

Cerium Chloride as a Histochemical Marker of Hydrogen Peroxide in Reperfused Ischemic Hearts

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M. SHLAFFER, K. BROSAMER, J. R. FORDER, R. H. SIMON, P. A. WARD AND C. M. GRUM. Cerium Chloride as a Histochemical Marker of Hydrogen Peroxide in Reperfused Ischemic Hearts. *Journal of Molecular and Cellular Cardiology* (1990) 22, 83-97. Hydrogen peroxide (H_2O_2) has been implicated in cardiac damage due to ischemia and reperfusion. We adapted an electron microscopic, histochemical method for demonstrating H_2O_2 produced by isolated cells to isolated, buffer-perfused rabbit hearts. The method involves formation of an electron-dense precipitate when H_2O_2 reacts with cerium chloride ($CeCl_3$). We perfused hearts retrograde via the aorta with well-oxygenated bicarbonate-buffered solution, followed by one in which bicarbonate was replaced with imidazole (IPSS) to prevent precipitation of bicarbonate and $CeCl_3$. Some hearts were made globally ischemic (30 min, $37^\circ C$), reperfused 5 min with well-oxygenated IPSS containing 1 mM $CeCl_3$, then processed for electron microscopy. Others were perfused with IPSS containing catalase (300 U/ml) or albumin before ischemia and upon reperfusion, followed by $CeCl_3$ administration. Nonischemic control hearts perfused with IPSS (\pm catalase) were also studied. Electron micrographs were assessed visually and by computer for precipitate localization and amount. There was abundant precipitate on the luminal face of the coronary vascular endothelium in ischemic-reperfused, cerium-treated hearts, including those treated with albumin. There was significantly less in reperfused catalase-treated or nonischemic control hearts. X-ray microbeam analysis of the endothelial precipitate indicated the presence of Ce. This appears to be the first visual demonstration of a $CeCl_3$ - H_2O_2 -dependent reaction product in intact isolated ischemic hearts. The data indicate that at the time of reperfusion some H_2O_2 is accessible to the vascular space, and that its amount can be reduced by perfused catalase. Further modifications this technique may be useful for assessing the sites and pathways by which H_2O_2 is generated by hearts or other buffer-perfused organs subjected to stresses such as ischemia or hypoxia.

KEY WORDS: Cerium; Cytochemistry; Electron microscopy; Hydrogen peroxide; Ischemia; Oxygen radicals; Reperfusion.

Introduction

Many studies of blood- and buffer-perfused hearts subjected to ischemia or hypoxia have led to the conclusion that cytotoxic oxygen metabolites, including H_2O_2 , are produced, especially upon reperfusion or reoxygenation. Such oxidants are thought to contribute to cardiac damage. Most data to support these general hypotheses have been obtained from experiments showing that exogenous interventions that reduce oxidant generation or reactivity can reduce contractile or biochemical manifestations of damage. Electron spin resonance and biochemical techniques applied to intact hearts provide more direct support

for free radical generation, but they still make it difficult to localize sites of oxidant production or the sites that diffusible oxidants such as H_2O_2 might reach.

Previously we tested the ability of superoxide dismutase (SOD), catalase, allopurinol, or deferoxamine, to reduce creatine kinase loss from hypoxic/reoxygenated rabbit hearts (Myers *et al.*, 1985), or contractile dysfunction in globally ischemic and reperfused hearts (Shlafer *et al.*, 1982a, b; Myers *et al.*, 1986). Of the interventions tested singly by us (Myers *et al.*, 1985, 1986), only catalase gave some evidence of protection in both models, so we postulated that H_2O_2 played a role in the

damage process. Catalase is a large protein. When perfused, it should be restricted mainly to the vascular space and should be accessible there to the H_2O_2 which it degrades. We now report visual evidence of H_2O_2 formed in isolated, buffer-perfused hearts, and that it is present at such catalase-accessible sites.

Cerium chloride was used as the histochemical probe because it reacts with H_2O_2 to form an electron-dense precipitate (Briggs *et al.*, 1975), and this reaction can be inhibited by catalase. An isolated, buffer-perfused rabbit heart model was used to eliminate circulating neutrophils (Lucchesi and Mullane, 1986) and xanthine oxidase (Grum *et al.*, 1986, 1987; Downey *et al.*, 1987) as potential sources of H_2O_2 .

Materials and Methods

The overall protocol from heart perfusion to postfixation processing is summarized in Figure 1. We used healthy New Zealand White rabbits weighing approximately 1.5 kg. Animal procurement, care, and use followed all applicable guidelines. The protocol for heart isolation, perfusion, and instrumentation has been described previously (Myers *et al.*, 1986); modifications are noted below. Hearts were initially perfused retrogradely via the aorta for 20 min with a phosphate-free bicarbonate-buffered physiologic saline solution (BPSS; 37°C, 75 mm Hg perfusion pressure, equilibrated via direct bubbling with 95% O_2 + 5% CO_2 through fritted glass). This was to ensure that baseline coronary flow and contractile function (measured with a left intraventricular balloon) were comparable to values obtained in previous studies in which this perfusate was used (Schlafer *et al.*, 1982a, b; Myers *et al.*, 1985, 1986; Grum *et al.*, 1987; Schlafer *et al.*, 1982a, b; Myers *et al.*, 1985, 1986; Grum *et al.*, 1987). The composition of the BPSS was (mM): NaCl 118, KCl 4, NaHCO_3 25, MgCl_2 1.2, CaCl_2 2.4, D-glucose 11.

Since the BPSS contained bicarbonate, which precipitates with Ce, the perfusate was switched to one in which bicarbonate was omitted, and 15 mM imidazole (IPSS) was used for buffering. Sodium concentration and osmolality were restored by adding an additional 25 mmol/l NaCl. Imidazole was selec-

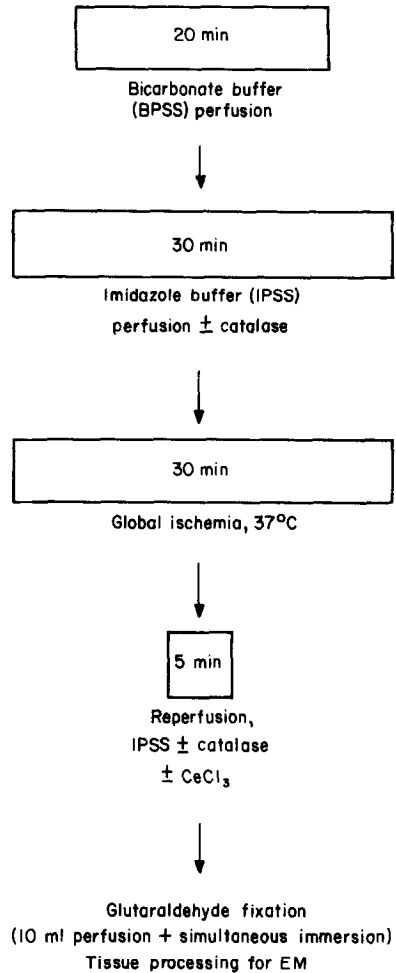


FIGURE 1. Schematic diagram of experimental protocol, from perfusion to fixation.

ted based on the results of contractile studies in nonischemic hearts which revealed that, compared with several other buffers (e.g. *N*-2-hydroxyethyl piperazine *N*-2-ethanesulfonic acid; Tris[hydroxymethyl] aminomethane), it caused the least alteration of contractile function and coronary flow upon switching from BPSS, and it was chemically compatible with CeCl_3 . The pH of the IPSS was adjusted to 7.4 with HCl, and the solution was bubbled with pure O_2 . Perfusion pressure, which rose slightly upon the switch from BPSS to IPSS (see Results), was readjusted to 75 mm Hg by reducing coronary flow with the perfusion pump.

Ischemia studies

Equilibration continued for another 30 min with IPSS perfusion, at which time the perfusion pump was stopped to cause 30 min of complete, normothermic global ischemia. Five hearts were studied in this manner.

Since the anticipated histochemical product was due to H₂O₂, for five hearts we added 300 U/ml of catalase (thymol-free, from bovine liver; Sigma) to the IPSS that was perfused before ischemia. The hypothesis was that catalase would metabolize H₂O₂ and thereby prevent or at least significantly reduce the ability of H₂O₂ to react with cerium.

Another group of hearts was perfused before ischemia with IPSS to which we added bovine serum albumin (0.027 mg/ml; weight equal to that used in catalase experiments; Fraction V, Sigma; $n = 5$). Perfusate gas tension was maintained as described below for catalase.

We studied another group of ischemic hearts ($n = 5$) to which cerium was not administered.

When either catalase or albumin was used, the perfusate was first pH-adjusted and equilibrated with O₂ by direct bubbling through fritted glass for at least 30 min. Direct gassing was stopped, protein was added with continual stirring, and gas tension was maintained by placing a tight-fitting inverted funnel and plastic wrap over the container, then passing O₂ through the funnel stem. This prevented frothing and potential inactivation or loss of the added protein. Solid CeCl₃ was added slowly to the oxygenated protein-containing perfusate with continual stirring immediately before use.

Nonischemic controls

Two other groups of hearts ($n = 5$ each) were nonischemic controls that were perfused for a time equal to that of the ischemia experiments. One of these had catalase in the perfusate, the other did not. We have perfused nonischemic hearts for up to 1.5 h with the IPSS, and have noted no significant functional alteration different from that normally encountered with prolonged perfusion with bicarbonate buffer. In addition, pilot studies showed that IPSS had no unique effect on functional recovery following global ischemia,

compared with the results obtained with bicarbonate-buffered perfusate.

Timing of cerium administration

At the end of ischemia (or after 30 min IPSS perfusion in controls) most hearts were reperfused for 5 min with oxygenated IPSS containing 1 mM CeCl₃. Thus cerium administration occurred at a time when oxidant production was expected to be great. When catalase or albumin was added to the pre-ischemic IPSS they were included in the reperfusate at the same concentration. Cerium chloride perfusion was limited to 5 min because cerium caused negative inotropy and, if perfusion was continued beyond that time, ventricular contracture occurred (see Results).

Tissue fixation and preparation

Hearts were fixed by retrograde aortic perfusion with approximately 10 ml of buffered glutaraldehyde (4% in 0.1 M Na cacodylate, pH 7.5, plus 5% w/v sucrose) to remove residual intravascular perfusate and begin intravascular fixation. They were immersed simultaneously in approximately the same volume of the same fixative to begin homogeneous fixation of the epicardium. Hearts were then removed from the cannula and samples of left ventricular papillary muscles and the free wall were removed with a scalpel. They were quickly minced into pieces of approximately 1 mm³ or less, and fixed at room temperature with gentle rotation for 90 min. Samples were rinsed three times in sucrose-cacodylate buffer (5% sucrose, 0.1 M sodium cacodylate, pH 6) to remove precipitate formed artifactually and nonspecifically during incubation (Briggs *et al.*, 1975). Samples were then rinsed twice with sucrose-cacodylate buffer at pH 7.5, and stored overnight at 4°C in this solution. Subsequent processing involved 2% OsO₄ postfixation at 4°C, then room temperature ethanol dehydration, propylene oxide infiltration, and embedding in EMBED 812® (Electron Microscopy Sciences, Ft. Washington, PA).

Gold or silver sections from the blocks (see sampling details below) were mounted on copper grids. Most were counterstained with uranyl acetate and lead citrate according to

standard protocols. Sections were examined and photographed in a JEOL JEM 1200 EX or Zeiss EM10-A transmission electron microscope, with a typical accelerating voltage of 60 keV. Most exposures were taken at 15 000 \times to achieve some consistency for later analysis of prints. Others were taken at 2000 to 3000 \times to show larger areas of myocardium and endothelial cells for better assessment of the homogeneity of treatment-related effects.

Microbeam analysis

Thin sections (some counterstained, others not) from hearts in all groups were carbon-coated and subjected to electron microbeam (X-ray) analysis in a JEOL 2000FX electron microscope equipped with a Tracor TN5500 Series II X-ray analyzer. This method was used to assess ion content at sites of obvious precipitation on the luminal face of the endothelium (see Results), as well as at other sites where there was less or no precipitate evident from visual inspection. Accelerating voltages of approximately 200 keV, and acquisition times of 500 to 2000 s, were found to optimize signal acquisition yet minimize sample destruction or movement. At the nominal microscope magnification we used, 15 000 \times , the probe beam focused on an area having an approximate diameter of 200 nm.

Sampling, assessment of micrographs, and data analysis

Eight to 10 blocks were prepared from each heart. Two to five grids prepared from each block were randomly selected, examined, and photographed. Four to five photomicrographs were taken from each grid. The person responsible for electron microscopy and photography (K.B.) sequentially numbered prints of all negatives and did not participate in their subsequent evaluation.

Prints were evaluated by the remaining authors, and by other persons familiar with cardiac ultrastructure, using a semiquantitative score to assess the precipitate. Initial inspection of prints from both counterstained and unstained sections readily showed that the majority of obvious precipitation was at the luminal face of the endothelium, so further assessment emphasized that site. Scores

(“visual scores”) were assigned as follows: 0 = no precipitate, clean and/or normal; 1 = minor (patchy or diffuse) precipitate, mostly clean or normal; 2 = moderate (patchy or diffuse) precipitate; 3 = heavy, diffuse or localized precipitate, few normal areas; 4 = heavy and widespread or confluent precipitate, no clean areas. The grading scale was explained to each investigator, and a micrograph from each grade (not used for computation of final data) was shown for orientation, before all other micrographs were graded in blinded fashion. Grades from each evaluator, and for each micrograph, were averaged.

For corroborative quantitative data we used computer-based morphometry to analyze 53 electron micrographs selected randomly from three Ce-treated groups: ischemia-reperfusion; ischemia-reperfusion plus catalase; and nonischemic controls without catalase. Four micrographs of ischemic-reperfused hearts that were not perfused with CeCl_3 were also analyzed. Images were digitized from contact prints (all having a magnification of approximately 15 000 \times) using a video camera and a Zeiss SEM-IPS[®] Image Analyzer. Images were shade-corrected and segmented based on gray level to separate the material of interest (the precipitate) from the background. Once adjusted for the original image magnification, the analyzer calculated the length of the luminal face of the endothelium (in μm), the area occupied by precipitate (in μm^2), and the area/length ratio. This ratio estimates the hypothetical depth of precipitate (in μm) if it were distributed over the luminal face in uniform thickness. The person who performed the image analysis was unaware of the groups to which the micrographs belonged.

Visual and computer-derived scores for each micrograph in each group were averaged (and reported as ± 1 s.e.m.), tabulated, and subjected to analysis of variance. A regression coefficient was calculated to assess for a relationship between data obtained with visual vs. computer-derived analysis. For one analysis, computer-derived scores were used as the independent variable, visual scores as the dependent variable. The variables were reversed for the second analysis, which gave statistically identical results. We used Scheffe's *F*-test, which accounts for multiple-group com-

parisons, to compare data between the non-ischemic and ischemic-reperfused groups. Unless noted otherwise all statistics were obtained using this test.

Treatment-related effects on overall myocyte or endothelial morphology were not analyzed quantitatively.

Results

Contractile effects of cerium perfusion

Figure 2 shows the effects on a nonischemic heart of switching from BPSS to IPSS, and subsequent perfusion with CeCl₃. The buffer switch quickly caused modest bradycardia, a slight fall of peak left ventricular pressure development, and a slight rise of perfusion pressure. Once perfusion pressure was lowered manually by reducing flow, contractile parameters remained constant throughout the duration of IPSS perfusion. Upon perfusion with 1 mM CeCl₃ there was a prompt negative inotropic response and a fall of perfusion pressure. The surface electrogram of the heart (measured in several separate experiments;

data not shown) persisted, indicative of excitation-contraction uncoupling. With continued CeCl₃ perfusion left ventricular end-diastolic pressure rose. If coronary (perfusion pump) flow was left alone, perfusion pressure rose also. As noted above, hearts were fixed after only 5 min of CeCl₃ perfusion, well before significant rises of end-diastolic pressure and frank ventricular contracture occurred.

Electron microscopic results

The general morphology of sections of ischemic-reperfused hearts showed changes typical of ischemic damage, but we did not conduct a formal morphometric evaluation of this. Such changes included clarification of the endothelium and/or variable amounts of separation from the myocytes beneath. Varying degrees of sarcoplasmic clarification, myofibrillar contraction and occasional contracture, and swelling of the sarcotubular network and mitochondria, were observed. Formed elements of the blood were not found in any of

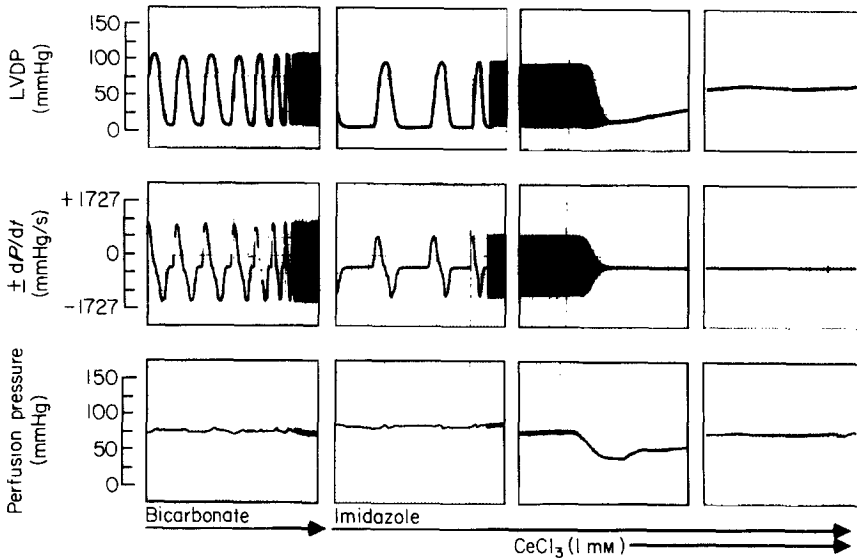


FIGURE 2. Effects of switching from bicarbonate-buffered PSS to one containing imidazole (IPSS), and administration of CeCl₃. The panels show (top to bottom) left ventricular developed pressure (LVDP), its electronically derived first derivative (dP/dt), and perfusion pressure during constant-flow perfusion. Switching to IPSS (second vertical panels from left) caused a slight fall of LVDP and dP/dt, and a rise of perfusion pressure that was corrected by slight lowering of the perfusion pump rate. Administering CeCl₃ caused negative inotropy, excitation-contraction uncoupling (electrogram data not shown) and, over 15 min (last vertical panels on right), contracture. Recorder speed was 25 mm/min except for the left-hand portions of the first and second vertical panels, which was 25 mm/s.

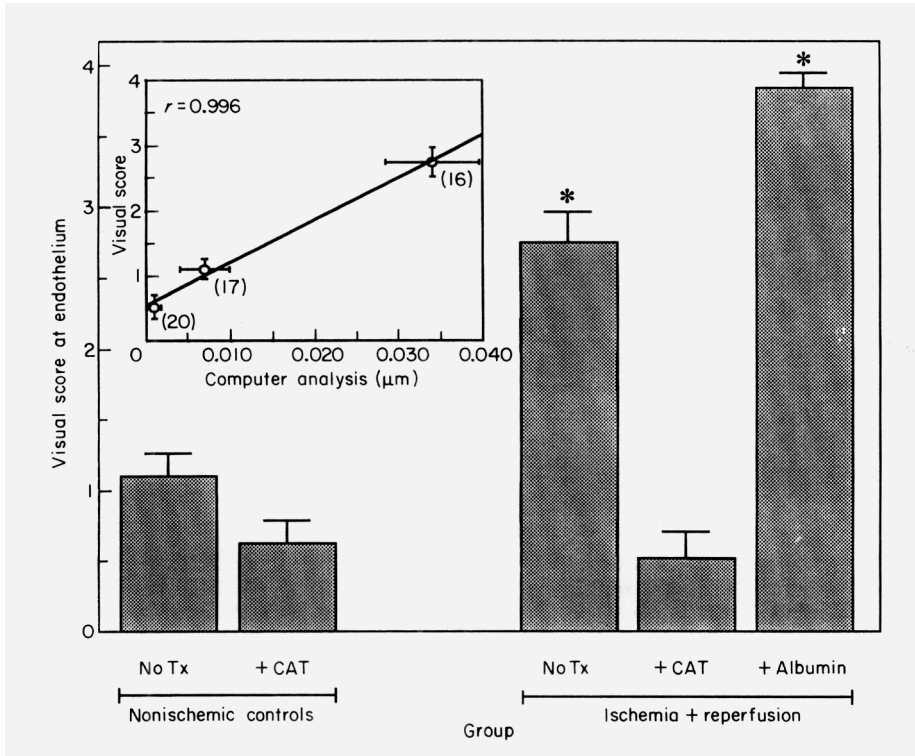


FIGURE 3. Graph summarizing mean visual scores (± 1 s.e.m.) for endothelial cerium precipitate in nonischemic and ischemic-reperfused hearts. Asterisk indicates significant difference ($P < 0.05$) from value for nonischemic control. Inset: Computerized morphometric assessment of electron micrographs from three CeCl_3 -treated groups [ischemia + reperfusion with catalase (CAT; 20 micrographs, indicated in parentheses) or without catalase pretreatment (No Tx; 16 micrographs); nonischemic controls (17 micrographs)] correlated significantly ($r = 0.996$; $P < 0.0001$) with visual scores for the same photos. Abscissa values are in μm , the hypothetical depth of the precipitate if it were uniformly distributed over the luminal surface of the endothelium in the field (see text). Values are arithmetic means ± 1 s.e.m.

the microscope fields, whether from nonischemic or ischemic-reperfused hearts.

When data using the two methods for grading endothelial-luminal face precipitate were compared, we obtained a correlation coefficient of 0.996 ($P < 0.001$; Figure 3 inset), indicating excellent agreement between methods. We found statistically significant differences ($P < 0.001$ based on analysis of variance of all groups' data; Figure 3) in the amount of endothelial precipitation in the various groups, regardless of the grading method used. The results were very consistent for each grid, block, heart, and evaluator, and did not differ statistically when data from counterstained or unstained sections were compared.

Figure 4 shows three histochemical features seen in hearts reperfused with CeCl_3 but with-

out catalase pretreatment. Routinely there was dense and abundant precipitation (group mean visual score: 2.75 ± 0.22 ; computer: $0.0342 \pm 0.056 \mu\text{m}$) almost exclusively at the luminal face of endothelial cells. Occasionally precipitate of comparable density was found in what appeared to be pinocytotic vesicles in the endothelium, and in what appeared to be tight junctions between endothelial cells. When the endothelial cell was closely apposed to the sarcolemma, we could not detect material of equivalent density or appearance at other sites, whether in high or low power fields or in sections that were or were not counterstained.

Electron microbeam analysis of precipitate at the luminal endothelial sites (Fig. 5) revealed three peaks at 4.8, 5.2, and 5.6 keV. These correspond to Ce (Bruijn *et al.*, 1980).



FIGURE 4. Localization of precipitate in $CeCl_3$ -treated ischemic-reperfused hearts without catalase or albumin pretreatment (lead citrate and uranyl acetate counterstained). Precipitate was observed mainly on the luminal face of endothelial cells. Expected consequences of ischemic damage on myocyte morphology (e.g. mitochondrial swelling, myofibrillar derangements, sarcoplasmic clarification) are apparent. The mean visual endothelial (luminal face) precipitate score for this group was 2.75 ± 0.22 ($P \ll 0.05$ vs. nonischemic control group; Scheffe's F -test). For this and all other electron micrographs, the scale bar indicates true image magnification.

Spectral peaks for lead and uranium appeared with counterstained sections. When there was a visually distinct separation between the endothelium and the adjacent myocyte (Fig. 5), we occasionally observed additional electron-dense material over or on the ab-luminal face of the endothelium, as well as at the sarcolemmal glycocalyx. When present it was always visually smaller, more diffuse, and less dense than what was observed at the luminal endothelial face. These characteristics may explain why, when the X-ray beam was moved over these sites, Ce peaks could not be detected. Peaks for lead and uranium persisted. Thus, we cannot state definitively whether this material contained cerium.

The visual score for albumin-treated hearts (Fig. 6) was 3.84 ± 0.11 (computerized morphometry was not used for this group). This value was not significantly different ($P > 0.05$) from values for the ischemic-reperfused hearts that received no catalase. Microbeam

data were comparable for hearts in these two groups.

Mean precipitate scores in catalase-treated, ischemic-reperfused hearts (visual: 0.52 ± 0.19 ; computer: $0.0014 \pm 0.0008 \mu m$; Fig. 7) were significantly lower ($P < 0.0001$) than scores for the other ischemic-reperfused, cerium-treated groups. We found no evidence of additional fine granular material in the interstitial spaces, as seen in sections of hearts not treated with catalase.

The morphology of nonischemic control, cerium-treated hearts (Fig. 8) appeared characteristically normal and showed good fixation. Their ultrastructure did not differ from that of nonischemic controls that were not perfused with $CeCl_3$, suggesting that $CeCl_3$ caused no observable morphologic effects of its own during brief treatment. The luminal-endothelial precipitate scores for this group (visual: 1.10 ± 0.16 ; computer: $0.0067 \pm 0.0029 \mu m$; Fig. 8) were significantly

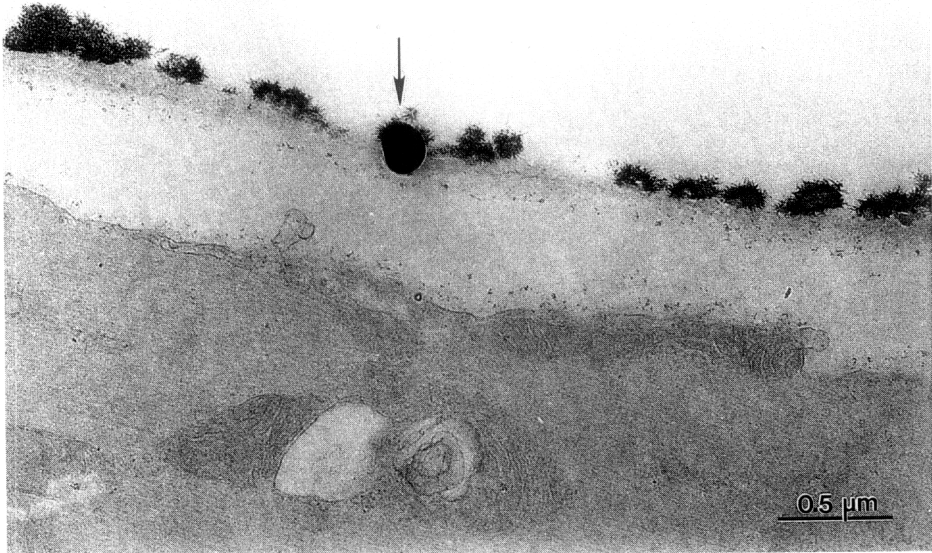
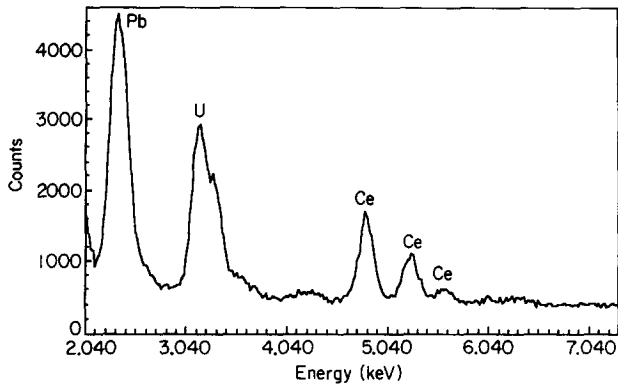


FIGURE 5. Electron micrograph from ischemic-reperfused CeCl_3 -treated heart (no catalase or albumin; from same group as shown in Fig. 4), and microbeam spectrum obtained on luminal face of endothelium (shown below). The sampling site for microbeam analysis is the dark spot marked with an arrow. The nominal diameter of this spot, 200 nm, and the acquisition time (784 s), are comparable to those used to evaluate other sites. Accelerating voltage of 200 keV and use of carbon-coated grid accounts for the decreased contrast. Note the fine electron-dense material on the abluminal face of the endothelium, and over the sarcolemmal basement membrane. It was less dense and more diffuse than that seen on the luminal endothelial face. With the maximum spectral acquisition times we could use without destroying the specimen or contaminating the microscope, we were unable to detect cerium (Ce) at these loci, or at other sites (e.g. inside myocytes) where no precipitate was apparent. Other abbreviations on spectrum: Pb, lead; U, uranium (used as counterstains).

less than those obtained in the nontreated and albumin-treated ischemic-reperfused groups ($P < 0.0001$). This score was not significantly different ($0.50 > P > 0.25$) from that for the catalase-treated reperfused hearts. Examination of the nonischemic controls also revealed

some fine and dispersed electron-dense material in the interstitial space, sometimes restricted to the basement membrane on the abluminal side of the endothelium and over the sarcolemma (Fig. 8, inset), suggesting that some cerium-peroxide reaction occurred.

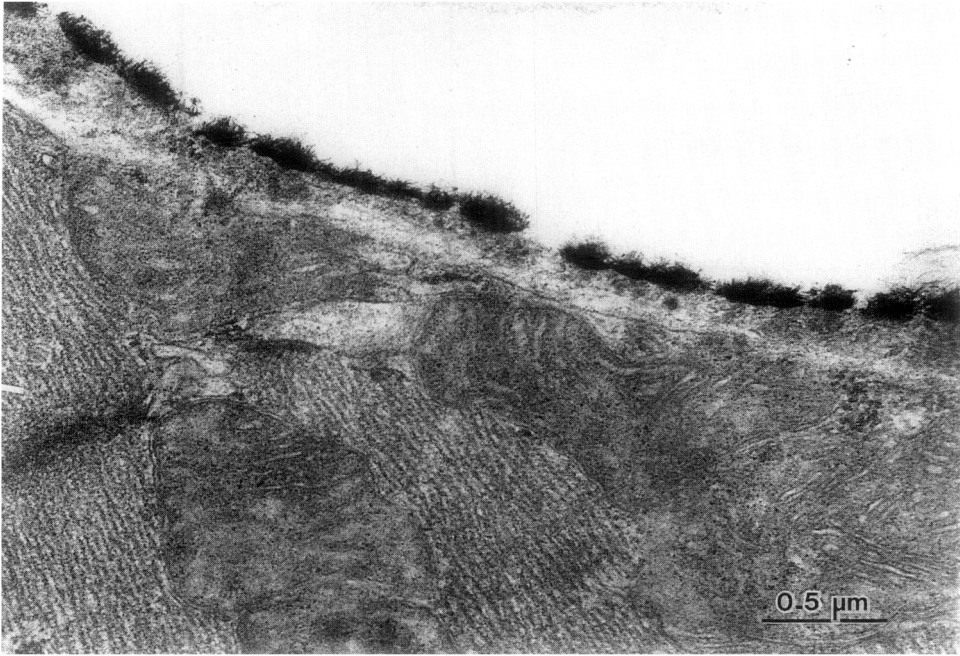


FIGURE 6. Electron micrograph of heart pre-treated with albumin (weight per ml equal to that of catalase) then reperused with CeCl₃. Albumin failed to reduce precipitate intensity or location compared with results obtained with catalase. The mean luminal endothelial precipitate score for this group was 3.84 ± 0.11 ($P > 0.05$ vs. ischemic control; $P \ll 0.05$ vs. nonischemic controls; Scheffe's F -test).

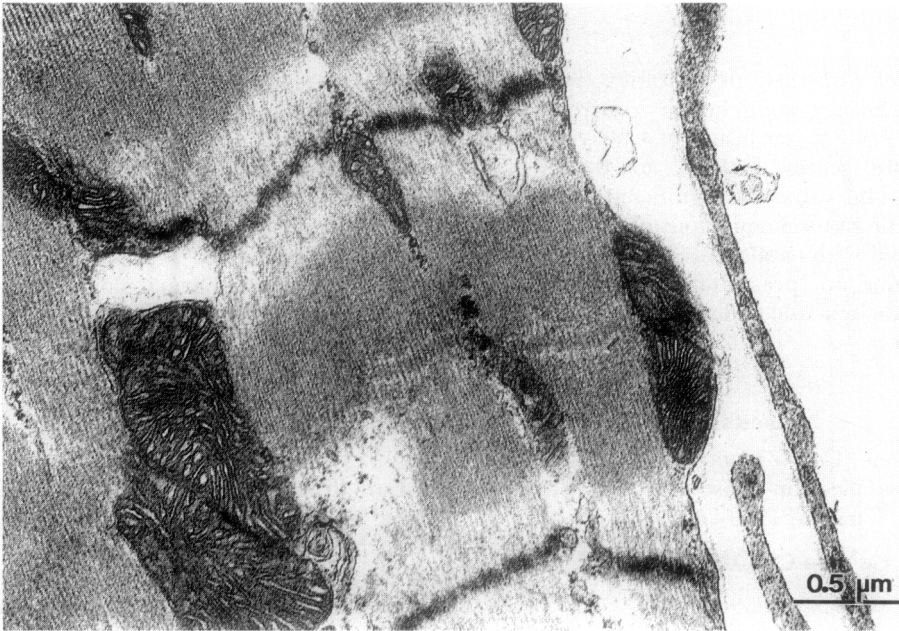


FIGURE 7. Pre-treating hearts with catalase (300 U/ml) significantly reduced and in some cases eliminated precipitate at the luminal face of the endothelium. It completely eliminated fine densities seen on the abluminal face and in the interstitial space [cf. Figs 5 and 8 (inset)]. The visual score in this micrograph is zero; the mean score for all micrographs in this group was 0.52 ± 0.19 ($P > 0.05$ vs. nonischemic control group; $P \ll 0.05$ vs. ischemic controls; Scheffe's F -test).

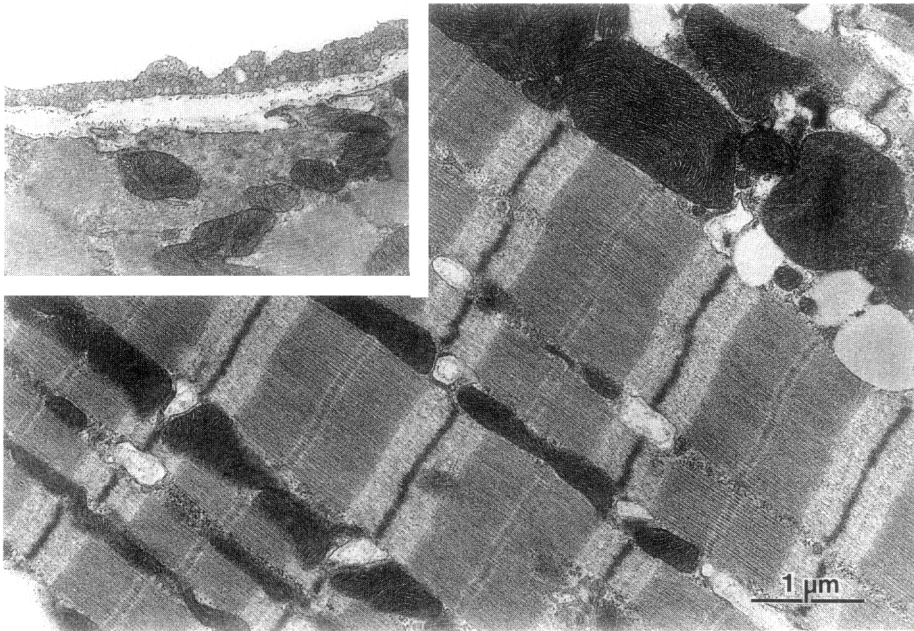


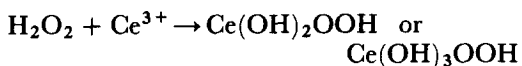
FIGURE 8. Myocytes and endothelium (inset) from nonischemic controls perfused with CeCl_3 , but without catalase pretreatment. The morphology is typical of well-fixed, nonischemic myocardial cells. The inset (same magnification as large micrograph) shows small amounts of fine diffuse electron-dense material on both the luminal and abluminal sides of the endothelium, as well as on the basement membrane over the sarcolemma (cf. Fig. 5). The mean precipitate score, assessed visually at the luminal face of the endothelium, was 1.10 ± 0.16 .

Consistent with this interpretation, we found that perfusing nonischemic controls with catalase prior to perfusion of CeCl_3 reduced precipitate scores further, to 0.63 ± 0.15 (Fig. 3). This value was significantly less than that of the nonischemic controls which were not treated with catalase.

We found no "precipitation" in hearts that were made ischemic and then reperfused without cerium.

Discussion

Cerous ions (Ce^{3+}) react with H_2O_2 to form one or two electron-dense products (Briggs *et al.*, 1975; Kuroda, 1989):



Through this reaction CeCl_3 has been used as a capture agent for the ultrastructural cytochemical demonstration or localization of H_2O_2 produced by such cells or tissues as

neutrophils (Briggs *et al.*, 1975; Butterick *et al.*, 1983; Vissers *et al.*, 1985), macrophages (Wozencraft *et al.*, 1985; Kuroda, 1986), thyroid (Labato and Briggs (1985), liver (Veenius and Wendelaar Bonga, 1979), lung (Warren *et al.*, 1989) and heart (Christie and Stoward, 1982; Vandeplassche *et al.*, 1989). These studies often use pharmacologic concentrations of exogenous substrates or inhibitors to stimulate or inhibit peroxide production, and rely on incubation of the biologic material in cerium-containing media.

Of the myocardial studies, Vandeplassche *et al.* (1989) incubated fixed, sectioned myocardium from regionally ischemic hearts in media containing CeCl_3 , with or without added NADH. Although they did not assess the effects of catalase on the cytochemical reaction, their data suggest that even after a modest ischemic insult mitochondrial NADH oxidase activity can produce H_2O_2 . Christie and Stoward (1982) incubated small pieces of myocardium in media containing CeCl_3 and a

variety of metabolic substrates and inhibitors, and demonstrated a putative H₂O₂-cerium reaction product thought to arise from an enzyme with the properties of diamine oxidase. Although they used unfixed tissue, their study was not done under conditions that preserve or allow measurement of cell function.

Our study differed methodologically in several ways from those cited above. We administered cerium by perfusion to intact isolated hearts that, up to the time of histochemical intervention and fixation, had some measurable level of function. The stimulus for increased H₂O₂ formation, reperfusion and concomitant reoxygenation following ischemia, was a pathophysiologically relevant one, not the administration of an exogenous chemical. Cerium administration was timed to coincide with reperfusion. Importantly, the method allowed us to show that a component of H₂O₂ which is produced (regardless of its sites or biochemical pathways of origin) gains access to the vascular compartment, which might be expected from data showing that perfused catalase can alter functional and biochemical consequences of ischemic or hypoxic myocardial damage (Myers *et al.*, 1985, 1986).

Several questions arise from this study. (1) Was the reaction product we observed both cerium- and H₂O₂-dependent, and (2) did its amount reflect the amount of H₂O₂ produced? (3) If H₂O₂ was involved, what are its sources? (4) What factors may have influenced precipitate localization? Finally (5), what are the biologic implications of the findings?

We cannot prove whether the precipitate arose from a direct reaction between Ce³⁺ and H₂O₂, but it is reasonable to postulate that it was, at least, both cerium- and peroxide-dependent. Electron microbeam data showed that the dense endothelial deposits we observed in hearts treated with CeCl₃ indeed contained cerium. No comparable deposits were observed in hearts not perfused with CeCl₃, but otherwise treated similarly. Thus, the densities were cerium-dependent.

We did not measure H₂O₂ directly, as with a biochemical technique, so we cannot prove that peroxide was formed. However the likelihood that H₂O₂ was produced by some

cell(s) within the heart is supported by the histochemical data plus other direct and indirect evidence (Christie and Stoward, 1982; Shlafer *et al.*, 1982a, b; Myers *et al.*, 1985, 1986).

The significantly greater amount of precipitate seen in ischemic-reperfused, cerium-treated hearts, indicates that ischemia and/or reperfusion increased the availability of a constituent in the histochemical reaction. Perfusate CeCl₃ concentration was constant in all experiments, and there is no reason to assume that any of the treatments used or interventions imposed would increase cerium reactivity. This suggests that more H₂O₂ was available, contributing to the greater amount of precipitate. The ability of submicromolar concentrations of catalase to reduce the amount of reaction product significantly also supports the involvement of H₂O₂. It is conceivable that reduced precipitation in the presence of catalase was due to nonspecific adsorption of cerium to the protein. However the well-known ability of catalase to quickly and effectively degrade H₂O₂ enzymatically, plus the inability of albumin to inhibit precipitate formation, argue against this alternative explanation. Catalase administration did not yield mean scores of zero at the endothelium, whether assessed in nonischemic hearts (mean score 0.62) or ischemic hearts (mean score 0.52). One interpretation of this is that a small component of the precipitate was either nonspecific, or represented precipitation between Ce³⁺ and phosphate, as discussed below.

The reaction product we measured probably underestimates the amount of H₂O₂ produced. Some of the H₂O₂ may have reacted with cellular constituents, or with O₂⁻ and Fe³⁺ to form OH[·], before it could react with CeCl₃. Another portion may have been degraded by endogenous catalase and glutathione peroxidase, whether in the myocyte or endothelium. Ischemia-induced partial loss or inactivation of myocyte glutathione peroxidase, with the potential concomitant reduction in the cell's ability to degrade H₂O₂, has been reported (*see* Shlafer *et al.*, 1987). Such diminished defenses may explain why we were able to detect any precipitate (H₂O₂) at all. The underestimate also probably reflects restricted access of cerium to H₂O₂, given the apparently limited ability of perfused cerium to

cross the sarcolemma. As discussed later, this may be especially true for myocyte-derived H_2O_2 .

Cerous ions also form an electron-dense precipitate with phosphate. This is the basis for using $CeCl_3$ in cytochemical studies of several enzymes that hydrolyze phosphate-containing substrates (Robinson and Karnovsky, 1983a, b; Recharadt and Hervonen, 1985; Dort *et al.*, 1987), including those found in the heart (Borgers *et al.*, 1971a, b; Schulze *et al.*, 1986). Thus a portion of the precipitate in nonischemic hearts might involve phosphate liberated during normal cell metabolism; the greater amount of precipitate in ischemic-reperfused hearts could reflect greater amounts of phosphate arising from cell damage, which in turn might be reduced by a protective intervention such as catalase. However the phosphate-liberating enzymes that have been studied with cerium are not inhibited by catalase, nor is there evidence that catalase inhibits the reaction between cerous ions and phosphate. This provides further support for the postulate that if the majority of precipitate we detected was not specifically one between Ce^{3+} and H_2O_2 , it was at least H_2O_2 -dependent. Studies of neutrophil 5'-nucleotidase activity indicate that the amount of cerium phosphate reaction product is proportional to enzyme activity in a soluble *in vitro* system, suggesting that precipitate density seen in cytochemical preparations also reflects enzyme activity (Robinson and Karnovsky, 1983a). This relationship may also apply to the interaction between Ce^{3+} and H_2O_2 . However a reaction between Ce^{3+} and phosphate, perhaps released through peroxide-independent processes, may account for the small amounts of precipitate seen in catalase-treated nonischemic or ischemic hearts.

It seems apparent from the current data that some H_2O_2 is present at the luminal face of the endothelium. The most difficult question to answer with the current data is from where the H_2O_2 arose. One possibility is that the endothelium is an important site of H_2O_2 generation in this model, based on the predominant loci of precipitate. Endothelial xanthine oxidase is not a likely H_2O_2 source (Jarasch *et al.*, 1981, 1986), since the rabbit heart lacks xanthine oxidase activity (Grum *et*

al., 1986, 1987; Downey *et al.*, 1987) at levels that are likely to be (patho)physiologically relevant and at substrate concentrations near the enzyme's K_m value (*see* Wajner and Harkness, 1989). Human myocardium is similar (Eddy *et al.*, 1987; Grum *et al.*, 1989). Moreover, we were unable to alter endothelial precipitation by pre-treating rabbit hearts with 1 mM allopurinol, a xanthine oxidase inhibitor, administered at times identical to those used here for catalase or albumin (unpub. obs.). Nevertheless the endothelial cell has several other biochemical pathways that could produce oxidants, including H_2O_2 (Hennig and Chow, 1988; Rubanyi, 1988; Halliwell, 1989), and they might account for the results. A technique involving cerium, plus the use of appropriate enzyme substrates and inhibitors method, may prove useful for evaluating them.

Another possibility is that the luminal face of the endothelium is merely the major site of reactivity between myocyte-derived H_2O_2 (e.g. from mitochondria Christie and Stoward, 1982; Schlafer *et al.*, 1987a, b) and perfused cerium. However the amount of precipitate we found over the sarcolemma, abluminal endothelial face, and within the interstitial space, appeared small compared with that found on the luminal face of the endothelium. That they were not eliminated completely by catalase might be explained by a substantial inability of this large protein (MW \sim 240 000) to diffuse across the endothelium from its vascular delivery site and intercept myocyte-derived H_2O_2 .

Regardless of the cellular source(s), a fraction of the H_2O_2 that is not metabolized or does not react with cell constituents first could diffuse into the extracellular space, which in blood-free systems lacks an H_2O_2 source. Our data suggest that some H_2O_2 does reach the extracellular spaces, including the vascular compartment. Flow upon reperfusion would increase the H_2O_2 concentration gradient towards the vascular lumen, where cerium is being delivered simultaneously. This should favor diffusion of H_2O_2 . Although diffusion-related factors may affect precipitate localization, it is difficult to envisage how they could negate the involvement of H_2O_2 in precipitate formation.

The endothelium does not appear to be a

total permeability barrier to cerium diffusion. This is based on the presence of diffuse densities in the interstitial space, even though their amount was too low to detect cerium with X-ray analysis. The prompt negative inotropic effect and concomitant excitation-contraction uncoupling upon perfusing CeCl₃ through otherwise functional nonischemic hearts also suggests that cerium reaches, at least, the sarcolemma or its glycocalyx during the brief administration period used for the histochemical reaction. Such responses resemble those caused by La³⁺ (Sanborn and Langer, 1970; Mezon and Bailey 1995).

Despite contractile and electrophysiologic similarities between La³⁺ and Ce³⁺, and between their crystal ionic radii (Ce³⁺ = 1.034 Å; La³⁺ = 1.061 Å), it appears that cerium's histochemical reactivity and localization are affected by factors other than, or in addition to, those affecting lanthanum. When cells with normal sarcolemmas (i.e. nonischemic cells) are exposed to ionic or colloidal lanthanum (Langer and Frank, 1972; Hoffstein *et al.*, 1975; Burton *et al.*, 1977), whether by incubation or perfusion, lanthanum deposits are intense but they are predominantly localized at the interstitial face (glycocalyx) of the sarcolemma. When CeCl₃ was perfused under largely similar conditions, the electron-dense material was slight and not found preferentially at the sarcolemma. When La³⁺ is administered to ischemically-damaged hearts, the substance can then be detected intracellularly, owing to disruption of the glycocalyx and sarcolemma that allows greater diffusion. Often this is accompanied by lesser amounts appearing on extracellular membrane faces. Moreover, when myocytes exposed to La³⁺ are pre-treated with some protective intervention prior to an insult such as ischemia, intracellular deposition is reduced. However there is no simultaneous and nearly complete reduction of lanthanum deposition on membrane faces exposed to the extracellular milieu. Cerium chloride did not behave in these ways in the presence or absence of ischemia or of catalase treatment.

It is difficult to envisage a process by which ischemia and reperfusion would dramatically intensify an alleged nonspecific Ce³⁺ binding,

especially preferentially at the luminal face of the endothelium, compared with what was seen in nonischemic controls. It is also difficult to envisage how catalase could significantly reduce such deposition, other than through its ability to degrade H₂O₂. Thus, if a portion of the precipitate merely reflects nonspecific binding of cerous ions to anionic sites on basement membranes (myocyte or endothelium), that portion is likely to be small compared with what occurred through the postulated Ce³⁺-H₂O₂ interaction.

Our data have several biologic implications for the postulate that H₂O₂ and other oxidants participate in damage to hearts during ischemia and/or reperfusion.

We have provided histochemical evidence to support a postulate that H₂O₂, regardless of its cellular origin(s), gains access to the extracellular space upon reperfusion after 30 min of normothermic ischemia. The data are also consistent with the hypothesis that the pool of H₂O₂ we detected is accessible to degradation by catalase administered by vascular perfusion. It is not possible, however, to state whether the H₂O₂ we detected was responsible for a component of injury incurred by hearts subjected to comparable ischemic conditions.

In summary, a cerium histochemistry technique applied to intact, buffer-perfused hearts, appears to have potential as a useful probe of H₂O₂ production and fates in this organ. It may be particularly useful when used with pharmacologic interventions thought to affect oxidant metabolism. The method may be applicable to the study of other buffer-perfused organs as well.

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