

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