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Relationship Between Intracellular Adenosine Triphosphate, Cytosolic-Free Calcium and Cytotoxicity in Hepatocytes Exposed to Anoxia/Reoxygenation

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An increase in the concentration of the intracellular calcium ion within hepatocytes has been proposed by several investigators as the explanation for the pathologic events that occur during anoxic and reperfusion injury. Normally the concentration of cytosolic-free calcium (Ca_i^{2+}) is maintained at a physiologic low level by energy-dependent transport systems. Anoxic conditions, which are known to deplete intracellular adenosine triphosphate (ATP) levels, are likely to cause a disruption of calcium homeostasis as a consequence of impaired calcium transport across endoplasmic reticulum, mitochondria, and plasma membranes. In these conditions calcium can be assumed to be released from either intracellular stores or to have entered the cell from the extracellular space or both. As a result, a sustained elevation of Ca_i^{2+} occurs which results in the activation of calcium-dependent degradative enzymes within the cells which produce cell damage and eventually death. Although some studies have supported this hypothesis, others have denied any role of calcium in anoxic cell injury. This controversy has stimulated a debate whether or not an elevation of Ca_i^{2+} is involved in the liver cell damage that occurs during anoxic injury. The objective of the present investigation was to determine the precise sequence of events that occurs in rat hepatocytes exposed to a 2-hour period of anoxia followed by 1 hour of reoxygenation.

Materials and Methods

Hepatocytes

Freshly isolated hepatocytes were prepared from adult male Sprague-Dawley rats weighing between 200 and 250 g. The cells were isolated from fed animals using collagenase by the perfusion method described previously.[1] Cell viability assessed by trypan blue exclusion averaged 90% and was never less than 85%. The cells were imbedded in low temperature gelling agarose and were perfused at a rate of 0.6 mL/min with standard Krebs-Henseleit bicarbonate buffer (KHB) at 37°C. In all experiments, the hepatocytes were first perfused for 1 hour in KHB saturated with 95% O_2–5% carbon dioxide (Co_2). During the 2-hour experimental anoxic period, the cells were perfused with KHB saturated with 95% N_2–5% Co_2. After 2 hours of anoxia, the cells were reperfused with oxygenated KHB for 1 hour. In some experiments, the cells were exposed to Ca^{2+}-free media (+0.1 mmol/L EGTA) during the period of anoxia. The control and recovery perfusates always contained 1.3 mmol/L Ca^{2+}.

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Intracellular ATP

Intracellular ATP was measured by ^{31}P-nuclear magnetic resonance spectroscopy in real time in freshly isolated hepatocytes imbedded in agarose gel threads and perfused with KHB.[2] The ATP content was expressed as the ratio of βATP peak to the peak of the external standard methylene diphosphonic acid sealed in a spherical glass bulb positioned within the threads.

Cytosolic Ionized Calcium

Ca_i^{2+} was measured with the Ca^{2+}-sensitive photoprotein aequorin[3] which was incorporated into the isolated hepatocytes by gravity-loading. The aequorin-loaded cells were imbedded in agarose gel threads, placed in the cuvette of an aequorin luminescence photometer, and perfused at a rate of 0.6 mL/min with KHB at 37°C, as previously described.[3]

Lactate Dehydrogenase

Cell injury was monitored by measuring lactate dehydrogenase (LDH) release from the cells into the effluent perfusate before, during, and after the anoxic period. Because the concentration of perfused cells imbedded in the gel threads varied slightly between experiments, LDH release was expressed as the percent increase over the control value measured during the 1-hour control period of each experiment.

Results

During the first hour of anoxia, the intracellular ATP content of rat hepatocytes fell 60%. It was unmeasurable after 2 hours of anoxia. The resting cytosolic-free calcium in the cells ranged between 130 and 180 nmol/L. Anoxia increased Ca_i^{2+} in two distinct phases: a first rise occurred within 15 minutes and reached a mean value of 389 ± 35 nmol/L ($P < .001$). A second peak reached a maximum value of 1450 ± 120 nmol/L ($P < .001$) after 1 hour of anoxia. Lactate dehydrogenase was barely increased during the first hour of anoxia (+80%); however LDH release increased sixfold during the second hour of anoxia ($P < .001$). During reoxygenation the intracellular ATP level, Ca_i^{2+}, and LDH returned to control levels within 45 minutes. To determine whether the increased LDH release was related to the rise in Ca_i^{2+} and whether or not the increased Ca_i^{2+} was caused by Ca^{2+} influx from the extracellular space, the cells were perfused with Ca^{2+}-free KHB during the anoxic period. After 2 hours of anoxia in a Ca^{2+}-free medium, the ATP content of hepatocytes fell over 90%, but the Ca_i^{2+} was less than under control condition (< 10 nmol/L) and LDH release by the liver cells increased 2.7-fold.

Discussion

These studies provide direct evidence for a massive increase in cytosolic-free calcium in isolated rat hepatocytes produced by simple anoxia. The initial increase in Ca^{2+} observed after the institution of anoxia occurred even in the absence of Ca^{2+} in the medium. This finding strongly suggests that the source of this Ca^{2+} increase is an intracellular (endoplasmic reticulum and/or mitochondria) site. The second larger peak in Ca^{2+} was abolished totally when calcium was removed from the perfusate. This suggests that this second massive increase in Ca_i^{2+} occurs as a consequence of an influx of Ca^{2+} from the perfusate to the cytosol. This sustained elevation of Ca_i^{2+} produced during anoxia leads to an activation of calcium-dependent degradative enzymes that produce cell injury as measured by the release of cytosolic LDH. Because the loss of cytosolic LDH is 2.5 times less in Ca^{2+}-free media than in the presence of extracellular Ca^{2+}, it can be concluded that cell injury produced by anoxia is directly related to the rise in cytosolic-free calcium.

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