Irreversible injury in anoxic hepatocytes precipitated by an abrupt increase in plasma membrane permeability

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ABSTRACT

Using low-light digitized video microscopy, the onset, progression, and reversibility of anoxic injury were assessed in single hepatocytes isolated from fasted rats. Cell-surface bleb formation occurred in three stages over 1-3 h after anoxia. Stage I was characterized by formation of numerous small blebs. In stage II, small blebs enlarged by coalescence and fusion to form a few large terminal blebs. Near the end of stage II, cells began to swell rapidly, ending with the apparent breakdown of one of the terminal blebs. Breakdown of the bleb membrane initiated stage III of injury and was coincident with a rapid increase of nonspecific permeability to organic cationic and anionic molecules. On reoxygenation, stages I and II were fully reversible, and plasma membrane blebs were resorbed completely within 6 min of reoxygenation without loss of viability. Stage III, however, was not reversible, and no morphological changes occurred on reoxygenation. The results indicate that onset of cell death owing to anoxia is a rapid event initiated by a sudden increase of nonspecific plasma membrane permeability caused by rupture of a terminal bleb. Anoxic injury is irreversible until this event occurs. — HERMAN, B.; NIEMINEN, A.-L.; GORES, G. J.; Lemasters, J. J. Irreversible injury in anoxic hepatocytes precipitated by an abrupt increase in plasma membrane permeability. FASEB J. 2: 146-151; 1988.

Key Words: blebbing • cell death • ischemia • reperfusion • digitized video microscopy

Recent evidence has focused attention on alterations in plasma membrane structure and function as a major factor in the evolution of ischemic and hypoxic injury and, in particular, the conversion of reversible to irreversible injury. Results of morphological, functional, and biochemical studies indicate that plasma membrane alterations and consequent dysfunction are important early features of hypoxic injury. For example, ischemia results in a degradation of membrane phospholipids (1), an increase in plasma membrane permeability (2), and a decrease of Na⁺,K⁺-ATPase activity (3) and the appearance of small membrane defects after fixation of cells for transmission electron microscopy (4, 5). In kidney, liver, heart, and other cell types, cell surface blebbing is an early morphological expression of ischemic and hypoxic injury (6-10). Blebbing of the plasma membrane is also characteristic of cellular injury with various toxins (11). Thus, a major site of hypoxic and toxic injury is the plasma membrane, and this injury is manifested as plasma membrane blebbing.

There is no clear picture of the critical event or events that leads to the transition from reversible to irreversible injury. We have recently developed methods to assess the onset of cell death in relation to the structure and physiology of single hepatocytes with multiparameter digitized video microscopy (12). In a model of chemical hypoxia with cyanide and iodoacetate (12), we observed the rapid development of cell surface blebs in cultured hepatocytes culminating in a loss of cell viability associated with the breakdown of one of these plasma membrane blebs. The results suggested the possibility that cell death occurred as a consequence of rupture or lysis of a surface bleb and a resulting increase in nonspecific plasma membrane permeability.

Here, we report the onset, progression, and reversibility of injury in hepatocytes under conditions of true anoxia. Early anoxic injury was characterized by the formation, fusion, and enlargement of plasma membrane blebs. Subsequently, a sudden breakdown of the plasma membrane permeability barrier occurred. Changes observed before this breakdown were reversed entirely by reoxygenation; however, after breakdown occurred, the injury was irreversible.

MATERIALS AND METHODS

Hepatocyte isolation and culture

Hepatocytes were isolated from 24- or 48-h fasted rats (200-300 g) by collagenase digestion (13). Briefly, livers

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were perfused for 5 min via the portal vein with a basic medium containing 115 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 0.5 mM EGTA, and 25 mM sodium HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] buffer, pH 7.4, saturated with 100% oxygen at 37°C. Subsequently, the livers were rinsed with EGTA-free basic medium containing 1 mM CaCl₂ and 0.2 mg/ml of collagenase for 10-15 min in situ. The perfusion was stopped and the livers were rinsed gently with a stainless steel comb in EGTA-free basic medium containing, in addition, 1.2 mM MgSO₄, 2 mM CaCl₂, and 1% bovine serum albumin. The cell suspension was centrifuged through a 60-μm nylon mesh gauze (Tetco, Elmsford, NY), incubated for 20 min at 37°C, and filtered again. The filtrate was centrifuged for 2 min at 50 × g and the supernatant was resuspended three times. Viability of the hepatocytes was routinely more than 90% by trypan blue exclusion. Isolated cells were cultured on glass coverslips coated with rat tail collagen in Waymouth medium MB-752/2 containing 5% fetal calf serum and 100 nM insulin.

Preparation of submitochondrial particles

Rat liver mitochondria were isolated in 0.25 M sucrose, 2 mM potassium HEPES buffer, pH 7.4, by differential centrifugation (14). Submitochondrial particles were prepared by disruption as described previously (15) with the exception that the digitonin step was omitted. The submitochondrial particles were stored at −65°C before use.

Digitized video microscopy

Experiments were carried out with a low-light digitized video microscope (DVM) as described in detail elsewhere (12, 16). Briefly, our DVM consists of a Zeiss IM35 microscope connected to a ISIT-66 camera (DAGE-MIT, Michigan City, IN) and an IP-512 board set (Imaging Technologies, Woburn, MA) housed in an 11/23 based Q bus microcomputer (Scientific Micro System, Mountain View, CA). Excitation light for fluorescence is provided by a 100-W mercury lamp and, routinely, the excitation intensity was attenuated 100- to 1000-fold before reaching the cells, which prevented photobleaching of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and phototoxicity. The output from the DVM was recorded continuously with a ½-in video cassette recorder (VCR). By recording images with the VCR, we were able to monitor alterations in bleb morphology and BCECF fluorescence at video frame rates (33 ms). BCECF fluorescence was excited at the pH-insensitive wavelength of 440 nm and imaged with a 510-nm dichroic reflector and a 520- to 550-nm interference filter. Propidium iodide fluorescence was imaged with a 554-nm exciter filter, 580-nm dichroic reflector, and 580-nm long pass filter. Playback images were photographed from a video monitor with Kodak Technical Pan film (Rochester, NY).

Labeling and anaerobic incubation of cells

Hepatocytes on round 22-mm coverslips were loaded with 5 μM BCECF-AM (acetoxymethyl ester) for 30 min at 37°C in growth medium, washed three times in Krebs-Henseleit-bicarbonate buffer supplemented with 20 mM sodium HEPES buffer, pH 7.4, and mounted into an airtight perfusion chamber. The concentration of intracellular BCECF was estimated to be 115 ± 10 μM based on digitonin releasable absorbance from suspensions of hepatocytes loaded under identical conditions.

The anaerobic chamber permitted infusion of media with a syringe pump. Total exchange of medium required about 2 min at the infusion rates employed in the experiments. Temperature was maintained at 37°C with a Sage air curtain incubator. In a typical experiment, hepatocytes were loaded with BCECF, mounted into the anaerobic chamber, and placed on the microscope stage. Fresh Krebs-Henseleit-bicarbonate-HEPES buffer containing 1 μM propidium iodide and saturated with 95% O₂, 5% CO₂ was then infused. After collecting baseline phase and fluorescence images, Krebs-Henseleit-bicarbonate-HEPES buffer containing 1 μM propidium iodide, 1.0 mg protein/ml submitochondrial particles, and 5 mM succinate was infused to establish and actively maintain anaoxia (P_O₂<<1 torr). Under these conditions, the maximum velocity of oxygen uptake by the submitochondrial particles was about 100 natalm·min⁻¹·ml⁻¹ (15). Inasmuch as the submitochondrial particles were premixed with succinate-containing medium, anaerobiosis was established as soon as the submitochondrial particle-containing medium entered the chamber. Reoxygenation was achieved by infusion of fresh, oxygenated Krebs-Henseleit-bicarbonate-HEPES buffer containing 1 μM propidium iodide but no submitochondrial particles or succinate.

Materials

BCECF-AM was purchased from Molecular Probes (Eugene, OR), propidium iodide and bovine serum albumin fraction V from Sigma Chemical Company (St. Louis, MO), Waymouth's medium from Gibco Laboratories (Grand Island, NY), and collagenase from Cooper Biomedical (Malvern, PA). Other reagents were obtained from standard commercial sources.

RESULTS

Stages of bleb formation in anoxia

Hepatocytes cultured 2 h on collagen-coated coverslips maintained a rounded morphology (Fig. 1A). After initiating anoxia by infusion of submitochondrial particles and succinate, hepatocytes underwent marked morphological changes (Fig. 1). Within 25 min, numerous small, clear blebs formed on the surface of the cells (Fig. 1B). These blebs continued to enlarge and coalesce by fusion until one to three large terminal

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blebs remained (Fig. 1, C–G). We classify the initial phase of small bleb formation as stage I, whereas the subsequent growth and coalescence of the blebs is classified as stage II. The fusion of adjacent blebs resembled the fusion of soap bubbles and was complete within 33 ms, the time between consecutive video frames (data not shown).

Toward the end of stage II, bleb growth accelerated and cell volume increased rapidly. In the experiment shown in Fig. 1, the hepatocyte was nearly covered with broad and relatively shallow blebs after 54 min of anoxia (Fig. 1E). Over the next 80 s, the blebs more than doubled in cross-sectional area (Fig. 1, F, G, and I). Subsequently, in the time between consecutive video frames of the time-lapse recording (200 ms), one of the blebs abruptly enlarged (Fig. 1, J and K). Further bleb growth ceased completely after this event, suggesting that lysis or rupture of the bleb had taken place. Propidium iodide, an impermanent cationic dye that labels the nuclei of nonviable cells, was excluded by anoxic hepatocytes up to and including the stage of rapid enlargement of terminal blebs (Fig. 1H). However, within 1 min of apparent rupture of a terminal bleb, propidium iodide labeled the cell nuclei (Fig. 1K). This membrane breakdown delineates the beginning of stage III of the anoxic injury.

Increased plasma membrane permeability and the onset of cell death

The acetoxymethyl ester of BCECF is hydrolyzed by endogenous cytoplasmic esterases to trap free BCECF in the cytosol of the hepatocytes. BCECF is retained by the cells because of four net negative charges that cannot pass the plasma membrane permeability barrier. To assess nonspecific plasma membrane permeability during the transition from stage II to stage III injury, we monitored loss of BCECF fluorescence in relation to propidium iodide nuclear labeling during anoxia.

Figure 2 illustrates a typical experiment. After 166 min of anoxia, propidium iodide had labeled one of two cells in the field (Fig. 2A). BCECF fluorescence was retained by the cell excluding propidium iodide and was absent from the cell labeled by propidium iodide (Fig. 2B). The cell retaining BCECF fluorescence showed
blebbing characteristic of late stage II (Fig. 2C). In the next 35 s, BCECF fluorescence was lost—first from the blebs and nuclear area (Fig. 2, D–F) and then after another 75 s from the rest of the cell (Fig. 2I). The phase image taken after BCECF had begun to exit the cell showed that one of the terminal blebs had increased sharply in size (Fig. 2G) and the subsequent fluorescent image showed nuclear labeling by propidium iodide (Fig. 2H). Thus, the transition from stage II to stage III of anoxic injury again seemed marked by an apparent breakdown of the plasma membrane permeability barrier. The nonspecific increase of permeability was indicated by loss of trapped, anionic BCECF and uptake of extracellular, cationic propidium iodide, which occurred simultaneously.

Reversibility of the three stages of anoxic injury

The preceding experiments suggested that a sudden increase in plasma membrane permeability was associated with the onset of cell death. To determine whether this event was synonymous with the onset of irreversible injury, hepatocytes in various stages of anoxic injury were reoxygenated (Fig. 3). Plasma membrane blebbing was reversed within 6 min for cells in either stage I or stage II of anoxic injury (Fig. 3, A–C, E–G). There was no reperfusion paradox, i.e., reoxygenation did not cause cells to lose viability (Fig. 3, D and H). In cells that had already taken up propidium iodide (stage III), reoxygenation did not cause any reversal of blebbing or other change in cell morphology (Fig. 3, I–L). The plasma

Figure 2. Leakage of a trapped fluorescent probe (BCECF) from anoxic hepatocytes in relation to the uptake of propidium iodide. Hepatocytes from 48-h fasted rats were cultured 2 h, loaded with BCECF-AM, incubated with propidium iodide, and made anoxic by infusion of submicochondrial particles. A) Propidium iodide fluorescence after 165 min of anoxia. A cell to the lower right takes up propidium iodide. No other cells in the field are propidium iodide positive. B) BCECF fluorescence after 166 min of anoxia. BCECF fluorescence is diffuse and extends into blebs. The nonviable cell has no BCECF fluorescence. C) Phase image after 166 min of anoxia. Note large terminal blebs. D–F) BCECF fluorescence disappears from blebs and the nucleus within about 30 s. G) Phase image after 167 min of anoxia. Bleb to upper right is larger than in C. No further bleb growth occurred. H) Propidium iodide fluorescence after 168 min of anoxia. Nuclear propidium iodide is now evident. I) BCECF fluorescence after 169 min of anoxia. BCECF fluorescence is nearly absent.

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DISCUSSION

In cell death, there is a breakdown of the plasma membrane permeability barrier: cytosolic enzymes are released, trapped cytoplasmic markers such as fluorescein diacetate leak out, and normally impermeant dyes such as trypan blue and propidium iodide enter the cell (12). What is not known is whether this loss of the plasma membrane permeability barrier is gradual or abrupt, whether it precipitates cell death or merely follows as a postnecrotic event, and whether any structural changes accompany loss of the permeability barrier. Here, we have examined alterations in hepatocyte morphology and plasma membrane permeability in relation to the onset of irreversible injury. Our findings indicate: 1) anoxia induces formation of numerous small blebs of plasma membrane (stage I) (Fig. 1B), which enlarge and coalesce to form a few large terminal blebs (stage II) (Fig. 1, C-G); 2) at the end of stage II, anoxic cells swell rapidly until one of the blebs apparently ruptures (Fig. 1K); 3) this bleb breakdown leads to loss of the plasma membrane permeability barrier and is synonymous with cell death (stage III) (Fig. 2); and 4) reoxygenation reverses injury of stages I and II but not of stage III (Fig. 3).

Our data indicate that progression through the various stages of blebbing is highly variable from cell to cell. Loss of viability in different cells occurred after less than an hour or more than 3 h. This emphasizes the importance of studying single cells in delineating a sequence of events leading to cell death.

Several mechanisms may underlie bleb formation, cellular swelling, and membrane hyperpermeability observed during anoxic and ischemic injury. An attractive hypothesis based on these findings has been that decreases in cellular ATP levels lead to disruption of cellular ion homeostasis and an increase of cytosolic free calcium. This leads in turn to activation of cellular phospholipases, proteases, and other degradative enzymes, resulting in the increase of plasma membrane permeability and onset of cell death. However, recent
measurements of cultured hepatocytes exposed to cyanide and iodoacetate failed to demonstrate an increase in cytosolic free calcium preceding bleb formation or the onset of cell death (12). Thus, a role for increased cytosolic free Ca\(^{2+}\) as a key regulator of cell injury during ATP depletion was not demonstrable.

Other alterations of cellular function have been documented during ischemic or hypoxic injury, but the significance of these changes to plasma membrane structure and permeability is not known. For example, ATP hydrolysis and anaerobic glycolysis produce a fall of cytosolic pH (17), which may influence crucial enzymes and protein-lipid interactions. Superoxide and other free radicals may be formed during reperfusion of ischemic tissues and may attack plasma membrane phospholipids and cause membrane damage (18). Alterations of cell volume regulation (19) and membrane-cytoskeleton interactions (19, 20) occur in anoxic injury. Such changes may be important to bleb formation and eventual lysis. Indeed, high-molecular-weight solutes can protect ischemic renal proximal tubule cells against blebbing and irreversible injury presumably by preventing alterations of cell volume (21). The remarkable rapidity with which bleb resorption occurs after reoxygenation suggests that damage by the above mechanisms can be reversed until very late in the course of anoxic injury. Future studies addressing regulation of plasma membrane structure and function immediately before loss of the plasma membrane permeability barrier and after reoxygenation may reveal the mechanisms responsible for the onset of irreversible anoxic injury.

In summary, our findings indicate that the transition to an irreversible stage of injury occurs simultaneously with the onset of cell death as assessed by nuclear dye uptake and release of trapped cytoplasmic probes. Further, these events are precipitated by a sudden rupture of the plasma membrane and associated increase of nonspecific plasma membrane permeability.

This work was supported, in part, by grants AG07218, DK37034, and HL35490 from the National Institutes of Health and by a grant-in-aid (86-1299) from the American Heart Association. J. J. L. is an Established Investigator of the American Heart Association.

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Received for publication July 31, 1987. Accepted for publication October 28, 1987.