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Oxidative Stress Occurs in Perfused Rat Liver at Low Oxygen Tension by Mechanisms Involving Peroxynitrite

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ABSTRACT

Ethanol increases free radical formation; however, it was recently demonstrated that it also causes extensive hypoxia in rat liver in vivo. To address this issue, it was hypothesized that peroxynitrite formed in normoxic periportal regions of the liver lobule has its reactivity enhanced in hypoxic pericentral regions where the pH is lower. Via this pathway, peroxynitrite could lead to free radical formation in the absence of oxygen. Livers from fed rats were perfused at low flow rates for 75 min. Under these conditions, periportal regions were well oxygenated but pericentral areas became hypoxic. Low-flow perfusion caused a significant 6-fold increase in nitrotyrosine accumulation in pericentral regions. During the last 20 min of perfusion, the spin-trap α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone was infused and adducts were collected for electron-spin resonance analysis. A six-line radical adduct signal was detected in perfusate. Direct infusion of peroxynitrite produced a radical adduct with identical coupling constants, and a similar pattern of nitrotyrosine accumulation was observed. Retrograde perfusion at low rates resulted in accumulation of nitrotyrosine in periportal regions. Although the magnitude of the radical in perfusate was increased by ethanol, it was not derived directly from it. Both nitrotyrosine accumulation and radical formation were reduced by inhibition of nitric oxide synthase with *N*-nitro-L-arginine methyl ester, but not with the inactive D-isomer. Radical formation was decreased nearly completely by superoxide dismutase and *N*-nitro-L-arginine methyl ester, consistent with the hypothesis that the final prooxidant is a derivative from both NO· and superoxide (i.e., peroxynitrite). These results support the hypothesis that oxidative stress occurs in hypoxic regions of the liver lobule by mechanisms involving peroxynitrite.

In rodent models of ethanol-induced liver injury, oxidative stress occurs by mechanisms involving increased formation of α-hydroxyethyl-free radical (Knecht et al., 1995). Furthermore, ethanol causes a hypermetabolic state in the liver (Videla et al., 1973), resulting in hypoxia in pericentral regions of the liver lobule in vivo (Tsukamoto and Xi, 1989; Oshita et al., 1992). It has been proposed that hypoxia caused by ethanol leads to oxidative stress via a classical hypoxia/reoxygenation pathway (Knecht et al., 1995). When hypoxia occurs, mitochondria can no longer produce ATP, leading to the accumulation of breakdown products such as xanthine and hypoxanthine. Upon reintroduction of oxygen, superoxide is produced, using purines as substrates for xanthine oxidase. In the presence of trace metals such as iron, reactive-free radicals (e.g., hydroxyl radicals) could be produced, leading to the formation of lipid radicals. However, during chronic ethanol exposure with the Tsukamoto-French enteral feeding model, hypoxia occurs very early and appears to be independent of blood alcohol concentrations (Arteel et al., 1997a). Therefore, it is feasible that alcohol causes chronic hypoxia in the liver. Because reoxygenation is difficult to envision during chronic hypoxia, hypoxia/reoxygenation mechanisms seem less likely to be responsible for the oxidative stress observed.

Results from previous in vivo and in vitro studies suggest the involvement of low cellular oxygen tension in oxidative stress in the liver. For example, chronic hypoxia in vivo caused by hypobaric breathing results in lipid peroxidation (Nakanishi et al., 1995). Furthermore, in rat liver perfused at 25% of normal flow rates (low-flow, reflow model), where periportal regions are well oxygenated but pericentral regions remain hypoxic (Bradford et al., 1986), reactive oxygen species have been detected by photoemission (Suematsu et

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[1] Unless otherwise stated, the term peroxynitrite is used to refer to both peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH); the International Union of Pure and Applied Chemistry names are oxoperoxynitrate (1−) and hydrogen oxoperoxonitrate, respectively.

ABBREVIATIONS: SOD, superoxide dismutase; L-NAME, *N*-nitro-L-arginine methyl ester; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; ESR, electron spin resonance; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; NO·, nitric oxide.

al., 1992). Furthermore, allopurinol, an inhibitor of superoxide generation by xanthine oxidase, protects against cellular damage in this model (Suematsu et al., 1992). Although it has been proposed that the source of free radicals in the hypoxic liver is the marginally oxygenated cells in midzonal regions (Bradford et al., 1986), the mechanism of free radical formation has not yet been elucidated.

Peroxynitrite[1] ($ONOO^-$) is formed by a diffusion-limited reaction of nitric oxide and superoxide and has a half-life of about 0.01 s, which is about 100 times shorter than that for nitric oxide (NO·) (Beckman and Koppenol, 1996). $ONOO^-$ is proposed to be involved in inflammation, atherosclerosis, renal failure, and allograft rejection (Murphy et al., 1998). Furthermore, a role for nitric oxide-dependent pathways in ischemia-reperfusion injury has been demonstrated (Matheis et al., 1992). Here, it is hypothesized that $ONOO^-$ leads to oxidative stress in hypoxic liver (Fig. 1). Specifically, it is proposed that the reactivity of $ONOO^-$ is enhanced by decreases in pH caused by hypoxia, leading to formation of hydroxyl-like free radical species in the absence of oxygen. Therefore, the purpose of this study was to test the hypothesis that hypoxia leads to oxidative stress by mechanisms involving $ONOO^-$ and to determine whether this effect is altered by ethanol. Preliminary results of this study have been presented elsewhere (Arteel et al., 1997b).

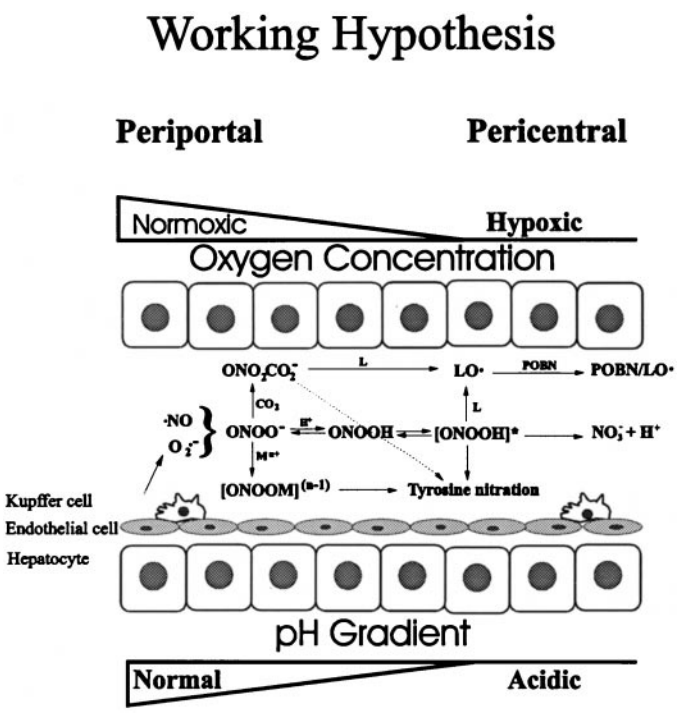

Fig. 1. Working hypothesis for the role of $ONOO^-$ in oxidative stress produced in regions of the liver lobule with low oxygen tension. NO· and superoxide (O_2^-) in liver react rapidly to form $ONOO^-$. The acidic pH in hypoxic regions of the liver would favor protonation of $ONOO^-$ (pKa = 6.8) in this region to peroxynitrous acid (ONOOH), leading to a reactive intermediate ([ONOOH]*) that can attack surrounding molecules such as lipids (L), forming lipid-derived free radicals (LO·). Free radicals, such as LO·, can be trapped with the spin-trap POBN and quantitated by ESR spectroscopy. Nitrated tyrosine residues can be detected immunohistochemically and quantitated with image analysis. $ONOO^-$ also reacts with carbon dioxide at physiologic concentrations to form nitrosoperoxycarbonate ($ONOOCO_2$). This intermediate can also form free radicals via one-electron oxidation pathways and enhances nitration of tyrosine residues on proteins.

Materials and Methods

Chemicals and Reagents. Sodium pentobarbital (Nembutal) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Superoxide dismutase (SOD), *N*-nitro-L-arginine methyl ester (L-NAME), and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) were obtained from Sigma Chemical Co. (St. Louis, MO). Racemic pimonidazole hydrochloride was synthesized according to published procedures (Smithen and Hardy, 1982) and monoclonal antibodies against reduced, protein-bound pimonidazole were prepared as described previously (Raleigh et al., 1994). Chemicals used in the preparation of formaldehyde-fixed, paraffin-embedded tissue sections were of reagent-grade purity from local suppliers. The avidin-biotin complex peroxidase Vectastain kit, avidin-biotin blocking kit, rat-adsorbed horse anti-mouse antibodies, and the diaminobenzidine peroxidase substrate were purchased from Vector Laboratories Inc. (Burlingame, CA). $ONOO^-$ synthesis was performed by conversion of a solid KO_2/quartz sand mixture with NO· gas diluted with argon as described by Koppenol et al. (1996). The solid was then dissolved in 0.01 M aqueous sodium hydroxide, and hydrogen peroxide was eliminated by treatment with MnO_2 powder and the mixture was filtered. $ONOO^-$ was concentrated by freeze fractionation, and the final concentration was determined spectrophotometrically ($\epsilon_{302\ nm} = 1700\ M^{-1}\ cm^{-1}$).

Isolated Rat Liver Perfusion. All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals. Female Sprague-Dawley rats were given standard laboratory chow and water ad libitum. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and their livers were isolated and perfused with an oxygen-saturated Krebs-Henseleit bicarbonate buffer (pH 7.4; 37°C; 4 ml/min/g) by a cannula inserted in the portal vein. The perfused liver is generally considered a good predictive tool of in vivo liver responses because it maintains ultrastructure and many physiologic functions of the intact organ (e.g., bile secretion; Brouwer and Thurman, 1996). Effluent perfusate was collected by a cannula placed in the vena cava and flowed past a Clark-type oxygen electrode. Rates of O_2 uptake were calculated from the influent-effluent concentration difference, flow rate, and liver wet weight. Perfusate pH was determined with an in-line electrode. Aliquots of perfusate were collected every 5 min during normal flow and every 15 min during low-flow to determine carbohydrate (lactate and glucose) release enzymatically.

After basal oxygen uptake was determined (20 min), flow rates were decreased to 0.7 to 0.8 ml/g/min (low-flow) for 75 min. As oxygen tension decreases during low-flow perfusion, so does the rate of oxygen consumption (Wu et al., 1990). Therefore, this model reflects hypoxia, a finding confirmed with surface O_2 electrodes (Matsumura et al., 1986). In some livers, L-NAME or its enantiomer D-NAME (0.5 mM final concentration), SOD and/or ethanol (50 mM) were infused 10 and 5 min before low-flow perfusion, respectively, and continued throughout the low-flow perfusion period. In parallel experiments, the hypoxia marker pimonidazole (400 μM) was infused for 20 min during low-flow perfusion. $ONOO^-$ (initial concentration ~30 μM in the perfusate) was infused in some livers; during these experiments, livers were made anoxic by perfusion with nitrogen-saturated buffer to prevent endogenous radical formation. To ensure delivery of peroxynitrite into the liver, the tubing from the syringe containing peroxynitrite (in ice-cold 0.1% KOH) was placed adjacent to the liver so that it was infused directly into the sinusoidal space. Given the rapid decay rate of $ONOO^-$, its steady-state concentration at the site of infusion was obviously much lower than the levels infused. At the end of the experiment, tissue samples were collected and immediately fixed in formaldehyde solution for subsequent immunohistochemical analysis.

Determination of 3-Nitrotyrosine and Protein-Bound Pimonidazole by Immunohistochemistry. Paraffin blocks of formaldehyde-fixed liver tissue were sectioned at 6 μm, and 3-nitrotyrosine or pimonidazole was detected with a biotin-streptavidin-peroxidase indirect immunostaining method with diaminobenzidine

as a chromogen as described previously (Arteel et al., 1997a). After the immunostaining procedure, a counterstain of hematoxylin was applied. An Image-1/AT image acquisition and analysis system (Universal Imaging Corp., Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY) was used to capture and analyze tissue sections immunostained for nitrotyrosine and pimonidazole at 400× and 40× magnifications, respectively (Arteel et al., 1997a). Specificity of nitrotyrosine staining was determined by incubating an antinitrotyrosine antibody with 3-nitrotyrosine (Sigma Chemical Co.) before immunostaining.

Detection of Free Radical Adducts. To assess free radical formation, the spin-trapping agent POBN (5 mM) was infused during the last 20 min of the low-flow perfusion period. The role of superoxide was investigated in some livers by infusing SOD (3800 U/min) concomitantly with POBN. Perfusate containing spin-trapped adducts was collected in dipyridyl (10 mM) to prevent trace-metal catalyzed ex vivo radical formation. Immediately after collection, perfusate (100 ml) was extracted with a mixture of chloroform/methanol (2:1; 30 ml) and the organic layer was placed on dry ice until analysis on the same day. Sample volume was reduced to 1 ml by bubbling with nitrogen, and then the sample was placed in a quartz electron spin resonance (ESR) cell and bubbled with nitrogen for an additional 5 min to remove dissolved oxygen. Free radical adducts were detected with a Varian E-109 ESR spectrometer. Instrument conditions were as follows: 40-mW microwave power; 80-G sweep width; 1.25-G modulation amplitude; 1-s time constant; and 16-min sweep time. Spectra were recorded on an IBM-compatible computer interfaced with the spectrometer. Hyperfine coupling constants were determined with a spectral simulation program written by Duling (1994). The magnitude of the six-line signal was measured at identical gains and expressed in arbitrary units (1 U = 1 inch on chart paper).

Statistics. Results are expressed as mean ± S.E. unless otherwise indicated. Statistical analysis was performed by ANOVA with Tukey's post hoc tests. *P* values less than 0.05 were considered to be significantly different.

Results

Effect of Low-Flow Perfusion on O_2 Tension, Glycogenolysis, and pH. After 20 min of normal flow (4 ml/g/min), flow rates were decreased to 0.7 to 0.8 ml/g/min for 75 min. Figure 2 depicts the effect of low-flow perfusion on outflow oxygen tension, glycogenolysis, and pH for livers infused with L-NAME. After low-flow was initiated, outflow oxygen tension of the perfusate decreased rapidly and was below the limits of detection within 5 min (Fig. 2, top). Glycogenolysis, determined from the rate of glucose plus lactate release into the perfusate, decreased significantly, from 140 ± 27 to 60 ± 5 μmoles/g/h during low-flow and remained suppressed for 70 min (Fig. 2, middle). This effect is most likely due to an increase in consumption of carbohydrates by the liver, leading to less release into the perfusate. Perfusate pH decreased from approximately 7.30 during normal flow to approximately 7.15 during low-flow. Infusion of L-NAME had no effect on the above parameters. To determine whether low-flow perfusion causes hypoxia, the hypoxia marker pimonidazole (400 μM) was infused. During perfusion at normal flow rates, pimonidazole binding was localized to pericentral regions of the liver lobule (Fig. 3A) and comprised about 15% of the total cellular area (Fig. 4). Low-flow perfusion increased pimonidazole binding by a factor of about 4, with staining appearing in midzonal regions (Figs. 3B and 4). L-NAME had no significant effect on the increase in pimonidazole binding caused by low-flow perfusion (Figs. 3C and 4).

Low-Flow Perfusion Increased Nitrotyrosine Accumulation in Pericentral Regions of the Liver Lobule. At normal flow rates, nitrotyrosine staining was light and comprised less than 1% of the total cellular area (Figs. 5A and 6) in pericentral regions of the liver lobule. In the absence of ethanol, low-flow perfusion did not have a dramatic effect on nitrotyrosine staining compared to normal flow (data not shown); however, low-flow perfusion with ethanol increased nitrotyrosine accumulation significantly in pericentral regions by about a factor of 7 (Fig. 6). Nitrotyrosine staining was localized predominantly in nonparenchymal cells, most likely Kupffer and endothelial cells (Fig. 5B). Infusion of L-NAME under these conditions blunted nitrotyrosine accumulation significantly by a factor of 3 (Figs. 5C and 6). Moreover, it was localized in the periportal regions of livers when perfused in the retrograde direction at low-flow rates but predominantly in the pericentral regions if $ONOO^-$ was infused directly (data not shown). When anti-nitrotyrosine antibody was preincubated with nitrotyrosine before

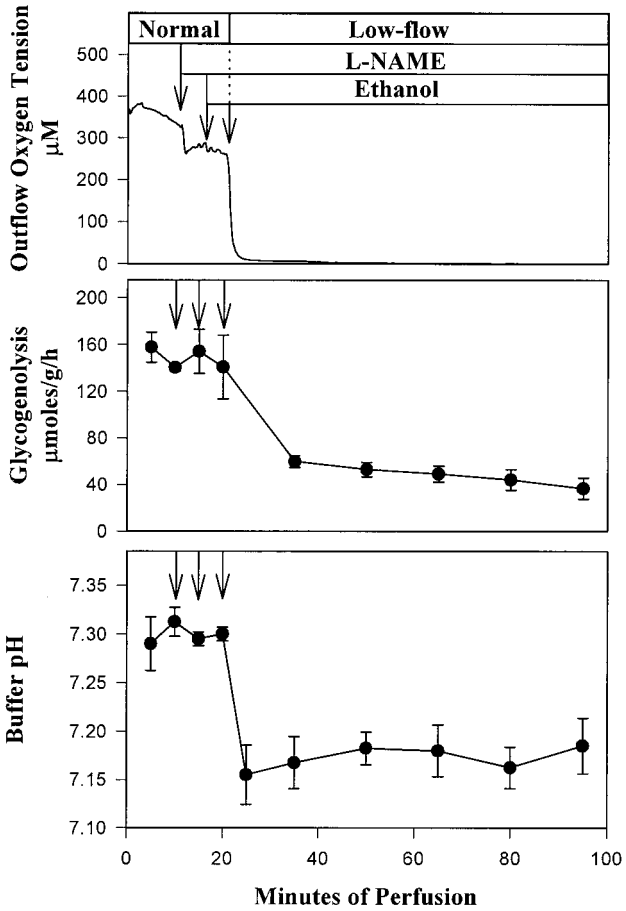

Fig. 2. Effect of low-flow perfusion on oxygen tension, glycogenolysis, and pH. Livers were perfused at normal flow rates (4 ml/g/min; normal flow). The NO· synthase inhibitor L-NAME (0.5 mM) in this example was infused beginning 10 min into the experiment and maintained throughout the procedure. Ethanol (50 mM) infusion was initiated at 15 min. After 20 min of perfusion, rates were decreased to 0.7 to 0.8 ml/g/min (low-flow). Outflow O_2 tension and pH were determined with electrodes as detailed in *Materials and Methods*. Glycogenolysis was determined enzymatically by glucose and lactate release into the perfusate. A typical O_2 trace is shown (top). Results are mean ± S.E. (n = 4) for glycogenolysis (middle) and perfusate pH (bottom).

experiments, no staining was observed, confirming the specificity of the observed immunoreactivity (data not shown). Given the recent attention that the Kupffer cell has received with respect to alcoholic liver disease (Thurman, 1998), these data are consistent with the hypothesis that nonparenchymal cells are involved in alcoholic liver injury.

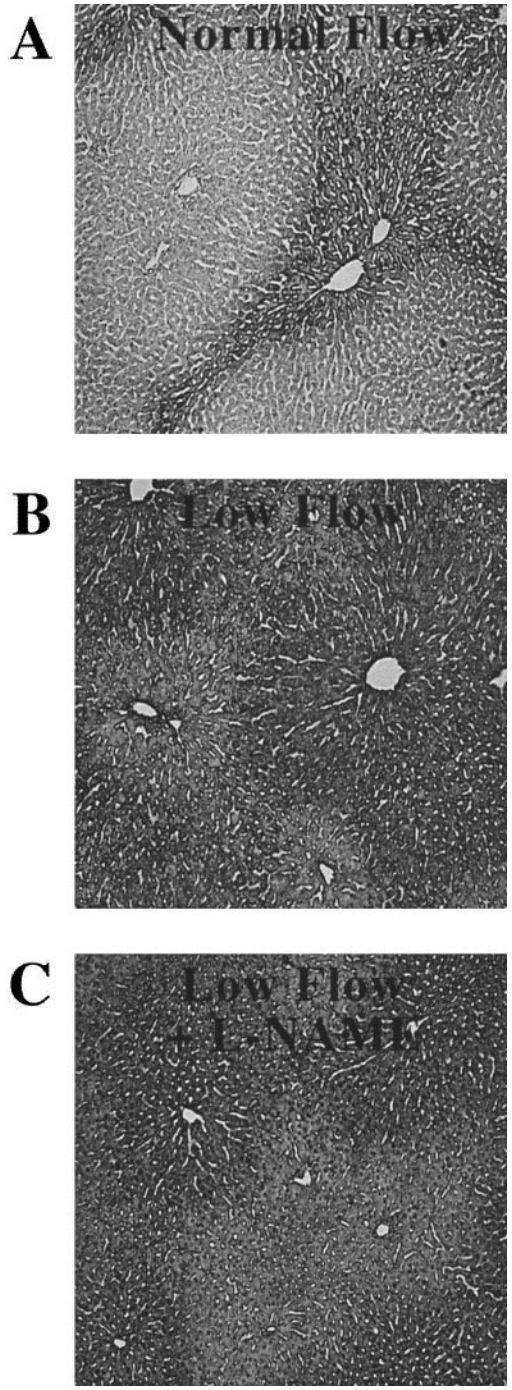

Fig. 3. Effect of low-flow perfusion on the sublobular pattern of pimonidazole binding in liver. Livers were treated as detailed in Fig. 2. Representative photomicrographs (40 ×) of pimonidazole binding (black) against a hematoxylin counterstain (gray) in livers after infusion of 400 μM pimonidazole for 15 min are depicted. Immunohistochemistry was performed as described in *Materials and Methods*. Representative photomicrographs from livers perfused at normal flow rates (A), livers perfused at low flow rates in the presence of ethanol (low-flow, B), and at low-flow rates in the presence of ethanol and L-NAME (low-flow + L-NAME, C) are shown.

Low-Flow Perfusion Causes Formation of Free Radicals. In addition to nitration reactions (e.g., nitrotyrosine formation), $ONOO^-$ also can cause oxidation by decomposing to hydroxyl radical (Gatti et al., 1998). Because hydroxyl-like radicals can form secondary radicals, free radical adducts were detected directly here with ESR and the spin-trapping technique. Figure 7A depicts a representative ESR spectra of a radical formed in organic extracts of perfusate during low-flow. No radical adduct signal was detected in the aqueous fraction of perfusate (data not shown). Low-flow perfusion in the presence of ethanol led to formation of a six-line radical spectrum detected in the organic phase of the perfusate. Computer simulation of the spectrum (Fig. 7B) revealed two radical species. When $ONOO^-$ was infused (Fig. 7C), a radical with analogous coupling constants to those detected under low-flow conditions was detected (Fig. 7D). Direct infusion of decomposed $ONOO^-$ resulted in a small background spectrum (data not shown) similar to one observed from the perfusion system in the absence of liver (see Fig. 8H). Furthermore, SOD had no effect on the magnitude of the radical spectrum obtained after direct infusion of $ONOO^-$ (data not shown).

When L-NAME (Fig. 8B), SOD (Fig. 8C), or both (Fig. 8D) were infused during low-flow perfusion with ethanol, the magnitude of the radical spectrum was decreased almost completely to near background levels (Fig. 8H) compared with low-flow perfusion with ethanol alone (Fig. 8A). When D-NAME, an L-arginine analog that does not inhibit NO·

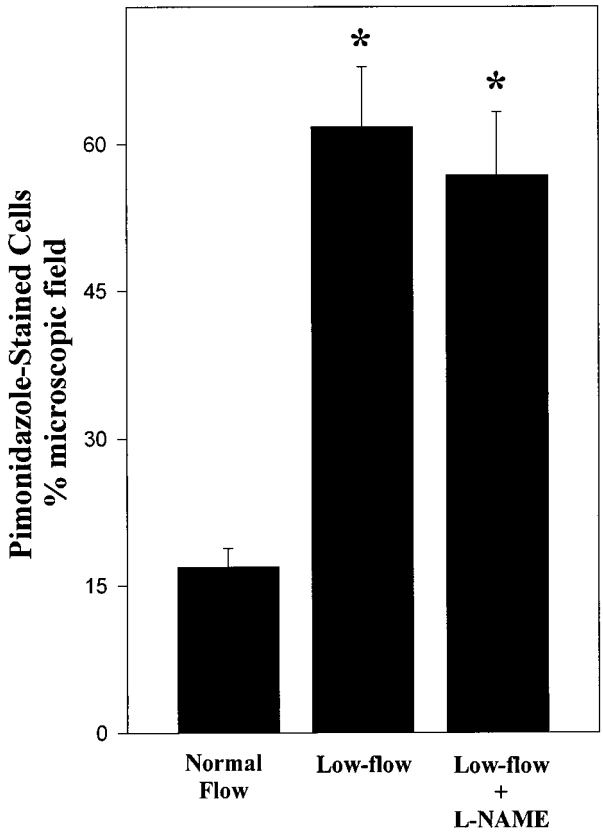

Fig. 4. Quantitation of pimonidazole accumulation in perfused liver. The effects of normal-flow, low-flow, and low-flow + L-NAME perfusion on pimonidazole accumulation in liver are summarized. Conditions are as in Fig. 3. Results are mean ± S.E. (n = 4). *$p < .05$ compared with controls by ANOVA with Tukey's multiple comparison test.

synthase, was infused in the presence of ethanol, the magnitude of the radical spectrum was similar to ethanol-only controls (data not shown). If the radical adduct spectrum was directly due to ethanol (e.g., α-hydroxyethyl radical), the added coupling caused by infusion of [^{13}C]ethanol should split the spectrum from 6 to 12 lines. However, under these conditions, [1-^{13}C]ethanol did not change the spectrum (Fig. 8E), indicating that the radical adducts were not derived directly from ethanol per se. Moreover, in vitro experiments with both [1-^{13}C] and [2-^{13}C]ethanol in the presence of $ONOO^-$ (30 μM added as a bolus under constant vortexing) did not lead to α-hydroxyethyl radical formation (data not shown). However, the magnitude of the radical spectrum was decreased when livers were perfused at low-flow rates in the absence of ethanol (Fig. 8F), indicating that radical formation was partly ethanol-dependent. The magnitude of the free radical signal produced in the absence of ethanol was also decreased by infusion of L-NAME and/or SOD (data not shown). No radical spectra were formed in the absence of POBN (Fig. 8G), and only a small background spectrum was observed from the perfusion system in the absence of liver (Fig. 8H).

Discussion

Hypoxia Is Involved in Alcohol-Induced Liver Injury. It was recently demonstrated with hypoxia markers that both acute and chronic ethanol consumption cause hypoxia at the cellular level in vivo in rat liver (Arteel et al., 1997a), verifying previous conclusions based on changes in oxygen uptake by the liver and blood oxygenation measurements (Videla et al., 1973). Because hypoxia precedes tissue injury in the Tsukamoto-French model of alcohol-induced liver damage (Arteel et al., 1997a), it may play a critical early step in liver damage caused by ethanol. Although long-term hypoxia may kill cells outright, hypoxia-induced oxidative stress via free radical formation could also be important. It was previously shown that the hypermetabolic state due to acute ethanol exposure was dose dependent (Wendell and Thurman, 1979). Because blood alcohol levels cycle in humans consuming alcohol as well as in rats on the Tsukamoto-French protocol (Tsukamoto et al., 1985), it is predictable that hypoxia would occur at high blood-alcohol levels with normoxia returning when blood levels decline. For this reason, it has been proposed that hypoxia and subsequent reoxygenation are the source of free radicals in livers from rats treated with alcohol (Knecht et al., 1995). However, recent work with 2-nitroimidazole hypoxia markers demonstrated

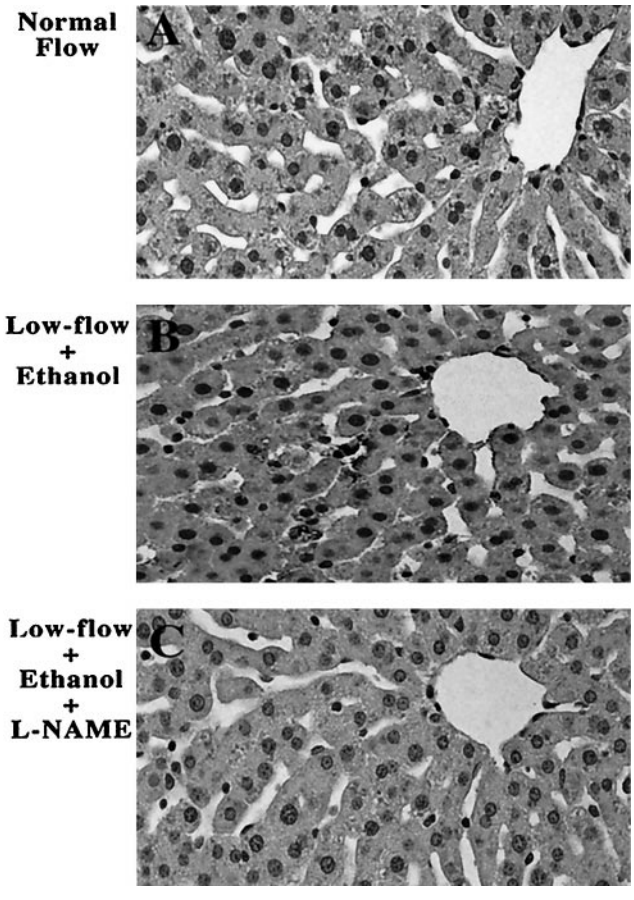

Fig. 5. Effect of low-flow perfusion and L-NAME on 3-nitrotyrosine accumulation in liver. Conditions are as in Fig. 2. Nitrotyrosine accumulation was determined immunohistochemically as detailed in *Materials and Methods*. Representative photomicrographs (400 ×) of nitrotyrosine (brown) against a hematoxylin counterstain (blue) in livers are shown. No nitrotyrosine staining was observed in periportal regions.

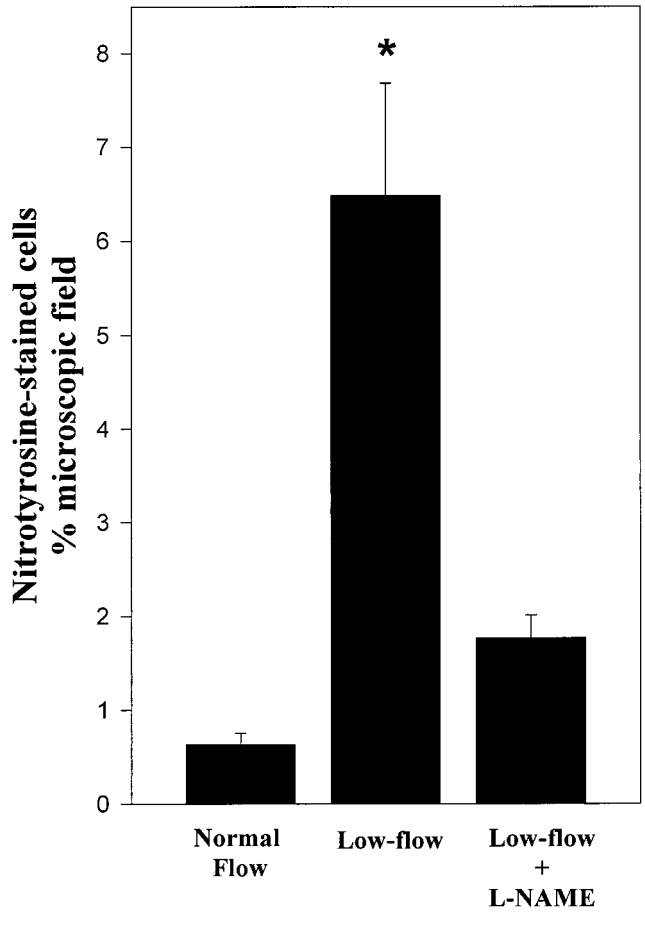

Fig. 6. Quantitation of nitrotyrosine accumulation in pericentral regions in the perfused liver. The effect of normal-flow, low-flow, and low-flow + L-NAME perfusion on nitrotyrosine accumulation in pericentral regions of the liver lobule are summarized. Conditions as in Fig. 5. Results are mean ± S.E.; $n = 4$ to 7. *$p < .05$ compared with controls by ANOVA with Tukey's multiple comparison test.

that hypoxia occurs early and remains during long-term ethanol exposure in rats (Arteel et al., 1997a). Under these conditions, free radical formation by a classical hypoxia/reoxygenation pathway seems unlikely.

Is $ONOO^-$ Involved in Oxidative Stress Due to Ethanol? Although $ONOO^-$ is a potent prooxidant in its own right (Murphy et al., 1998), when it is protonated to peroxynitrous acid, it becomes highly reactive, yielding oxidizing (Gatti et al., 1998; Lymar and Hurst, 1998) and nitrating (Ischiropoulos et al., 1992; Ischiropoulos, 1998) species by an excited intermediate. Furthermore, $ONOO^-$ reacts rapidly with carbon dioxide to form nitroperoxycarbonate in bicarbonate buffers (Denicola et al., 1996); oxidizing and nitrating reactions can also be mediated by this intermediate (Fig. 1). Cellular pH in liver declines rapidly during hypoxia (Desmoulin et al., 1987). Because glycolysis and lactate production predominate in pericentral regions of the liver lobule (Jungermann and Thurman, 1992), the observed decreases in pH caused by low-flow perfusion (Fig. 2) most likely predominate in this area. Under these conditions, the reactivity of $ONOO^-$ could increase via peroxynitrous acid as well as by nitrosoperoxycarbonate (see Fig. 1 for scheme).

Hypoxia and Free Radical Formation. In livers perfused at 25% of normal flow rates (~0.7–0.8 ml/g/min), making pericentral regions hypoxic (Fig. 4), oxidative stress has been detected by photoemission (Suematsu et al., 1992). Furthermore, low-flow perfusion followed by a return to normal flow leads to ESR-detectable free radical formation in liver (Bremer et al., 1994). Here, it was demonstrated that low-flow perfusion leads to ESR-detectable free radical adducts (Figs. 6 and 9). Furthermore, the radical signal was decreased by both L-NAME and SOD (Figs. 8 and 9). These data are consistent with the hypothesis that the final prooxidant is a derivative of both superoxide anion and NO· (e.g., $ONOO^-$; see Fig. 1). Moreover, because exogenously administered SOD does not enter the cell, these data suggest that the prooxidant detected with ESR was localized in the extra-

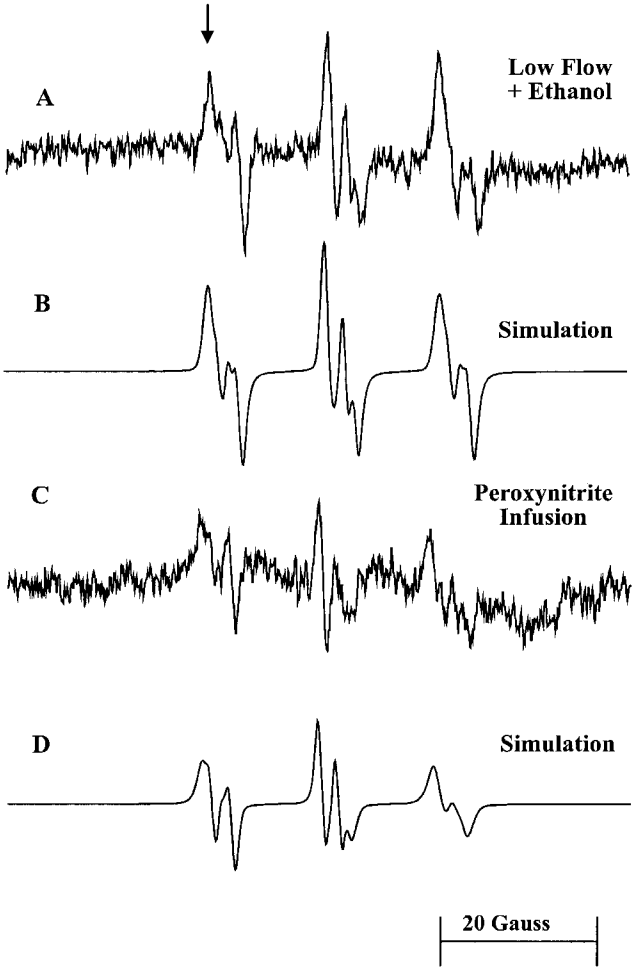

Fig. 7. Effects of low-flow perfusion on ESR spectra of free radical adducts in perfusate. Livers were treated as detailed in Fig. 2. During the last 20 min of perfusion, the spin-trap POBN (5 mM) was infused and effluent perfusate was collected. Extraction of perfusate and analysis of ESR spectra were performed as detailed in *Materials and Methods*. Typical (A) and computer-simulated spectra (B) are shown; simulation indicated that two radical species were present. Also, a radical spectrum detected when $ONOO^-$ was infused directly into liver under anoxic conditions is shown (C). When vehicle (0.01 M NaOH) was infused under these conditions, no radical adduct signal was detected (data not shown). Simulation of this radical spectrum (D) determined that the two radical species due to $ONOO^-$ were indistinguishable from those detected with low-flow perfusion. Typical spectra are shown from experiments repeated at least twice. Arrow indicates line used to quantitate the magnitude of the radical signal (see Fig. 9).

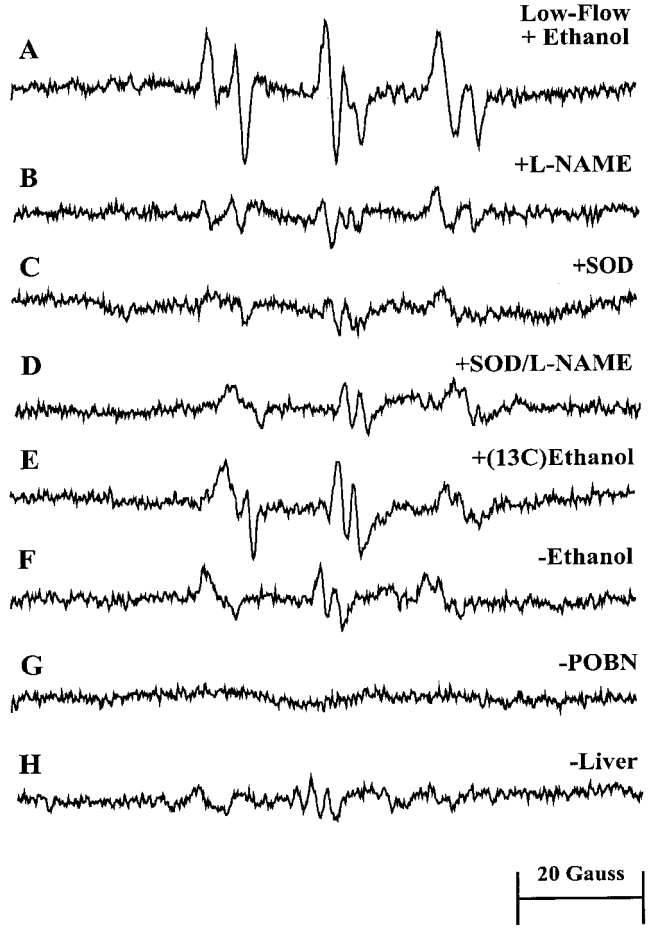

Fig. 8. Radical adducts in perfusate. Livers were treated as detailed in Fig. 2. The effect of L-NAME (B), SOD (C), and SOD and L-NAME together (D) on radical signal intensity of low-flow perfusion with ethanol (A) are shown. The effect of removing ethanol on the ESR signal (E), or infusing [^{13}C]ethanol (F) are also shown. Radical signals formed while perfusing the system in the absence of the spin-trap POBN (G) or a liver (H) are also detailed. Typical spectra are from experiments repeated at least twice.

cellular space, a hypothesis supported by nitrotyrosine staining in nonparenchymal cells (see below).

The average coupling constants of the two radical species generated (from three separate experiments) during low-flow perfusion with ethanol were 1) a^N = 14.88 G, a_β^H = 3.55 G and 2) a^N = 14.34 G, a_β^H = 1.51 G (Fig. 7, A and B). When $ONOO^-$ was infused directly, similar coupling constants were obtained: 1) a^N = 14.88 G, a_β^H = 3.35 G and 2) a^N = 14.45 G, a_β^H = 2.06 G; Fig. 7, C and D). These experiments provide physical evidence that the free radicals observed during hypoxic perfusion are derived from $ONOO^-$.

Hypoxia and Nitrotyrosine Formation. The conclusion that $ONOO^-$ is involved in hypoxia-induced oxidative stress is also supported by nitrotyrosine formation under these conditions (Fig. 6). Furthermore, similar staining was observed when $ONOO^-$ was directly infused into liver. However, alternative hypotheses exist to explain the observed results. For example, it is known that other pathways involving NO· can lead to the formation of free radicals and nitrotyrosine adducts (Gunther et al., 1997). These pathways may also be enhanced by hypoxia in liver and may play a role in the observed nitrotyrosine staining. On the other hand, no significant nitration of free tyrosine was observed under conditions of constant simultaneous generation of NO· and superoxide in vitro (Pfeiffer and Mayer, 1998). Therefore, the results of immunohistochemical detection of nitrotyrosine must be interpreted carefully.

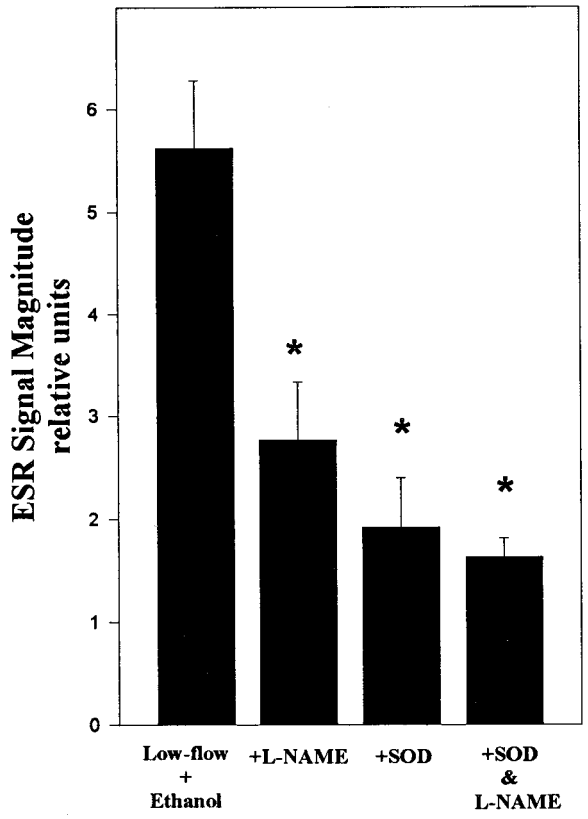

Fig. 9. Effect of L-NAME and SOD on free radical formation caused by low-flow perfusion. The effects of low-flow perfusion and L-NAME, SOD, and L-NAME + SOD on ESR-detectable free radicals are summarized. The amplitude of the first peak on the left on the ESR radical signal was used for comparisons. Results are mean ± S.E. (n = 3–4). *p < .05 compared with controls by ANOVA with Tukey's multiple comparison test.

It is also possible that hypoxia increases the formation of $ONOO^-$ by stimulating NO· and/or superoxide production. However, if hypoxia-induced increases in $ONOO^-$ formation are responsible for the results observed here, one would expect to observe nitrotyrosine staining in periportal regions of the liver lobule where Kupffer cells, the most likely source of both superoxide and NO·, predominate (Muto, 1975). However, nitrotyrosine staining was localized almost exclusively in the pericentral regions; therefore, enhanced formation of $ONOO^-$ seems an unlikely explanation for the results observed here. Moreover, perfusion in the retrograde direction at low-flow rates resulted in accumulation of nitrotyrosine in periportal regions of the liver lobule.

Ethanol Increases Free Radicals Formed during Hypoxic Perfusion. When livers were perfused under hypoxic conditions in the absence of ethanol, the magnitude of the radical signal was decreased by about a factor of 2 (Fig. 8). These data suggest that ethanol plays a role in radical formation under hypoxic conditions. Indeed, Pou et al. (1995) reported that α-hydroxyethyl radical adducts can be formed from direct attack of $ONOO^-$ on ethanol, suggesting that it plays a role in free radical formation with $ONOO^-$. However, infusion of [1-^{13}C]ethanol did not produce a 12-line spectrum (Fig. 8E) as would be expected if the radicals were derived directly from labeled ethanol (e.g., α-hydroxyethyl radical) (Knecht et al., 1995).

Alternatively, ethanol may enhance the decline in pH caused by hypoxic perfusion. Under analogous experimental conditions, Desmoulin et al. (1987) demonstrated that ethanol significantly lowered intracellular pH caused by low-flow perfusion with ^{31}P-NMR. Under these conditions, the enhanced decline in pH would favor the formation of reactive peroxynitrous acid, leading to more free radicals. Although some studies with animals have not shown a decrease in cellular pH after alcohol, other studies have (Cunningham et al., 1986); little work has been done in humans regarding this issue. However, it is well known that hypoxia, the model studied here, causes a decrease in intracellular pH. Indeed, the ability of the fed hepatocyte to produce lactic acid under hypoxic conditions far outweighs the buffering capacity of the cell (reviewed in Bücher, 1970). Furthermore, increased lactate/pyruvate ratios have been shown in humans after alcohol consumption (Volpi et al., 1997), demonstrating that lactic acidosis occurs in vivo.

Conclusions. Regardless of the mechanism, however, these findings in the perfused liver illustrate a possible new pathway of free radical formation caused by hypoxia. However, these experiments were performed with a model system that may or may not be relative of the situation in vivo. On the other hand, chronic hypoxia in vivo may enhance oxidative stress via this pathway. First, chronic hypoxia decreases the ability of cells to detoxify free radicals (Jones, 1985; Nakanishi et al., 1995). Second, chronic hypoxia may also increase baseline production of prooxidants. For example, the activity of NADPH oxidase and xanthine oxidase, known sources of superoxide anion, are increased by hypoxia (Brass et al., 1991), as well as NO· synthase gene expression (Arnet et al., 1996). Taken together, it is concluded that pericentral cells undergoing chronic hypoxia in vivo are exquisitely prone to oxidative stress from $ONOO^-$ formed in regions where oxygen exists. Although NO·-dependent pathways previously have been demonstrated to be involved in ischemia/

reperfusion injury (Matheis et al., 1992), the data presented here are consistent with the hypothesis that $ONOO^-$ formed in oxygenated periportal regions can damage hypoxic pericentral areas of the liver lobule. Therefore, the results of these studies suggest a possible alternative pathway involving $ONOO^-$ in free radical formation due to hypoxia in liver.

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