In situ determination by surface chemiluminescence of temporal relationships between evolving warm ischemia-reperfusion injury in rat liver and phagocyte activation and recruitment.

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(Article begins on next page)
Liver ischemia-reperfusion is characterized by an increased oxygen-dependent free radical chain-reaction rate and an increased steady-state concentration of reactive oxygen species. The aim of this study was to evaluate the in situ generation of reactive oxygen species and its relationship with phagocyte activation and recruitment in reperfused rat liver. Rat livers were subjected to 2 hours of selective lobular ischemia and reperfusion for up to 12 hours. The following parameters were determined: in situ liver chemiluminescence, understood to reflect the tissue steady-state concentration of singlet oxygen \( \left( \text{O}_2^* \right) \); myeloperoxidase tissue activity; the number of neutrophils; and the degree of necrosis. An early chemiluminescence burst was measured after 30 minutes of blood reflow (early phase of oxidative stress), followed by a relapse and a further increase after 4 to 12 hours of reperfusion (late phase of oxidative stress). Both early and late phases were modified by pretreatment with gadolinium chloride (GdCl\(_3\)), pointing to a key role of the Kupffer cells. Neutrophils infiltrated the liver, myeloperoxidase activity, in situ chemiluminescence, and necrosis were found to be strongly correlated over the 4- to 12-hour reperfusion period \( (r = .960; \text{average of the 4 correlation coefficients}) \). Together with resident phagocytes, neutrophil recruitment and activation appear to provide a major contribution to the increase of oxygen-dependent free-radical reactions and amplification of liver reperfusion damage. Surface chemiluminescence appears to properly describe the in situ and in vivo progressive organization of the acute inflammatory response with phagocyte-mediated liver injury. (Hepatology 2000;31:622-632.)

Oxygen free radicals have long been indicated as being responsible for ischemia-reperfusion-induced liver damage.\(^1\)-\(^3\) The increased production of oxygen radicals and the postischemic injury of the liver were subsequently recognized to have a 2-phase time course: an early phase, from reflow up to about 30 to 60 minutes of reperfusion, and a late phase, from approximately 3 to 24 hours of blood reflow.\(^4\)-\(^8\) The first phase is understood as an overproduction of oxygen radicals in activated Kupffer cells\(^4\),\(^7\) and in the mitochondria of hepatocytes and of endothelial cells, where oxygen reflow encounters highly reduced respiratory chains.\(^6\),\(^8\) The second phase appears to follow the infiltration and activation of neutrophils in the liver parenchyma.\(^4\),\(^5\),\(^9\)-\(^16\) The first phase, which includes increased nitric oxide (NO) production by liver mitochondria\(^17\) and Kupffer cells,\(^18\),\(^19\) initiates the signal transduction processes that promote neutrophil accumulation in the liver.\(^9\),\(^10\),\(^20\)

Although many studies have reported neutrophil infiltration causing liver injury after ischemia-reperfusion, the contribution of activated neutrophils together with resident phagocytes to the in situ generation of oxygen free radicals has not yet been clarified in the reperfused liver. In this study, the levels of singlet oxygen \( \left( \text{O}_2^* \right) \) in the tissue were monitored by chemiluminescence in the in situ–reperfused liver as a marker of oxidative stress, and myeloperoxidase activity was taken as a marker of neutrophil activation. Surface organ chemiluminescence, by measuring the photons emitted by the tissue, determines: 1) directly, the steady-state concentration of \( \text{O}_2^* \) and the electronically excited state of molecular oxygen; and 2) indirectly, the rate of free radical chain reactions occurring in the tissue.\(^21\)-\(^23\) Surface chemiluminescence has been used to assess the increased steady-state concentration of \( \text{O}_2^* \), after rat liver ischemia-reperfusion,\(^6\),\(^8\) and mouse liver necrosis after acetaminophen\(^24\) and fenofibrate\(^25\) administration.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 180 to 220 g were used. Animals were fed a chow diet devoid of vitamins A, C, and other antioxidants, with the exception of vitamin E (50 mg/kg) (Cargill, Buenos Aires, Argentina), and with water ad libitum, and maintained under 12-hour light/dark cycles. To block Kupffer cell activity, one group of rats was pretreated with gadolinium chloride (GdCl\(_3\)) at 10 mg/kg body weight diluted in sterile 0.9% NaCl solution and administered through the tail vein 24 hours before ischemia-reperfusion. Another group of rats (controls) received an equal.
volume of saline solution. Animals received humane care in compliance with institutional guidelines.

Experimental Model. A model of lobar hepatic ischemia-reperfusion was used. The rats were injected with heparin (400 IU/kg body weight, intraperitoneally) and then anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally) diluted in sterile 0.9% NaCl solution. The liver was exposed by a midline laparotomy and the liver lobes moved gently to expose the hilum. Atraumatic Glober bulldog clamps (Roboz Surgical Instrument Co., Washington, DC) were used to interrupt the portal venous and the hepatic arterial blood supply to the right lobes, thus rendering ischemia about one third of the total hepatic mass. The medial, left lateral, and caudate lobes (nonischemic lobes), retaining an intact portal and arterial inflow and venous outflow, were used as control tissue. Hepatic ischemia was maintained for 2 hours with the abdominal cavity gently closed and the animals placed under warming lamps to keep body temperature constant. After declamping, the abdomen was closed with 3-0 silk, and the animals were housed and provided with food and water ad libitum until a second laparotomy was performed 4, 8, or 12 hours after reperfusion.

Spontaneous Liver Surface Chemiluminescence. The spontaneous surface chemiluminescence of the right lateral (ischemic) and of the nonischemic lobes of the in situ liver was monitored using a photon counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA) (Fig. 1), with a model 9658 photomultiplier responsive over the range of 350 to 850 nm (Thorn EMI, Middlesex, England) as described elsewhere.21-23 Chemiluminescence in the intact organ is the result of different photoemissive reactions: 1) \( ^{1}\text{O}_2 \) dimol emission; and 2) formation of 1,2-dioxetane derivatives, either from the lipid peroxidation free radical process or from the reaction of \(^{1}\text{O}_2 \) with double bonds, facilitating the generation of excited carbonyl groups after rupture of the carbon-carbon and oxygen-oxygen bonds.21-23 Emission was expressed in counts per second per square centimeter of liver surface (cps/cm²). Spectral analysis of liver chemiluminescence was performed with cut-off Kodak Wratten filters (Eastman Kodak, Rochester, NY) as described elsewhere.26

Sample Handling. At the end of each experimental period, the animals were killed by exsanguination under pentobarbital anesthesia. Samples from the ischemic right lateral lobes and the nonischemic lobes (controls) were taken in terminal procedures and bisected for biochemical and morphological assays.

Myeloperoxidase Activity. Myeloperoxidase activity was measured by a modification of the technique described by Krawisz et al.27 Briefly, liver samples (0.5-1.0 g wet weight) were freeze-thawed and homogenized (1:9, wt/vol) in 50 mmol/L potassium phosphate buffer (pH 6.0), containing 0.5% hexadecyltrimethylammonium bromide. The homogenates were sonicated during 90 seconds, centrifuged (6,000 rpm/30 min at 4°C) and the supernatant assayed for myeloperoxidase activity. An aliquot (0.25-0.75 mL) was added to 2.9 mL of 50 mmol/L potassium phosphate buffer (pH 6.0), containing 40 mmol/L of o-dianisidine-dihydrochloride and 40 mmol/L H₂O₂. The changes in absorbance at 460 nm were spectrophotometrically measured at room temperature. One unit of myeloperoxidase activity was defined as the amount of enzyme that degrades 1 µmol peroxide per minute at 25°C. The results were expressed as units per gram of wet tissue. Liver tissue contains substantial amounts of glutathione peroxidase, catalase, as well as hemoglobin, which compete with myeloperoxidase for H₂O₂. The use of hexadecyltrimethylammonium bromide in the homogenization buffer allows for the solubilization of the myeloperoxidase bound to the granular membranes, and at the same time for the inhibition of the pseudoperoxidase activity of the above proteins by destroying their heme nucleus.28

Morphological Determinations. Fragments of liver (1.0 × 0.5 × 0.3 cm) were fixed overnight in 4% formaldehyde in 50 mmol/L potassium phosphate buffer (pH 7.4) at room temperature, dehydrated in graded alcohol solutions, and embedded in paraffin.

![Diagram of the photon-counting apparatus adapted for measuring in situ organ chemiluminescence. The lucite rod used as an optical coupler is placed in front of the liver exposed in situ. PMT, photomultiplier tube.](image-url)
Five-micrometer sections were stained with hematoxylin-eosin to determine the number of neutrophils and the degree of necrosis. Neutrophils were counted in 50 nonconsecutive 400× histological power fields in liver sections, to a depth of 0.5 cm below the surface, so that this number would be comparable with liver surface chemiluminescence. Neutrophils were identified by their size (10-12 µm) and their segmented nuclei. Only neutrophils that were present within sinusoids or had extravasated into the tissue were counted. Neutrophils in necrotic areas were not counted; the number was expressed as the average number of neutrophils per histological power field.

Samples of nonischemic and ischemic liver were taken 4 hours after reperfusion from rats treated with sterile 0.9% NaCl solution, then they were fixed with 2.5% glutaraldehyde solution in 0.1 mol/L sodium phosphate buffer (pH 7.4) for 3 hours at 4°C, postfixed in a 2% osmium tetroxide solution, dehydrated in graded ethanol solutions, and embedded in Epon-Araldite. Ultrathin sections stained with uranyl acetate and lead citrate were examined through a 410 Philips transmission electron microscope (Philips Electronic Instruments, Inc., Eindhoven, Holland) with an 80-kV acceleration voltage. The 4-hour time of reperfusion was chosen to avoid the interference of cell debris in the examination of the possible spatial relationship between hepatocytes and neutrophils.

Necrosis was graded using an arbitrary scale through the evaluation of 15 nonconsecutive acini as follows: 1 point, scattered necrotic hepatocytes distributed in the acinus; 2 points, presence of a focus of necrotic hepatocytes; 3 points, presence of 2 or more foci of necrotic hepatocytes; 4 points, total necrosis of the acinus (panacinar necrosis). The degree of necrosis of each liver sample was obtained by summing the points assigned to the individual acini. A maximum of 60 points was possible.

**Neutrophil Isolation and Spectral Analysis.** Human neutrophils were isolated by conventional procedures and suspended in 145 mmol/L NaCl, 1 mmol/L CaCl2, and 25 mmol/L phosphate buffer (pH 7.4). Phorbol 12-myristate 13-acetate (PMA) (0.15 µg/mL) was added to 3 × 10⁶ cells/mL cell aliquots, and spectral analysis of activated neutrophil chemiluminescence was performed with cut-off Kodak Wratten filters.26

**Statistical Analysis.** Tables and figures report means of four experiments ± SEM. Data were analyzed statistically by factorial ANOVA, followed by the Bonferroni test for other comparisons. Differences between 145-mmol/L NaCl- and GdCl3-treated rats were analyzed by the unpaired two-tailed Student t-test. Regression output analysis between determinations was performed using the Spearman correlation coefficient.

**Chemicals.** Hydrogen peroxide, GdCl3, α-dianisidinedihydrochloride, and hexadecyl-trimethyl ammonium ammonium were from Sigma Chemicals Co. (St. Louis, MO). Other reagents were of analytical grade.

**RESULTS**

**Liver Surface Chemiluminescence as Measurement of Oxidative Stress During Ischemia-Reperfusion.** The spontaneous surface chemiluminescence of the liver in situ, before ischemia-reperfusion, was 12 ± 2 cps/cm², which is consistent with a steady-state concentration of 1O2 of about 10⁻¹⁶ mol/L.30 In the first oxidative phase after reoxygenation, liver chemiluminescence increased markedly to 2.6 times the initial spontaneous chemiluminescence as a transient burst, with maximal emission at 30 minutes and a decline 60 minutes after reperfusion (Fig. 2). Increased chemiluminescence directly indicates an increased concentration of 1O2 and other excited

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**Fig. 3.** Rat liver chemiluminescence during ischemia-reperfusion and effect of GdCl3 pretreatment. Experimental details are given in Materials and Methods. Spontaneous chemiluminescence before the cycle of ischemia-reperfusion (0/0); after 2 hours of ischemia (2/0); after 2 hours ischemia and 0.5 hours reperfusion (2/0.5); after 2 hours ischemia and 1 hour reperfusion (2/1); after 2 hours of ischemia and 4 hours of reperfusion (2/4); after 2 hours of ischemia and 8 hours of reperfusion (2/8); and after 2 hours of ischemia and 12 hours of reperfusion (2/12). a, significantly different from 2/0.5 GdCl3 ischemic lobes (P < .01); b, significantly different from 2/1 GdCl3 ischemic lobes (P < .05); c, significantly different from 2/12 GdCl3 ischemic lobes (P < .01). (□), Rats and saline; (■), rats and GdCl3.

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**Fig. 4.** Spectral distribution of chemiluminescence of rat liver and stimulated neutrophils. (A) Spontaneous rat liver chemiluminescence. (B) Chemiluminescence of neutrophils (3 × 10⁶ cells/mL) suspended in 145 mmol/L NaCl, 1 mmol/L CaCl2, 25 mmol/L phosphate buffer (pH 7.4) stimulated with 0.15 µg/mL PMA. (C) Chemiluminescence of rat liver subjected to 2 hours’ ischemia and 12 hours’ reperfusion.
species, and indirectly indicates both a higher concentration of oxidative free radicals and an increased rate of free radical reactions in the liver. The increased steady-state concentrations of $^1O_2$, other excited species, and oxidizing free radicals constitute the chemicals basis of biological oxidative stress. Liver chemiluminescence increased again in the second oxidative phase after blood reflow, which began after 4 hours of reperfusion (Fig. 2) and continued for up to 12 hours of reperfusion (Fig. 2). Treatment with GdCl$_3$ abolished the first oxidative phase and partially decreased photoemission during the second phase, after 12 hours of reperfusion (Fig. 3).

Spectral analysis of spontaneous liver chemiluminescence (Fig. 4A) shows a main broad emission band in the red region, between 640 nm and the unresolved 715- to 850-nm band, which is consistent with $^1O_2$ dimol emission at 634 and 711 nm. In the interval of 440 to 640 nm, the emission of control liver, measured in arbitrary units, is very low (Fig. 4A). Spectral analysis of the chemiluminescence of PMA-activated neutrophils (Fig. 4B), in addition to a much more intense red emission band (640-715 nm), shows a second broad band with maximum emission at 520 to 535 nm and emission in the blue (440-520 nm) and yellow-green (535-640 nm) wavelengths. The blue emission is consistent with the production of excited carbonyl compounds, and the 520- to 535-nm band is consistent with the emission from a dioxetane intermediate compound type. Spectral analysis of

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**FIG. 5.** Number of neutrophils in the liver during the late reperfusion phase (pmn/hpf): number of neutrophils per histological power field. Before ischemia-reperfusion cycle (0/0); after 2 hours of ischemia (2/0); after 2 hours of ischemia and 4 hours of reperfusion (2/4); after 2 hours of ischemia and 8 hours of reperfusion (2/8); and after 2 hours of ischemia and 12 hours of reperfusion (2/12). a, significantly different from 0/0, 2/0, and 2/4 ischemic lobes ($P < .05$). (□), Nonischemic lobes; (■), ischemic lobe.

**FIG. 6.** Nonischemic lobes (used as internal control) after a complete cycle of ischemia-reperfusion. PS: portal space, where branches of portal vein, hepatic artery, and intrahepatic biliary tree are visible; THV, terminal hepatic venule. The hepatic cords are correctly distributed in fashion in both affluent and effluent vascular structures.
**FIG. 7.** Neutrophil accumulation in the ischemic lobes after 12 hours of reperfusion. The arrows indicate dilated sinusoids plugged by neutrophils. Hepatocytes of relatively normal appearance are visible (large asterisk), as well as other adjacent altered cells (small asterisks).

**FIG. 8.** (A) Rat liver activity of the myeloperoxidase enzyme during ischemia-reperfusion. Experimental details are given in Materials and Methods. Activity of myeloperoxidase before the ischemia-reperfusion cycle (0/0); after 2 hours of ischemia (2/0); after 2 hours of ischemia and 4 hours of reperfusion (2/4); after 2 hours of ischemia and 8 hours of reperfusion (2/8); and after 2 hours of ischemia and 12 hours of reperfusion. a, significantly different from 0/0 ischemic lobes ($P < .001$); b, significantly different from 2/0 ischemic lobes ($P < .01$); c, significantly different from 2/0 ischemic lobes ($P < .001$); d, significantly different from 2/4 ischemic lobes ($P < .001$); e, significantly different from 2/8 ischemic lobes ($P < .01$). ( ), Nonischemic lobes; ( ), ischemic lobe. (B) Rat liver activity of the myeloperoxidase enzyme during the late reperfusion phase and effect of GdCl$_3$ pretreatment. Myeloperoxidase activity after 2 hours of ischemia and 4 hours of reperfusion (2/4); after 2 hours of ischemia and 8 hours of reperfusion (2/8); and after 2 hours of ischemia and 12 hours of reperfusion (2/12). a, significantly different from 2/12 GdCl$_3$ ischemic lobes. ( ), Rats and saline; ( ), rats and GdCl$_3$. 

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**A**

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**B**

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the chemiluminescence of rat liver after 2-hour ischemia and 12-hour reperfusion (Fig. 4C) showed a significantly increased red emission at 640 to 715 nm with respect to control liver and a band at 520 to 535 nm not present in the control tissue; the latter band indicated the participation of activated neutrophils in organ chemiluminescence.

**The Inflammatory Response as an Amplification Mechanism of Oxidative Damage to Parenchymal Cells.** The number of neutrophils in the liver increased progressively in the postischemic lobes during the 4- to 12-hour period after blood reflow, showing approximately a 6-fold increase 12 hours after declamping (Fig. 5), while there was no change in the neutrophil number (3-6 per histological power field) in the nonischemic lobes. The latter lobes showed normal histology at the light-microscopic observation performed at the end of the ischemia-reperfusion cycle (Fig. 6). On the other hand, in those lobes subjected to ischemia and reperfusion, some of the numerous neutrophils present appeared to reach close contact with the surface of hepatocytes (Fig. 7).

Liver myeloperoxidase activity in the group of rats treated with 145 mmol/L NaCl solution, compared with that in nonischemic tissue (liver before ischemia and controls: 5.2 ± 0.5 U/g wet tissue), was significantly higher at all experimental time points of the late reperfusion phase, reaching 6.5 times above control values after 12 hours of reflow (Fig. 8A). On the contrary, in the liver of rats treated with GdCl₃, myeloperoxidase activity increased after 4 hours of reperfusion but without any further rise during the late reperfusion phase (Fig. 8B).

**Hepatocellular Damage During Reperfusion as a Consequence of Neutrophil Infiltration.** The liver acinar structure was mostly preserved after 2 hours of ischemia. However, there were some foci of hepatic cord disarrangement as a result of the presence of hepatocytes that were swollen to varying extents and of collapsed sinusoids, mainly in the periportal and midzonal regions of the acinus. Scattered necrotic hepatocytes were also observed (Fig. 9). During the late phase of reperfusion, yellowish-green zones appeared over the congested hepatic surface, corresponding histologically to severe hepatocellular necrosis, and mainly disposed around the portal triads. This necrotic zonal distribution was parallel to neutrophil infiltration (Fig. 10 and Table 1).

Assessed by electron microscopy, sinusoidal spaces appeared dilated, denuded, and containing several neutrophils. In some fields, neutrophils appeared in close contact with injured hepatocytes, without interposition of sinusoidal lining (Figs. 11 and 12). On the contrary, the nonischemic lobes showed a normal cytoarchitecture with regular appearance and mutual relationship of intracellular organelles (Fig. 13).

**Correlation of the Morphometric and Biochemical Parameters of Inflammatory Liver Injury After Reperfusion.** Regression analysis was performed for: 1) the number of neutrophils counted in the tissue; 2) homogenate myeloperoxidase activity; 3) in situ liver chemiluminescence; and 4) the necrotic score of the liver.
liver acini. The linear correlations between each pair of variables, with high statistical significance, support a basic role for neutrophils in postischemic liver injury (Table 2).

**DISCUSSION**

Liver injury induced by ischemia and reperfusion is a common event, which frequently follows circulatory shock with resuscitation, as well as many types of liver surgery, including transplantation and tumor resection.

Contributing to oxidative stress of the early phase of reperfusion, as assessed by organ chemiluminescence, is the production of reactive oxygen species as well as of NO by Kupffer cells, which can diffuse to hepatocytes and endothelial cells and cause mitochondrial dysfunction. NO markedly inhibits cytochrome oxidase activity and mitochondrial respiration, and increases the mitochondrial production of superoxide radicals and $H_2O_2$. Inhibition of the Kupffer cells with gadolinium chloride leads to disappearance of the oxidative stress in the early reperfusion phase. Accordingly, it seems that gadolinium blocks not only phagocytosis, but also NO production, the latter being inferred from the lack of a chemiluminescence burst after reflow.

The accumulation of neutrophils during the late phase of liver reperfusion is mediated by the local generation of chemotactic agents, which regulate neutrophil migration from the vascular compartment to the parenchymal cells. In this sense, there is a large body of evidence from different models of hepatic inflammation to indicate that oxygen free radicals play a central role in promoting neutrophil infiltration in the liver. For example, it has been claimed that the release by the postischemic liver of platelet activating factor and leukotriene B4, two potent agents that promote neutrophil chemotaxis, depends on the generation of oxygen radicals by the cells. Moreover, it has recently been suggested that hepatocytes, in response to an agonist stimu-
lus most probably released by activated Kupffer cells, such as tumor necrosis factor α and interleukin-1, initiate and amplify an acute inflammatory response through the regulated expression and secretion of specific proinflammatory cytokines, e.g., interleukin-8 and neutrophil activating protein-78.40

The activation of intact neutrophils is accompanied by the production of reactive species such as superoxide radical29,41 and NO,29,42 as well as by light emission.42 Because the thickness of liver tissue that decreases transmittance of light in the 650- to 700-nm wavelength band to one half is about 0.5 cm,21 activation of neutrophils located at less than 0.5 cm below the liver surface will be detected by organ surface photoemission.42 Organ surface chemiluminescence thus becomes an assay for organ inflammation in situ. The positive correlation between the number of infiltrated neutrophils counted in the liver parenchyma with 1) in situ liver chemiluminescence, and 2) liver necrosis indicates that the neutrophils are activated in reperfused hepatic tissue and are, at least in part, responsible for irreversible tissue damage. The observed alterations of sinusoidal spaces with even contact between the neutrophils and damaged hepatocytes (Figs. 11 and 12) confirm this hypothesis.

However, Kupffer cells still appear to contribute to the inflammatory response in the later phase of reperfusion; as shown in Figs. 3 and 8B, both surface chemiluminescence and tissue activity of myeloperoxidase at 12 hours of reperfusion were down-regulated by GdCl3 treatment. Kupffer cells may influence the later outcome of the inflammatory response through the release of by-products that attract and activate neutrophils.

There are several ways by which the activated neutrophils might induce tissue injury; these include activation of NADPH oxidase41,43 and NO synthase,29,42 and release of products of arachidonic acid metabolism (eicosanoids) and proteolytic enzymes (elastase, metalloproteinases, etc.).44 When neutrophils infiltrated into the liver parenchyma are activated, membrane-bound nicotinamide adenine dinucleotide phos-
phate, reduced form (NADPH)-oxidase and NO synthase initiate rapid oxygen uptake, known as the respiratory burst, in which superoxide anion radicals (O$_2^-$) and NO are produced in near-equimolar quantities and released into the extracellular space. The released primary products, O$_2^-$ and NO, react to produce H$_2$O$_2$ and peroxynitrite (ONOO$^-$). In the presence of superoxide dismutase in the extracellular space, more H$_2$O$_2$ is formed; in its absence, more ONOO$^-$ is formed. Both H$_2$O$_2$ and ONOO$^-$ are highly cytotoxic, the former being a direct powerful oxidant, and the latter acting indirectly, yielding the highly reactive hydroxyl radical (HO•) after homolysis by reaction with Fe$^{2+}$.

It is well known that the oxidants released by neutrophils during the respiratory burst effectively produce cell and tissue damage during the inflammatory response. Because the process is mediated by reactive species, cytotoxicity will be stronger when the chemoattracted neutrophils become activated and approach endothelial and even parenchymal cells.

Activated neutrophils produce O$_2^*$ that is detected chemically or by chemiluminescence. O$_2^*$ is an excited state of molecular oxygen with a half-life of about 2 to 3 microseconds that yields one photon from the molecular collision of two O$_2^*$ molecules (2 O$_2^*$ → 2 O$_2$ + hv). The photon given off by the dimol emission of O$_2^*$ affords the basis of the chemiluminescence assay used in this study to monitor oxidative stress in the liver in situ. Three chemical reactions, probably occurring simultaneously during neutrophil activation, can be indicated as sources of O$_2^*$. First, membrane lipid oxidation, caused by neutrophil oxidative burst, generates hydroperoxyl radicals (ROO•), which after bimolecular collisions yield O$_2^*$. Second, hypochlorous acid is formed in the myeloperoxidase-catalyzed oxidation of chloride anion by hydrogen peroxide (H$_2$O$_2$ + Cl$^-$ → HOCl + HO$^-$), and O$_2^*$ is formed in the reaction of hypochlorous acid with hydrogen peroxide (H$_2$O$_2$ + HOCl → H$_2$O + Cl$^-$ + O$_2^*$). Finally, it has recently been recognized that ONOO$^-$ reacts with H$_2$O$_2$ to yield O$_2^*$. The results of this study provide substantial evidence that Kupffer cell activation and neutrophil recruitment and activation play a central role in building up reperfusion damage in
the liver. In addition, they point to surface chemiluminescence as a useful technique to examine the temporal relationship between evolution of warm ischemia-reperfusion injury and phagocyte activation and recruitment in situ and in vivo.

**Table 2. Linear Correlation Among the Parameters Measured During the Ischemia-Reperfusion Cycle**

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<th>Significance</th>
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**Regression Equations**

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Abbreviations: CL, chemiluminescence; PMN, number of neutrophils; MPO, myeloperoxidase tissue activity.


45. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 1990;87:1620-1624.


