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HYPOXIA POTENTIATES KILLING OF HEPATOCYTE MONOLAYERS BY LEUKOTRIENES, HYDROPEROXYEICOSATETRAENOIC ACIDS, OR CALCIUM IONOPHORE A23187

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Potentiation of chemical toxicity by hypoxia was studied in confluent hepatocyte monolayers. Addition of either hydroperoxyarachidonic acid (50 μg), leukotriene C_4 (10 μg), or calcium ionophore A23187 (1.8 μg) to hepatocyte monolayers followed by incubation in 2% oxygen for 24 h killed 95% of the hypoxic cells, but was without effect on the normoxic cells. The greater than 10-fold increase in toxicity of A23187 suggests that hypoxic cells are less able to regulate intracellular calcium. The increased toxicity of hydroperoxyarachidonic acid and leukotriene C_4 may be due to a related reduction in activity of protective enzymes.

This study was performed to demonstrate the value of hepatocyte monolayers maintained under hypoxic conditions as a model system for examining the damage to liver cells induced by free radical metabolites. Our study of the reductive metabolism of halocarbons has led us to consider the interrelationship between the rate of free radical formation, extracellular oxygen availability, intracellular oxidation-reduction potential, and inhibition of intracellular protective mechanisms. Confluent hepatocyte monolayers prepared as described by Berry and Friend [1] and by Bissell [2] appear to be ideal for investigations of this interrelationship. In contrast to experiments performed in intact perfused liver, in which periportal cells may have extracellular oxygen concentrations of 100 μM while centrolobular cells have only 1–5 μM [3–5], experiments in hepatocyte monolayers allow every cell to experience an exactly defined external oxygen concentration. Moreover, the flat geometry of the monolayer should result in an equal extracellular to intracellular oxygen gradient in all cells [6]. Hepatocyte monolayers have stable intracellular concentrations of glutathione, ATP, and NADPH, as well as enzyme activities [2,6–8]. Monolayers are viable for up to 5 days, in contrast to hepatocytes that have not adhered to a support, which are rarely viable for more than 3 h [2]. In that the first ultrastructure changes occur 3 h after halocarbon metabolism and continue for 18 h [9], the long life and stable enzyme activities of hepatocyte monolayers are essential for our proposed studies.

It has been shown that the calcium ionophore A23187 causes hepatocyte death by allowing the rapid influx of calcium ions [10–12]. Control of intracellular calcium compartmentation as well as calcium transport to the extracellular space is performed by a Ca-ATPase [13,14]. Other studies have shown that the ATP/ADP ratio is decreased during hypoxia [15–17]. We found that after incubation of hepatocytes for 24 h in 2% oxygen, the combination of the oxygen gradients from the gas to incubation medium and from the incubation

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Abbreviations: HPETE, hydroperoxyeicosatetraenoic acid; DMSO, dimethylsulfoxide.

medium to the cytoplasm produced an intracellular oxygen concentration that caused 100% cell death in the presence of 1 μM A23187.

It is known that high concentrations of linoleic acid hydroperoxides destroy membrane-bound cytochrome *P*-450 [18,19] and that 1–4 mM concentrations of *t*-butylhydroperoxide is toxic to isolated hepatocytes [14]. A goal of this study was to determine if concentrations of arachidonic acid hydroperoxides as low as 1–50 μM would be toxic to hypoxic cells [15,16].

Leukotrienes have been shown to activate polymorphonuclear macrophages and to cause chemotaxis at concentrations as low as 1 nM [20]. However, concentrations of leukotriene C_4 100-times higher are required to cause release of lysosomal enzymes from neutrophils [21]. We have recently shown that leukotriene B_4 is rapidly metabolized by hepatic cytochrome *P*-450 [22], but that this metabolism does not occur in the absence of oxygen. Therefore, it is possible that a 1–10 μM concentration of leukotriene C_4 may enter hypoxic hepatocytes and remain active during a 24-h incubation period.

Hepatocytes were prepared from fed adolescent male Sprague-Dawley rats by in situ perfusion of collagenase (100 mg, Sigma type I) into the portal vein as described by Bissell [2]. The isolated and washed hepatocytes were suspended in 30 ml Gibco medium 199 with Earle's salts containing 100 U/ml penicillin, 20 mU/ml insulin, 1 μM dexamethasone and 2% rat serum (M-199), and aliquots containing $0.5 \cdot 10^6$ cells were allowed to attach to collagen-coated 60 mm Lux Permanox Contur culture dishes for 2 h at 37°C in 5% CO_2/95% air. The Permanox dishes facilitate O_2 exchange through the dish bottom and reduce cell pile-up at the edge. At this stage, more than 95% of the hepatocytes were viable as assayed by exclusion of Trypan blue. The attached cells were washed three times and then incubated in 3 ml of M-199 at 37°C under 5% CO_2/95% air for 24 h. The confluent monolayers were washed, 3 ml M-199 added to all dishes and then the dishes were divided into normoxic and hypoxic groups. The normoxic group was placed in a standard incubator with 5% CO_2/95% air at 37°C, while the hypoxic group was incubated in 2% O_2/5% CO_2/93% N_2. After 2 h of this pre-incubation, the test substances were added to both groups. At equilibrium, 2% O_2 will produce a 20 μM O_2 concentration in the medium. The incubator used for the hypoxic group of dishes was enclosed in a plastic glove box to allow the addition of test substances without altering hypoxic conditions.

Arachidonic acid hydroperoxides were prepared as previously described from arachidonic acid (Nu-Chek) and Cu/H_2O_2 [23]. The crude mixture from this reaction was chromatographed first on a C-18 reverse phase HPLC column (Altex) 10 mm $\times$ 25 cm with 2.5 ml/min of methanol/H_2O/acetic acid (90 : 10 : 0.5). The fraction that eluted from this column in 10–16 min was rechromatographed on the same column at 2.5 ml/min of methanol/H_2O/acetic acid (80 : 20 : 0.5). The fraction that eluted from the second column between 22–32 min contained the hydroperoxide (HPETE) mixture used in the hepatocyte experiments. This fraction was shown to consist of hydroperoxides by absorption spectroscopy and starch-iodine reaction. More than 95% of the mixture consisted of monohydroperoxyeicosatetraenoic acids of which the main components were shown to be 5-HPETE and 15-HPETE by reduction with triphenylphosphine followed by methylation, HPLC, and mass spectrometry (data not shown). The leukotrienes were a generous gift of Dr. Joshua Rokach of Merck-Frosst Canada Laboratories. Their purity and concentration were assayed as previously described [24]. The calcium ionophore A23187 was obtained from Calbiochem. Arachidonic acid and linoleic acid were repurified by preparative HPLC before use. Extinction coefficients used for quantitation were $2.7 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for hydroperoxides and $4 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for leukotrienes.

The HPETE, leukotriene C_4 and leukotriene B_4 were prepared for addition by adding the appropriate amount to a glass test tube, removing the solvent, adding 0.5 ml M-199, sonicating in a bath for 1 min, and then adding the 0.5 ml suspension to the 3 ml of M-199 already in the appropriate culture dish. In the case of the calcium ionophore A23187, the ionophore was dissolved in DMSO and added to 0.5 ml of M-199 by injection under the surface of the liquid. (0.25 μl of a stock solution of A23187 in DMSO produced a 1 μM final concentration in 3.5 ml.) The A23187 suspen-

sion was then sonicated for 1 min and immediately added to the appropriate culture dish. The DMSO alone was shown to have no effect on control cells. An additional 0.5 ml of M-199 was added to all control dishes in order to equalize the volumes. All incubations were performed in duplicate. Cell viability at the end of the 24-h incubation was assessed by adding Trypan blue, waiting 5 min, and photographing fields of approx. 200 cells at ×200 magnification on a Nikon phase-contrast inverted microscope with Kodak color negative film. The resulting color prints were used to look for surface blebs and irregularities as well as to allow counting of Trypan blue-stained nuclei versus unstained nuclei.

The results of this study are shown in Table I. It is seen that the viability of control hepatocytes in 2% oxygen was as good as that in 20% oxygen. Preliminary experiments at 0.5% oxygen showed that most cells would die in a 24-h period with such severe hypoxia. A 10 μM concentration of the calcium ionophore A23187 killed at least 95% of the cells at both oxygen concentrations. We used both light and scanning electron microscopy to verify that the ionophore caused the blebbing of our normoxic cells as has been reported by previous authors [10–12]. The normoxic hepatocytes survived exposure to 1 μM A23187 (3.5 nmol, 1.8 μg) with no signs of surface blebbing, whereas 95% of the hypoxic cells were killed. We suggest that in these hypoxic cells, the Ca-ATPase had insufficient activity to overcome the calcium leakage produced by the low concentration of ionophore. The 50 μg of mixed HPETE (149 nmol, 43 μM) was slightly toxic to normoxic cells as indicated by some increased nuclear staining and minor perturbation in surface morphology, whereas nearly all of the hypoxic cells were killed. This finding is consistent with a previous study [7] in which 1–4 mM of *t*-butylhydroperoxide was toxic to a hepatocyte suspension and the toxicity was exacerbated when glutathione peroxidase was inhibited. The addition of 10 μg leukotriene C_4 (16 nmol, 4.6 μM) had little effect on normoxic cells but caused killing of 95% of the hypoxic hepatocytes. The ten-fold lower concentration (460 nM) killed only half of the hypoxic hepatocytes under the conditions of our incubation, whereas the 0.1 μg addition (46 nM) had no observable effects. The mechanism by which leukotrienes could kill hepatocytes is a subject for future investigation. One possible explanation is that leukotrienes have been shown to act as calcium ionophores [25] and a killing process identical to that produced by A23187 may occur. A second possibility is that, since leukotriene B_4 has been shown to cause release of lysosomal enzymes in neutrophils exposed to cytochalasin [21], it may cause a similar activation of lysosomes in hepatocytes. A preliminary experiment on the effect of leukotriene B_4 was performed only in the case of normoxic hepatocyte monolayers. The addition of 50 μg (149 nmol, 43 μM) had no effect on these hepatocytes during a 7-h incubation. The lack of effect of this high concentration in the normoxic hepatocytes may be explained by our recent demonstration that leukotriene B_4 is rapidly metabolized by hepatic cytochrome $P-450$ [22]. The control addition of 50 μg oleic acid to hepatocyte monolayers was nontoxic to the hypoxic cells, whereas 50 μg of arachidonic acid showed some toxicity in both the

TABLE I

EFFECT OF 24 H EXPOSURE TO DIFFERENT TEST SUBSTANCES ON HEPATOCYTE VIABILITY

Confluent hepatocyte monolayers were maintained in 20% O_2 for 24 h to stabilize enzyme activities. Then the monolayers were washed, covered with fresh medium, and divided into two groups. The normoxic group was placed in an incubator under 20% oxygen while the hypoxic group was exposed to 2% oxygen. After 2 h of pre-incubation the test substances were added. After an additional 24 h, viability was assessed by staining with Trypan blue and by counting stained nuclei in color photomicrographs. The values presented are the range of duplicate dishes and are accurate to approx. ±5%.

Additions	Percent viable hepatocytes after 24 h	
	O_2 (20%)	O_2 (2%)
Control	95	95
Ionophore A23187 (10 μM)	0	0
Ionophore A23187 (1 μM)	95	5
HPETE (50 μg)	90–95	5
HPETE (10 μg)	95	40–60
Leukotriene C_4 (10 μg)	95	0
Leukotriene C_4 (1 μg)	95	40–60
Leukotriene C_4 (0.1 μg)	95	95
Oleic acid (50 μg)	95	95
Arachidonic acid (50 μg)	90	70–90

normoxic and hypoxic hepatocytes.

These hepatocyte monolayers appear to be an excellent system in which to study the potentiation of toxicity by hypoxia [5,6,15–17]. This system will be useful to study whether metabolically produced free radicals [26,27] lead to cell death.

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