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Mark Toraason , Ingrid Heinroth-Hoffmann , Donald Richards , Michael Woolery & Peter Hoffmann

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H_2O_2-INDUCED OXIDATIVE INJURY IN RAT CARDIAC MYOCYTES IS NOT POTENTIATED BY 1,1,1-TRICHLOROETHANE, CARBON TETRACHLORIDE, OR HALOTHANE

Mark Toraason

Cellular Toxicology Section, Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Cincinnati, Ohio, USA

Ingrid Heinroth-Hoffmann

Institute of Pharmacology and Toxicology, Martin Luther University, Halle, Germany

Donald Richards, Michael Woolery

Cellular Toxicology Section, Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Cincinnati, Ohio, USA

Peter Hoffmann

National Research Council Associate, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Cincinnati, Ohio, USA

Free radical-induced oxidative stress has been linked to ischemia-reperfusion injury of the myocardium. The $^{\bullet}OH$ radical is considered the most damaging radical and can be increased in cells by treatment in vitro with H_2O_2. The purpose of the present study was to determine if aliphatic halocarbons enhance H_2O_2-induced oxidative injury in isolated cardiac myocytes from neonatal rats. Oxidative damage was assessed by measuring release of thiobarbituric acid-reactive substances (TBARS) from lipid peroxidation, loss of lactate dehydrogenase (LDH) through damaged sarcolemmal membranes, and alterations in intracellular calcium ($[Ca^{2+}]_i$) transients in electrically stimulated (1 Hz, 10 ms, 60 V) myocytes. H_2O_2 increased TBARS release and LDH leakage in a concentration-dependent (20–200 μM) manner. Continuous suffusion with H_2O_2 first altered the configuration of $[Ca^{2+}]_i$ transients, then eliminated them, and finally caused $[Ca^{2+}]_i$ overload (basal $[Ca^{2+}]_i$ exceeded peak systolic $[Ca^{2+}]_i$ of control). The time to $[Ca^{2+}]_i$ overload was inversely associated with concentration, and the shortest time to overload was obtained with 100 μM H_2O_2. A 1-h

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Address correspondence to Mark Toraason, Cellular Toxicology Section, Division of Biomedical and Behavioral Science, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Cincinnati, OH 45226.

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preincubation of myocytes with the iron chelator deferoxamine inhibited all effects of H_2O_2. 1,1,1-Trichloroethane, carbon tetrachloride, or halothane at 1 mM significantly and reversibly reduced $[Ca^{2+}]_i$ transients but did not influence TBARS release or LDH leakage. Simultaneous exposure of myocytes to H_2O_2 and halocarbons did not affect the myocyte response to H_2O_2 exposure. Results indicate that the three halocarbons tested do not enhance H_2O_2-induced oxidative injury in isolated cardiac myocytes.

There is considerable evidence linking oxygen free radicals to myocardial ischemia-reperfusion injury (Blasig et al., 1990; Bolli et al., 1990; Reimer et al., 1990; Ohmi et al., 1992; Tavazzi et al., 1992). Experimental models that mimic the oxygen radical aspect of ischemia-reperfusion injury have been developed for cultured cardiac myocytes (Kaneko et al., 1989a, 1989b; Massey and Burton, 1990). Such systems frequently generate the highly reactive hydroxyl radical ($^{\bullet}OH$) by treatment of cells with xanthine-xanthine oxidase, hydrogen peroxide (H_2O_2), or iron. It is assumed that after the addition of H_2O_2, the $^{\bullet}OH$ is produced via the Haber-Weiss or Fenton reactions. Although there is some controversy as to the source (Semb et al., 1989), timing (Ohmi et al., 1992; Kirshenbaum et al., 1992), and damage caused by $^{\bullet}OH$ (Takemura et al., 1992), its concentration is significantly elevated during ischemia and reperfusion of the myocardium (Blasig et al., 1990; Bolli et al., 1990; Tavazzi et al., 1992).

Investigations of effects of $^{\bullet}OH$ have focused on lipid peroxidation (Janero et al., 1991; Kirshenbaum et al., 1992), thiol depletion (Bhatnagar et al., 1990; Lesnefsky et al., 1991), metabolic derangement (Ohmi et al., 1992), and alterations in intracellular calcium concentration ($[Ca^{2+}]_i$) (Josephson et al., 1991). Changes in myocardial $[Ca^{2+}]_i$ probably are the most significant in terms of cardiac arrhythmias associated with ischemia-reperfusion injury. Hess et al. (1984) first demonstrated that free radicals produced by xanthine-xanthine oxidase inhibited calcium uptake by sarcoplasmic reticulum vesicles. Since then a number of reports have described perturbations in myocardial calcium induced by free radicals. In beating myocytes, free radicals cause an increase in both diastolic and systolic $[Ca^{2+}]_i$ (Burton et al., 1990; Kihara et al., 1989). Contributing factors appear to be stimulation of sarcolemmal calcium uptake through inhibition of Na,K-ATPase (Kaminishi et al., 1989; Bhatnagar et al., 1990; Xie et al., 1990), leakage of calcium through ryanodine-sensitive channels of the sarcoplasmic reticulum (Okabe et al., 1991), and inhibited ATP-dependent calcium pump activity of the sarcolemma (Kaneko et al., 1989a, 1989b).

Perturbation of $[Ca^{2+}]_i$ has also been found to be a critical factor in the myocardial effects produced by aliphatic halocarbons such as the anesthetic agents halothane (HAL), isoflurane, and enflurane (Nelson and Sweo, 1988; Wheeler et al., 1988; Katsuoka et al., 1989; Eskinder et al., 1991) and the solvents 1,1,1-trichloroethane (TCE) (Hoffmann et al., 1992) and carbon tetrachloride (CT) (Toraason and Breitenstein, 1991). Several reports have recently demonstrated that halocarbons sensitize isolated cells to oxidative

injury. H_2O_2-induced lipid peroxidation was enhanced in erythrocytes from rats chronically exposed to halothane (Toker et al., 1990). Simultaneous exposure of vascular endothelial cells or arterial smooth muscle cells to iron-ADP and a variety of short-chain chlorinated hydrocarbons including TCE and CT shifted the concentration-response curve of iron-induced lipid peroxidation to the left, whereas the halocarbons alone had no effect (Tse et al., 1990). Halothane and isoflurane have also been reported to sensitize pulmonary artery endothelial cells to H_2O_2-induced cytotoxicity (Shayevitz et al., 1991) and increased $[Ca^{2+}]_i$ (Shayevitz et al., 1990).

In vivo, HAL has been reported to be protective, detrimental, and ineffective during an ischemic episode (Chakrabarty et al., 1991; Spahn et al., 1992). Spahn et al. (1992) attributed apparent inconsistent observations of halocarbon anesthetics on myocardial ischemia to their complex effects on hemodynamics and metabolism, which can impact an experimental ischemic episode in a myriad of ways, some of which are dependent on experimental design. Despite the lack of clear in vivo evidence, in vitro studies with vascular endothelial and smooth muscle cells indicate that the myocardium itself may have the potential to be sensitized to oxidative injury during halocarbon exposure. We tested this hypothesis by producing an oxidative stress in isolated cardiac myocytes from neonatal rats with H_2O_2 while simultaneously exposing cells to halocarbons. Trichloroethane was selected because it is found in a variety of industrial and commercial products and has been linked to sudden death, presumably due to cardiac arrhythmia stemming from accidental high-level exposure or recreational abuse (Bass, 1970; McCarthy and Jones, 1983; Siegal and Wason, 1990). Carbon tetrachloride is a highly metabolized halocarbon forming the reactive trichloromethyl radical in liver via cytochrome P-450 metabolism (Halliwell and Gutteridge, 1989). If formation of reactive intermediates of halocarbons contributes to oxidative injury in the present study, evaluation of CT would provide a contrast to the comparatively unreactive TCE (Kyrklund and Haglid, 1991). Halothane was selected because it is a widely used anesthetic.

MATERIALS AND METHODS

Preparation of Myocytes

Cardiac ventricular cells were isolated from 2- to 4-d-old Sprague-Dawley rats by a method previously described (Toraason et al., 1990). Rat pups were obtained from a breeding colony maintained in the animal quarters of the National Institute for Occupational Safety and Health, which is accredited by the American Association for Accreditation of Laboratory Animal Care. In order to reduce the percentage of fibroblasts in the cultures, ventricular heart cells were preplated at a density of 10^6 cells/ml in incubation flasks in M199 containing 10% newborn calf serum and 100 U penicillin-streptomycin/ml. After 1 h, flasks were gently swirled, which suspended unattached myocytes

and left the majority of fibroblasts attached. The cell suspension was then plated at 10^6 cells per 35-mm well for lipid peroxidation and cytotoxicity assessment 48 h after plating. For measurement of $[Ca^{2+}]_i$, cell suspensions of 0.5–1 × 10^6 cells/ml were plated as a convex meniscus of 3–6 drops of cell suspension on 19-mm, round, quartz cover slips held in 35-mm wells. After 24 h, cover slips were washed and incubated in 2 ml of serum-supplemented M199 and used 48–56 h later for measurement of $[Ca^{2+}]_i$.

Chemicals

Hanks balanced salt solution (HBSS) was purchased from Gibco, Grand Island, N.Y. Newborn calf serum was purchased from Hyclone, Logan, Utah. Dimethyl sulfoxide (DMSO), TCE, and CT were purchased from Fisher Scientific, Fair Lawn, N.J. Halothane was purchased from Halocarbon Laboratories, Inc., North Augusta, N.C. Purity levels of halocarbons were >99%. All other chemicals and reagents were purchased from Sigma, St. Louis, Mo.

Thiobarbituric Acid-Reactive Substances Assay

Lipid peroxidation was assessed by measuring TBARS released in culture dishes by the method of Yagi (1976) as modified by Casini et al. (1982). Test solutions obtained from cultured cells were cooled to 4°C and centrifuged for 10 min at 1000 × g to remove cells. Then 750 μl of the supernatant was combined with an equal volume of cold 12% trichloroacetic acid and centrifuged for 10 min at 1000 × g at 4°C to remove precipitated protein. One milliliter of supernatant was added to 1 ml TBA reagent (0.6% TBA, 0.01% BHT, 1.0 m*M* EDTA), and the mixture was heated at 100°C for 20 min. The mixture was allowed to cool, and TBARS were extracted with 3 ml of 1-butanol. A 1.0 m*M* stock solution of tetraethoxypropane in water was diluted in various amounts of 0.01 *N* HCl to produce malondialdehyde; these solutions were used as TBARS standards. The 1-butanol fractions and malondialdehyde standards were spectrofluorometrically analyzed with excitation wavelength of 520 nm and emission wavelength of 553 nm. The amounts of TBARS (nmol) were calculated using the linear regression obtained from the standards.

Lactate Dehydrogenase Determination

Lactate dehydrogenase (LDH) was measured with a Sigma kit (procedure 228-UV) in cell treatment buffer and in cells following a 10-min incubation in 2% Triton X-100 in HBSS solution at 37°C. The LDH activity is expressed as percentage of LDH in treatment buffer relative to total LDH in the cell culture dish.

Total Protein Determination

Protein was released from cells by adding 1 ml of 0.3 *N* NaOH containing 0.1% sodium dodecyl sulfate and incubating cells at 37°C for 1 h. Total protein was determined by the method of Bradford (1976).

Treatment of Cells for Assessment of Lipid Peroxidation and Cytotoxicity

Cardiac myocytes plated for 48 h in 35-mm wells were washed with HBSS and exposed to H_2O_2 with or without halocarbons in 1 ml of HBSS for 1 h at 37°C. Halocarbons were added to HBSS as a 0.1% DMSO aliquot. Controls received DMSO alone. After the 1-h incubation, buffer was removed from culture dishes and assayed for TBARS release and LDH leakage from myocytes. One milliliter of HBSS containing 2% Triton X-100 was added to culture wells, and cells were incubated for an additional 10 min at 37°C to release the remaining LDH from cells for determination of total LDH.

Preincubation of Myocytes with Deferoxamine

Cardiac myocytes were preincubated for 1 h with 1 m*M* deferoxamine prior to exposure to H_2O_2. Simultaneous treatment of myocytes with deferoxamine and H_2O_2 did not prevent cytotoxicity or lipid peroxidation. The 1-h preincubation may provide enough time for deferoxamine to enter the myocytes. Deferoxamine, in 100 μl of M199, was added directly to cultures containing serum-supplemented M199 to give a final concentration of 1 m*M*. The deferoxamine-supplemented M199 was removed prior to exposure to H_2O_2 and was not included in the treatment buffer.

Measurement of $[Ca^{2+}]_i$

Myocytes were loaded with fura-2, and $[Ca^{2+}]_i$ was measured as previously described (Hoffmann et al., 1992). In brief, myocytes were incubated for 10 min at 37°C with 2 μM of the acetoxymethyl ester dissolved in DMSO and added as 0.1% solution to culture medium. Cover slips containing myocytes loaded with fura-2 were transferred to a temperature-controlled (32°C) suffusion chamber on the stage of an inverted microscope (Nikon), which was coupled to a dual-excitation spectrofluorometer (Deltascan, Photon Technology International, South Brunswick, N.J.). Myocytes were suffused with HBSS at 2 ml/min and paced (1 Hz, 60 V, 10 ms) with 2 platinum electrodes, 3 mm apart. The data collection rate for all experiments was 20 Hz. Changes in $[Ca^{2+}]_i$ are represented by changes in the ratio of emission counts per second (cps) from 340/380 nm excitation. Treatment of myocytes with 10 μM digitonin caused a 90% loss of fura-2 fluorescence within 5 min, indicating the majority of measured fura-2 was cytosolic. H_2O_2-induced $[Ca^{2+}]_i$ overload and subsequent leakage of fura-2 precluded the determination of R_{min} and R_{max} for conversion of 340/380 ratios to $[Ca^{2+}]_i$. The extent and rate of decline of fura-2 fluorescence after H_2O_2 exposure was comparable to those produced by digitonin and were attributed to loss of sarcolemmal membrane integrity. Control experiments indicated that TCE, CT, or HAL (8 m*M*) did not quench or enhance fura-2 fluorescence, nor did they modify autofluorescence of myocytes. Therefore, changes in fura-2-fluorescence in

ventricular myocytes upon addition of halocarbons are considered to represent changes in $[Ca^{2+}]_i$.

Statistical Analysis

Data are presented as mean ± SD. Paired *t*-test, analysis of variance (ANOVA), and Duncan's multiple range test were performed using commercially available software (Statgraphics, Statistical Graphics Corp., Rockville, Md.).

RESULTS

Inverse Correlation Between Cell Density and H_2O_2-Induced Lipid Peroxidation

During the initial phases of this study, it became evident that H_2O_2-induced lipid peroxidation in cardiac myocytes was inversely dependent on cell density. To demonstrate this effect, heart cells were plated at 0.5, 1, or 2 million cells per 35-mm culture dish. Total protein was determined in each dish and found to reflect plating density. Figure 1 illustrates the inverse correlation between protein content and TBARS released following a 1-h exposure of cardiac myocytes to 100 μM H_2O_2. A comparable decrease in oxidative injury as cell density increased has been reported in human fibroblasts exposed to hypoxanthine-xanthine oxidase (Noel-Hudson et al., 1989). This association precluded the use of total protein for normalization of TBARS data. Therefore, TBARS are presented as nanomoles per 35-mm culture dish.

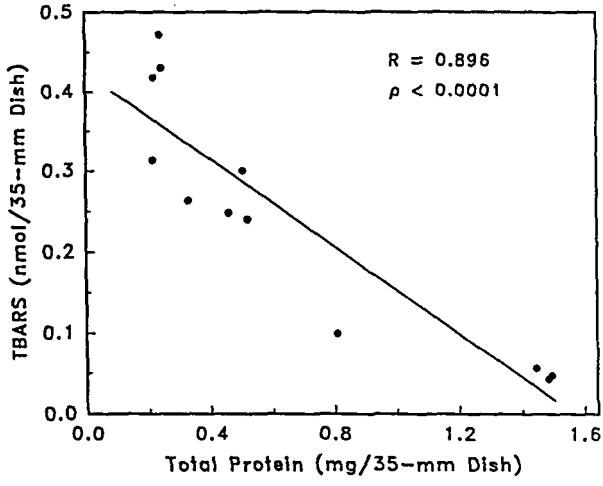

FIGURE 1. Inverse relationship between total protein per dish and TBARS released by treatment of cardiac myocytes with H_2O_2. Isolated myocytes were plated at 0.5, 1, or 2 million cells per 35-mm dish. After 2 d, cells were exposed for 1 h to 100 μM H_2O_2. The TBARS released from myocytes during exposure to H_2O_2 decreased as the total protein content of the dishes increased. Points are from individual dishes, and line is least-squares linear regression. Results are representative of two experiments.

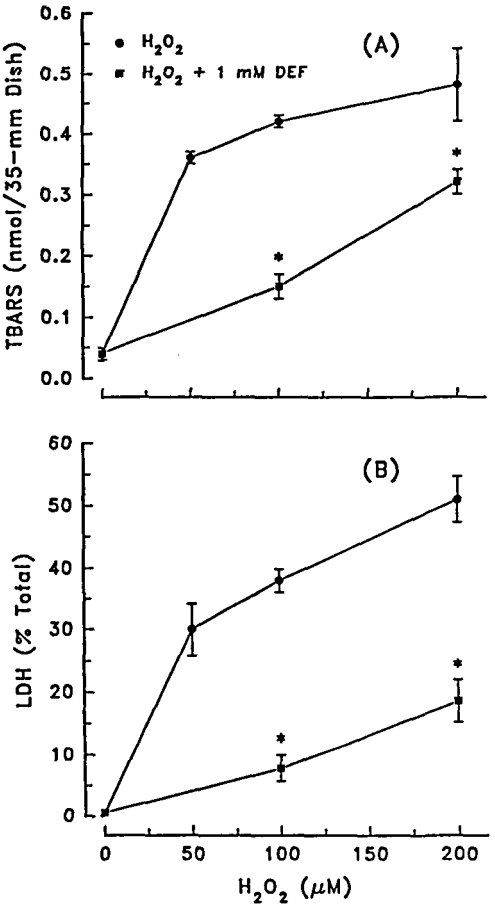

FIGURE 2. H_2O_2-induced lipid peroxidation and cytotoxicity in cardiac myocytes pretreated with deferoxamine. Myocytes were exposed to H_2O_2 for 1 h at concentrations shown; 50, 100, and 200 μM H_2O_2 significantly increased (A) TBARS and (B) LDH release (ANOVA, $p < .001$). Preincubation of cells for 1 h with 1 *mM* deferoxamine significantly (*$p < .001$) reduced TBARS and LDH released by exposure to 100 or 200 μM H_2O_2. Values are mean ± SD of four cultures, and representative of three experiments.

The inability to normalize TBARS data prevented combining replicate experiments from different cell platings, as small uncontrollable variations between platings were manifested as shifts in total TBARS released following a specific treatment. Therefore, TBARS and LDH results shown in figures are complete experiments conducted with a single cell plating and representative of two to four replicate experiments from different platings.

H_2O_2-Induced Lipid Peroxidation and Cytotoxicity

Figure 2 illustrates the TBARS and LDH released during a 1-h exposure of myocytes to 3 concentrations of H_2O_2. Release of TBARS was significantly

elevated by 50, 100, and 200 μM H_2O_2. A 1-h preincubation of cardiac myocytes with 1 mM deferoxamine significantly inhibited TBARS release induced by a 1-h exposure to 100 or 200 μM H_2O_2. The LDH release mirrored that of TBARS and was significantly increased by a 1-h exposure to 50, 100, or 200 μM H_2O_2. A 1-h preincubation with 1 mM deferoxamine protected cardiac myocytes from H_2O_2 cytotoxicity.

Effects of Halocarbons on H_2O_2-Induced Lipid Peroxidation and Cytotoxicity

Figure 3 illustrates the effect of 1 mM TCE, CT, or HAL on TBARS release and LDH leakage induced by 1-h exposure of cardiac myocytes to H_2O_2. The halocarbons alone did not cause a significant release in TBARS or

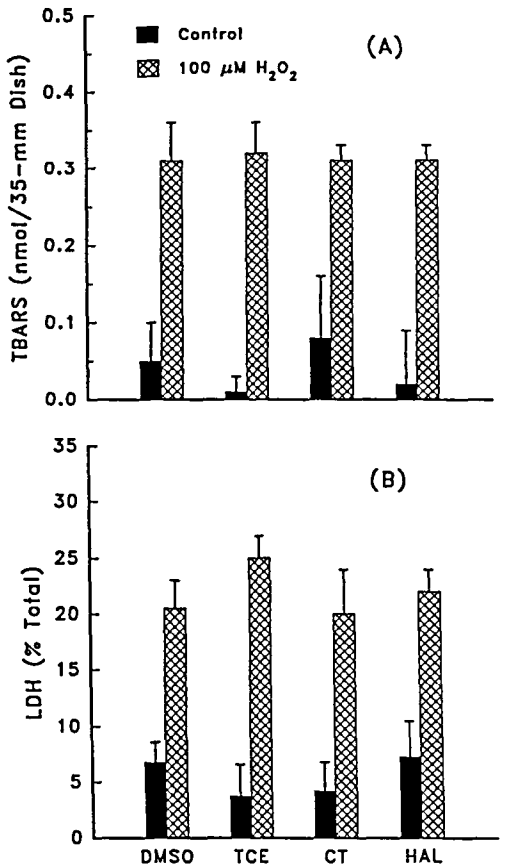

FIGURE 3. Absence of effect of TCE, CT, or HAL on H_2O_2-induced lipid peroxidation and cytotoxicity in cardiac myocytes. Myocytes were exposed to 0.1% DMSO (vehicle), or 1 mM TCE, CT, or HAL in the absence or presence of 100 μM H_2O_2. H_2O_2 significantly (ANOVA, $p < .0001$) increased (A) TBARS and (B) LDH released. TCE, CT, or HAL did not significantly affect TBARS release in the absence or presence of H_2O_2. Bars are mean ± SD of five cultures and representative of two experiments.

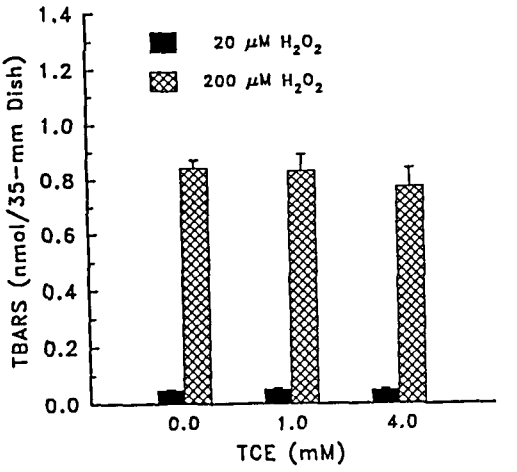

FIGURE 4. *Absence of effect of 1 or 4 mM TCE on 20 or 200 μM H_2O_2-induced lipid peroxidation in cardiac myocytes.* At 200 μM, H_2O_2 significantly (ANOVA, $p < .0001$) increased TBARS above that produced by 20 μM H_2O_2. TCE at 1 or 4 mM did not significantly affect TBARS released by H_2O_2. Bars are mean ± SD of four cultures and representative of two experiments.

LDH, nor did they modulate TBARS release or LDH leakage induced by H_2O_2.

Figure 4 illustrates that 0, 1, or 4 mM TCE resulted in comparable TBARS release in cells exposed simultaneously to either 20 or 200 μM H_2O_2.

Effect of H_2O_2 on $[Ca^{2+}]_i$

Following a 30-min equilibration period in the suffusion chamber, cardiac myocytes were suffused with HBSS containing 0, 20, 50, 100, or 200 μM H_2O_2. In control cells (0 μM H_2O_2), amplitude of $[Ca^{2+}]_i$ transients remained constant for more than 1 h (data not shown). Introduction of H_2O_2 resulted in changes in $[Ca^{2+}]_i$ that were categorized into three phases (Fig. 5). The first was identified as a change in the diastolic and/or systolic level of $[Ca^{2+}]_i$. Changes included decreased or increased peak systolic $[Ca^{2+}]_i$, increased diastolic $[Ca^{2+}]_i$, or combinations of the two. In the second phase, electrical stimulation no longer produced $[Ca^{2+}]_i$ transients. The third phase, referred to as $[Ca^{2+}]_i$ overload, was identified as that point where the static $[Ca^{2+}]_i$ level exceeded the peak systolic $[Ca^{2+}]_i$ prior to exposure to H_2O_2. Decisions regarding the time at which a myocyte moved from one phase to the next were made by computerized overlaying of corresponding data sets from individual myocytes. In the case of changes in systolic or diastolic $[Ca^{2+}]_i$, 10 consecutive $[Ca^{2+}]_i$ values would have to be greater or less than 10 consecutive corresponding values obtained prior to exposure. A summary of numerical data obtained from the experiments is in the upper portion of Table 1. Since none of the described effects were observed in untreated

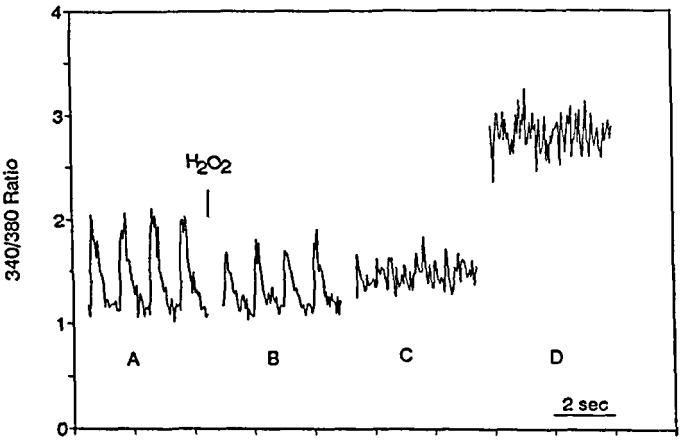

FIGURE 5. Effect of H_2O_2 on $[Ca^{2+}]_i$ transients in an electrically paced cardiac myocyte. $[Ca^{2+}]_i$ was monitored in a single myocyte by the emission ratio from excitation of fura-2 at 340/380 nm. (A) $[Ca^{2+}]_i$ transients in an electrically paced (60 V, 1 Hz, 10 ms) myocyte after 30 min of suffusion with HBBS and prior to H_2O_2 exposure. (B) Reduced $[Ca^{2+}]_i$ transient amplitude in the same myocyte after 10 min of continuous suffusion with 100 μM H_2O_2. (C) Absence of $[Ca^{2+}]_i$ transients after 16 min of suffusion with H_2O_2. (D) $[Ca^{2+}]_i$ overload after 20 min of H_2O_2 exposure.

cells, the response to 100 μM H_2O_2 was used as a basis for statistical comparison. Although trends are evident in the concentration-response data, statistically significant differences occurred only at 20 μM H_2O_2. Corresponding values from 50 and 200 μM H_2O_2 were not significantly different from values obtained with 100 μM H_2O_2. In addition, the effects of 20 μM H_2O_2 were highly variable; time to $[Ca^{2+}]_i$ overload ranged from 20 to 60 min. In 3 of 15 cell preparations treated with 20 μM H_2O_2 (2 of the 3 also exposed to 1 mM TCE), $[Ca^{2+}]_i$ overload was not observed during 70 min of exposure (not used in summary statistics in Table 1). A brief (2–4 min) $[Ca^{2+}]_i$ overload, followed by return to normal $[Ca^{2+}]_i$ was observed in 1 cell treated with 20 μM H_2O_2 alone and in 1 cell treated with 20 μM H_2O_2 and 1 mM TCE. For these cells, the initial brief $[Ca^{2+}]_i$ overload observed was used in calculating summary statistics in Table 1. Furthermore, at the termination of experiments, myocytes exposed to 20 μM H_2O_2 appeared normal, whereas myocytes exposed to higher concentrations exhibited blebs, increase in granules, and in some cases myocytes came off the quartz cover slips. The latter effect was observed invariably at 200 μM H_2O_2. No attempt was made to score and quantify these morphological changes.

Figure 6 illustrates the temporal sequence of $[Ca^{2+}]_i$ overload relative to loss of sarcolemmal membrane integrity in a cell treated with 100 μM H_2O_2. The $[Ca^{2+}]_i$ overload was monitored by the emission ratio from 340/380 nm excitation. Loss of sarcolemmal membrane integrity was monitored by measuring total fluorescence (total emission cps from 340 + 380 nm excitation). The loss of fura-2 is analogous to LDH leakage. Figure 6 illustrates that in a

control cell the 340/380 ratio remains constant despite a gradual decline in total fluorescence during 50 min of suffusion. After 22 min of suffusion with H_2O_2, a dramatic increase in 340/380 ratio occurs, indicating a rise in $[Ca^{2+}]_i$. This increase is apparently not due to a loss of sarcolemmal membrane integrity, since fura-2 remains at levels comparable to control for an additional 10 min. At 34 min, a precipitous loss of fura-2 is evident, indicating gross loss of sarcolemmal membrane integrity in cells suffused with 100 μM H_2O_2. Several attempts at this experiment failed because at the time of fura-2 leakage, the myocytes often hypercontracted or began to detach from the cover slip and move from the field of view. Comparable results to those shown in Figure 6 were obtained with three cell preparations.

Effect of Deferoxamine on H_2O_2-Induced $[Ca^{2+}]_i$ Overload

Preincubation of myocytes for 1 h with 1 mM deferoxamine significantly delayed the cessation of $[Ca^{2+}]_i$ transients and the onset of $[Ca^{2+}]_i$ overload induced by 100 μM H_2O_2 (Table 1).

TABLE 1. Effect of H_2O_2 and Halocarbons on $[Ca^{2+}]_i$ Transients in Cardiac Myocytes

			$[Ca^{2+}]_i$ Transients[a]		
H_2O_2 (μM)	Treatment	n	Altered[b]	Eliminated[c]	Overload[d]
		Concentration Response			
20	—	8	27 ± 7[e]	35 ± 12[e]	37 ± 11[e]
50	—	5	16 ± 5	21 ± 4	23 ± 4
100	—	13	11 ± 3	17 ± 3	20 ± 3
200	—	*7*	9 ± 3	15 ± 3	21 ± 6
		Deferoxamine Inhibition			
100	DEF	6	17 ± 6	23 ± 6[e]	27 ± 7[e]
		Effects of Halocarbons			
20	TCE	4	20 ± 5[e]	29 ± 4[e]	39 ± 15[e]
100	TCE	5	12 ± 2	15 ± 3	18 ± 3
100	HAL	5	11 ± 3	14 ± 3	19 ± 4
100	CT	5	11 ± 3	14 ± 3	19 ± 2

Note. TCE, HAL and CT (at 1 mM) were administered 5 min prior to and during exposure to H_2O_2. DEF indicates cells were preincubated with 1 mM deferoxamine for 1 h prior to exposure to H_2O_2.

[a]Values are mean ± SD of minutes after introduction of H_2O_2.

[b]Minutes after exposure to H_2O_2 when a change from preexposure diastolic and/or systolic $[Ca^{2+}]_i$ level was evident for 10 consecutive contractions.

[c]Minutes after exposure to H_2O_2 when electrical stimulation of cardiac myocytes failed to result in $[Ca^{2+}]_i$ transients.

[d]Minutes after exposure to H_2O_2 when basal $[Ca^{2+}]_i$ level in cardiac myocyte exceeded peak systolic $[Ca^{2+}]_i$ obtained prior to exposure.

[e]Values are significantly different from values obtained from cardiac myocytes treated with 100 μM H_2O_2 (ANOVA, $p < .01$).

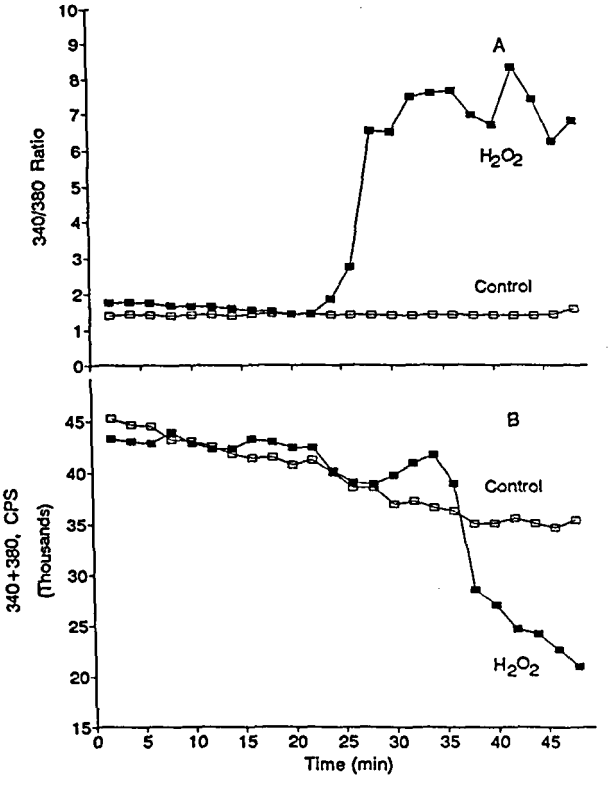

FIGURE 6. Increase in cardiac myocyte $[Ca^{2+}]_i$ and loss of sarcolemmal membrane integrity induced by 100 μM H_2O_2. (A) The emission ratio from excitation of fura-2 at 340/380 nm was monitored as an index of $[Ca^{2+}]_i$. In an untreated control cell, $[Ca^{2+}]_i$ remains constant during a 50-min suffusion, while a cell treated with 100 μM H_2O_2 exhibits a dramatic increase in $[Ca^{2+}]_i$ after 24 min of exposure. (B) The sum of emission counts per second from excitation of fura-2 at 340 and 380 nm was used as index of total fura-2 in the cell. In the same untreated control cell, gradual loss of fura-2 during 50 min of suffusion is evident. In the cell treated with 100 μM H_2O_2, there is a precipitous drop in total fura-2 at 34 min, indicating a loss of sarcolemmal membrane integrity. The effect of H_2O_2 illustrated here in a single cell is representative of three experiments.

Effect of Halocarbons and H_2O_2 on $[Ca^{2+}]_i$

Following a 30-min equilibration period, cardiac myocytes were suffused with HBSS containing halocarbons introduced into the buffer as DMSO aliquots. Figure 7 shows compressed 10-s data sets collected at 2-min intervals during exposure regimens identified on the figure. Suffusion of myocytes with 100 μM H_2O_2 caused a gradual decrease in $[Ca^{2+}]_i$ transients (Fig. 7A). After 20 min, a marked increase in $[Ca^{2+}]_i$, occurred that exceeded the systolic levels in preexposed cells ($[Ca^{2+}]_i$ overload). Reduction of $[Ca^{2+}]_i$, was evident within 2 min of exposure to TCE, and reduced transients shown in Fig. 7B were acquired within 4 min of initiation of exposure (see Hoffmann et al., 1992, for details of this effect). Comparable reductions in transients to

that shown for 1 m*M* TCE were produced by exposure of myocytes to 1 m*M* HAL or CT. Amplitudes of $[Ca^{2+}]_i$ transients in the presence of HC were 69 ± 7% (TCE), 66 ± 9% (CT), and 68 ± 7% (HAL) (mean ± SD, $n = 5$) of preexposed control values. The TCE, HAL, or CT did not significantly affect the temporal sequence of changes in $[Ca^{2+}]_i$ induced by 20 or 100 μM H_2O_2 (Table 1).

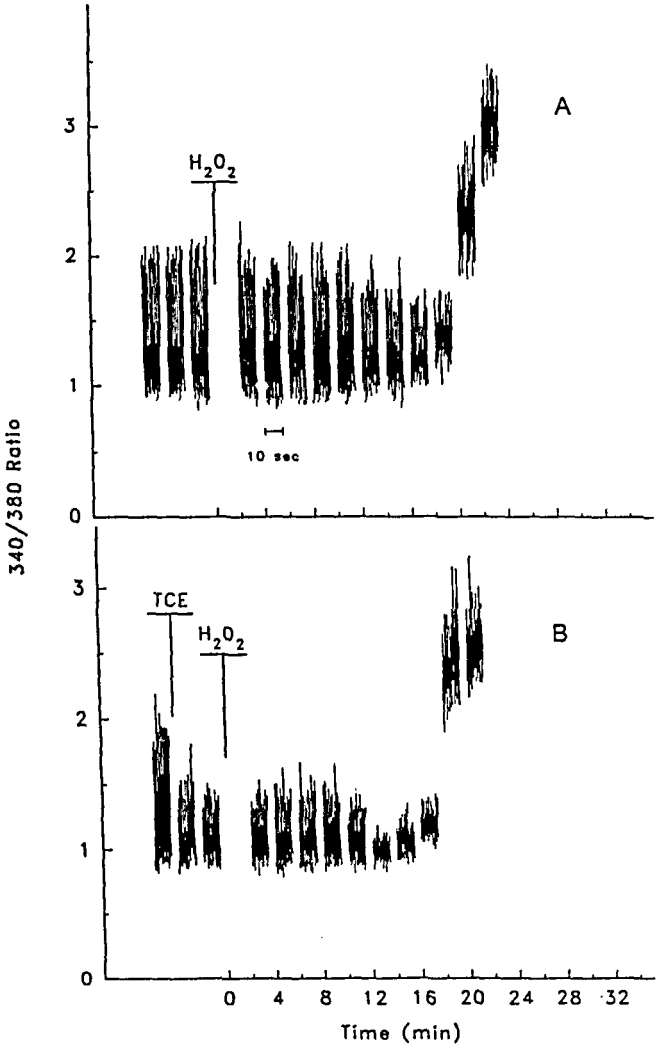

FIGURE 7. Complete $[Ca^{2+}]_i$ record (emission ratio from 340/380 nm excitation) of cardiac myocytes exposed to 100 μM H_2O_2 in (A) absence or (B) presence of 1 m*M* TCE. $[Ca^{2+}]_i$ was measured for 10 s every two min until $[Ca^{2+}]_i$ overload occurred (basal $[Ca^{2+}]_i$ exceeded peak systolic $[Ca^{2+}]_i$). TCE did not affect the temporal sequence of events resulting from exposure to 100 μM H_2O_2. A summary of these and other experiments is presented in Table 1.

DISCUSSION

H_2O_2-Induced Oxidative Injury in Cardiac Myocytes

H_2O_2-induced oxidative stress and subsequent injury of isolated cardiac myocytes observed in this study are consistent with previous reports. Janero et al. (1991) reported that 50–1000 μM H_2O_2 caused significant LDH leakage, TBARS release, ATP depletion, and myocyte mortality. These authors established 50 μM H_2O_2 as a threshold concentration, which is comparable to the effective concentration in our study, as is the temporal sequence of H_2O_2-induced events of 10–60 min. Deferoxamine was effective in protecting cells from H_2O_2-induced cytotoxicity and lipid peroxidation, which implicates Fenton chemistry in the iron-catalyzed metabolism of H_2O_2 to the highly reactive $^{\bullet}OH$ (Takemura et al., 1992; Zager and Foerder, 1992). The production of $^{\bullet}OH$ in cardiac myocytes treated with H_2O_2 has been confirmed with electron paramagnetic resonance (EPR) spectroscopy (Josephson et al., 1991).

In the present study, $[Ca^{2+}]_i$ overload occurred nearly 10 min prior to loss of sarcolemmal membrane integrity, presumably resulting from lipid peroxidation and made apparent by loss of fura-2 (Fig. 6). Myocardial ATP depletion has been proposed as a contributing factor in $[Ca^{2+}]_i$ overload (Janero et al., 1991). Depletion of ATP would reduce ATP-dependent calcium transport and lead to $[Ca^{2+}]_i$ overload. Josephson et al. (1991), however, reported that $[Ca^{2+}]_i$ overload precedes H_2O_2-induced ATP depletion in cardiac myocytes. Even if this is the case, $[Ca^{2+}]_i$ overload induced by H_2O_2 has also been attributed to the direct inhibition of ATP-dependent calcium transport by the plasma membrane and sarcoplasmic reticulum of coronary artery (Grover et al., 1992). In contrast, Beresewicz and Horackova (1991) reported that exposure of myocytes to 30 μM H_2O_2 resulted in $[Ca^{2+}]_i$ overload within 10 min of exposure. No change in peak inward Ca^{2+} current was found, and it was postulated that the $[Ca^{2+}]_i$ overload stemmed from Ca^{2+} loading of the sarcoplasmic reticulum as a result of increased sarcolemmal Na^+–Ca^{2+} exchange. Although our findings do not specifically address the source of the $[Ca^{2+}]_i$ overload, they are consistent with reports distinguishing $[Ca^{2+}]_i$ overload as a prior and separate event from nonspecific membrane damage caused by H_2O_2 (Janero et al., 1991; Josephson et al., 1991). Interestingly, Burton et al. (1990) reported a similar pattern of $[Ca^{2+}]_i$ overload in neonatal rat cardiac myocytes exposed to a xanthine-xanthine oxidase catalyzed free radical generating system. Presumably, H_2O_2 was produced by dismutation of the superoxide radical ($O_2^{\bullet-}$), which led to the formation of the $^{\bullet}OH$ and subsequent effects.

Effects of Halocarbons on Cardiac Myocytes

We have previously demonstrated that 1 mM TCE, CT, or HAL significantly inhibits contractility and intercellular communication in cardiac myocytes obtained from neonatal rats (Toraason et al., 1990, 1992). We have also

found that this concentration of TCE and CT significantly reduces $[Ca^{2+}]_i$ transients in electrically paced cardiac myocytes (Toraason and Breitenstein, 1991; Hoffmann et al., 1992). Furthermore, several studies have demonstrated that comparable concentrations of the anesthetic agents HAL, isoflurane, and enflurane inhibit sarcolemmal calcium entry and ATP-dependent calcium transport activity of sarcoplasmic reticulum in cardiac myocytes (Nelson and Sweo, 1988; Wheeler et al., 1988; Katsuoka et al., 1989; Eskinder et al., 1991). Therefore, concentrations used in this study were considered adequate. Despite the marked effects of TCE, CT, and HAL on the function of isolated cardiac myocytes, these compounds did not produce lipid peroxidation at the concentrations employed (1–4 m*M*).

Halocarbons Are Without Effect on H_2O_2-Induced Oxidative Injury

In vivo investigations and isolated perfused heart studies have reported that HAL protects, exacerbates, or is without effect on ischemia-reperfusion injury to the myocardium (Spahn et al., 1992). Such studies are complicated by the various protocols for inducing oxidative stress and the multitude of effects HAL has on the central nervous system, the vasculature, and the myocardium. The objective of the present study was to focus on the oxygen radical-mediated aspect of ischemia-reperfusion injury and examine cellular events devoid of systemic influence. The premise for this investigation was based on recent reports that halocarbons enhance oxidative injury in vascular endothelial and smooth muscle cells (Tse et al., 1990; Shayevitz et al., 1990, 1991). Oxidative injury in these studies was induced by activated neutrophils, Fe^{2+}, Fe^{3+}, and H_2O_2. Effects include increased cytotoxicity, lipid peroxidation, and elevated $[Ca^{2+}]_i$. These findings made it reasonable to postulate that an additive or synergistic effect of halocarbons and H_2O_2-induced injury would occur in isolated cardiac myocytes. The results of the present study, however, do not support this hypothesis. H_2O_2 concentrations employed in the present study ranged from the highly toxic 200 μM to the marginally toxic 20 μM, which in most cases caused $[Ca^{2+}]_i$ overload only after continuous suffusion for more than 30 min, but virtually no increase in TBARS release or LDH leakage during a 1-h exposure. Shayevitz et al. (1991) used H_2O_2 concentrations as high as 1 m*M* to demonstrate the effects of HAL, isoflurane, and enflurane on H_2O_2-induced reduction of plating efficiency—a much more sensitive assay than acute LDH leakage, which is an indication of gross damage. In the present study, 200 μM H_2O_2 resulted in a 50% loss of total LDH during 1 h, suggesting that the endothelial cells are resistant to H_2O_2 relative to cardiac myocytes. This relative resistance of endothelial cells was further demonstrated by the finding that 750 μM H_2O_2 increased $[Ca^{2+}]_i$ in endothelial cells only in the presence of HAL (Shayevitz et al., 1990). However, cardiac myocytes can also be resistant to H_2O_2. Josephson et al. (1991), in their investigation of adult rat cardiac myocytes discussed earlier, employed 1 and 10 m*M* H_2O_2. Nonetheless, the temporal

sequence of events including $[Ca^{2+}]_i$ overload was comparable to that observed in the present study with 50–200 μM H_2O_2. Rather than employ excessively high concentrations of oxidant, we limited H_2O_2 concentrations to those that were highly effective in inducing an oxidative injury and at the threshold for causing cell death.

The absence of an additive or a synergistic effect of H_2O_2 and halocarbons in neonatal rat cardiac myocytes suggests that findings with endothelial and smooth muscle cells may be specific to these cells types. Tse et al. (1990), however, argued against cell specificity based on observations that comparable halocarbon-oxidant synergistic injury occurred in both endothelial and vascular smooth muscle cells. They proposed that the synergistic interaction was cell independent and due to the lipophilicity of the halocarbons amplifying propagation of iron-induced lipid peroxidation in the membrane. Although the present study focused on H_2O_2, we also found that TCE, CT, or HAL did not enhance $FeSO_4$-induced lipid peroxidation or cytotoxicity in cardiac myocytes (M. Toraason, unpublished observation). The concept of nonspecific membrane effect is also consistent with the observation that preincubation and then removal of HAL just prior to introduction of H_2O_2 did not result in an enhancement of H_2O_2-induced oxidative injury in endothelial cells (Shayevitz et al., 1991). Halothane had to be present during H_2O_2 exposure to produce the synergistic effects. Furthermore, a nonspecific membrane effect of halocarbons appears to be responsible for our observations of immediate and reversible effects of halocarbons on intercellular communication, contractility, and $[Ca^{2+}]_i$ transients in cardiac myocytes (Toraason et al., 1990, 1992; Hoffmann et al., 1992). Differences in cell sensitivity due to variation in halocarbon metabolism also seem unlikely, since halocarbons alone do not induce significant lipid peroxidation in cardiac myocytes (present results), or in arterial endothelial or aortic smooth muscle cells (Tse et al., 1990). This, of course, contrasts with the metabolism of halocarbons to free radical intermediates and subsequent lipid peroxidation that occur in hepatic preparations (Cheeseman et al., 1985).

If cell specificity is discounted, and concentrations of H_2O_2 and halocarbons are independently effective, then the combinations of treatments utilized with cardiac myocytes in the present study may have been inadequate for replicating the findings of Shayevitz et al. (1990, 1991) and Tse et al. (1990) with endothelial and smooth muscle cells. Both groups reported concentration-dependent effects including noneffective combinations. However, we employed a wide range of independently effective concentrations of H_2O_2 and halocarbon and observed no interaction. Extending this investigation through utilization of greater concentrations of oxidant and/or halocarbon for the purpose of obtaining a positive interaction would have yielded results with questionable in vivo relevance. Therefore, present results demonstrate that 1–4 mM TCE, 1 mM HAL, or 1 mM CT in neonatal rat cardiac myocytes does not enhance oxidative injury induced by 20–200 μM H_2O_2.

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