Calcium-induced Cytotoxicity in Hepatocytes after Exposure to Extracellular ATP Is Dependent on Inorganic Phosphate

EFFECTS ON MITOCHONDRIAL CALCIUM*

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J. Paul Zoeteweij, Bob van de Water, Hans J. G. M. de Bont, Gerard J. Mulder, and J. Fred Nagelkerke

From the Division of Toxicology, Center for Bio-Pharmaceutical Sciences, University of Leiden, P. O. Box 9503, 2300 RA Leiden, The Netherlands

In isolated mitochondria extensive uptake of Ca\(^{2+}\) in the presence of an "inducing agent," e.g. inorganic phosphate (P\(_i\)), causes permeabilization of the mitochondrial inner membrane and a collapse of the mitochondrial membrane potential. In this study we tested whether the effect of phosphate occurs in intact hepatocytes. Rat hepatocytes were incubated with ATP to induce a sustained increase in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)), dissipation of the mitochondrial membrane potential, and cell death (Zoeteweij, J. P., van de Water, B., de Bont, H. J. G. M., Mulder, G. J., and Nagelkerke, J. F. (1992) Biochem. J. 288, 207-213). Omission of Pi, from the incubation medium delayed the loss of viability. The nonhydrolyzable ATP analog adenosine 5'-0-(3-thiotriphosphate) (ATP\(_\gamma\)S) had similar effects on [Ca\(^{2+}\)]\(_i\) and viability, but now omission of extracellular Pi, completely protected against cytotoxicity. Exposure to ATP or ATP\(_\gamma\)S induced a large cellular uptake of Pi. With the use of video-microscopy a significant increase in mitochondrial free calcium was observed before the onset of cell death. Accumulation of mitochondrial calcium was reduced when extracellular Pi was omitted. These results suggest that, after induction of high [Ca\(^{2+}\)]\(_i\), by ATP in hepatocytes, 1) mitochondria accumulate calcium which is associated with cell toxicity and 2) intracellular Pi, increases which stimulates mitochondrial calcium uptake. These observations support a calcium-dependent mitochondrial dysfunction, induced by phosphate, as a valid model for ATP-induced cytotoxicity in hepatocytes.

Intracellular free calcium in hepatocytes is kept at a resting level of 100-200 nM, in contrast to the millimolar concentration in the blood. Changes in [Ca\(^{2+}\)]\(_i\) serve as signals for activation or deactivation of various cellular processes (1-4). Cellular compartments, especially mitochondria and the endoplasmic reticulum, are important for the regulation of [Ca\(^{2+}\)]\(_i\), providing buffer capacity for short time regulation (5-8). For this purpose liver mitochondria possess an electroneutral uniport uptake system for calcium. Studies with isolated mitochondria showed that influx of calcium into mitochondria can be modified by a variety of compounds, in particular inorganic phosphate (8). Release of calcium from liver mitochondria back to the cytosol mainly occurs through Na\(^{+}\)-independent calcium efflux. In addition to the calcium uptake system the mitochondrial inner membrane can, under certain conditions, become permeable to calcium and other, structurally unrelated, small molecules and ions (8-11). An absolute requirement for this permeability transition is the presence of a micromolar concentration of calcium in the cytosol which leads to accumulation of calcium in the mitochondrial matrix (8). The presence of phosphate as an "inducing agent" will accelerate the initiation of the permeability transition (8, 9). In isolated mitochondria this permeabilization can be reversed by cyclosporin A (11-14).

A prolonged increase in [Ca\(^{2+}\)]\(_i\), is often associated with the development of cellular toxicity as occurs after exposure to toxic compounds or anoxia (15-23). The mitochondrial calcium uptake and extrusion system may be challenged by the increased Ca\(^{2+}\) and eventually become compromised, leading to mitochondrial dysfunction and cell death. In a recent paper we showed that cell death induced by high [Ca\(^{2+}\)]\(_i\), in hepatocytes, after exposure to extracellular ATP, is preceded by dissipation of the mitochondrial membrane potential (24). Influx of potassium, the major intracellular cation, into mitochondria, driven by the mitochondrial membrane potential, occurs when the mitochondrial inner membrane becomes more permeable (25, 26). A lowered [K\(^+\)]\(_i\), reduced dissipation of the mitochondrial membrane potential and cytotoxicity induced by high calcium (24).

Whereas studies in isolated mitochondria have shown that phosphate stimulates mitochondrial calcium uptake and permeabilization of the mitochondrial inner membrane (8), it is unknown if these findings with isolated mitochondria are also applicable to intact cells. Therefore we have evaluated the possible role of phosphate as inducing agent in calcium-dependent cellular dysfunction after exposure to ATP.

EXPERIMENTAL PROCEDURES

Materials—Collagenase, ATP, and ATP\(_\gamma\)S were obtained from Boehringer Mannheim, Germany. Bovine serum albumin (type V), poly-D-lysine hydrobromide, rhodamine 123, propidium iodide, digitonin, Fura-2/AM, and Firefly Lantern extract were obtained from Sigma. 3zPi was purchased from Du Pont de Nemours, Bad Homburg, Germany.

Isolation and Incubation of Hepatocytes—Liver parenchymal cells were isolated by collagenase perfusion as reported previously (27). Cells were incubated at a density of 3 x 10\(^6\) cells/ml in Hanks's solution/HEPES buffer (pH 7.4, 37 °C) composed of 120 mM NaCl, 5 mM KCl, 4.5 mM NaHCO\(_3\), 1.2 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 5 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin.
MgSO₄, 25 mM HEPES, supplemented with 1% (w/v) glucose and 1.5% (w/v) bovine serum albumin.

**Flow Cytometric Analysis of Cell Viability**—For determination of cell viability in flow cytometric studies 7 μl of a 25 μM aqueous propidium iodide solution were added to 100 μl of the cell suspensions, and the cells were directly analyzed for their fluorescence on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). A detailed description of the FACScan system has been given elsewhere (24). The number of cells which exhibited fluorescence due to propidium iodide uptake reflected the number of cells which had lost viability.

**Video Intensified Fluorescence Microscopy (VIFM) of [Ca²⁺]i, and Mitochondrial Free Ca²⁺ ([Ca²⁺]ₘₐₘ)**—For fluorescence measurements in single hepatocytes, the cells were attached to glass coverslips coated with poly-D-lysine. The attachment procedure as well as a detailed description of the VIFM system have been described recently (24). Briefly, the VIFM system consists of a Zeiss IM-35 inverted microscope (Oberkochen, Germany), Nikon Fluor 40x oil objective, and a CCD series 200 camera system (Photometrics, Tucson, AZ) controlled by a Compaq 386/20 computer (Compaq Computer Corporation, Houston, TX). Image analysis was performed with a Imagine system (Symptics, Cambridge, U. K.).

For measurement of [Ca²⁺]i, and [Ca²⁺]ₘₐₘ (24, 28), hepatocytes were loaded with 20 μM Fura-2/AM for 60 min, after which the cells were washed with Hanks’ solution/HEPES buffer, mounted in an incubation chamber, and transferred to the microscope. Digitized fluorescence imaging was performed at 340- and 380-nm excitation and 450- and 495-nm emission. The ratio image of 340/380-nm excitation images was used to calculate [Ca²⁺]i (29).

For determination of [Ca²⁺]ₘₐₘ (28) the Hanks’ solution/HEPES buffer was replaced by a sucrose buffer containing 210 mM sucrose, 3 mM EGTA, 20 mM KCl, 10 mM K2HPO4, 5 mM glutamate, 5 mM succinate, and 5 mM MgCl₂.

The plasma membrane was subsequently permeabilized by addition of digitonin (final concentration 150 μM). Irreplaceable samples treated with digitonin, permeabilization of the plasma membrane was confirmed by cellular uptake of trypan blue (30). A few seconds after permeabilization, 340/380-nm images of the remaining fluorescence were taken, and ratio imaging was performed for estimation of [Ca²⁺]ₘₐₘ. To minimize intercellular variations in the determination of [Ca²⁺]ₘₐₘ due to thickness of the cells, the ratios were always determined of mitochondria present in the focal plane of the nuclei.

To confirm that the remaining fluorescence was due to Pura-2 localized in the mitochondria, the mitochondria were uncoupled with CCCP (final concentration 5 μM), which causes release of mitochondrial calcium. Indeed, after addition of CCCP the measured [Ca²⁺]ₘₐₘ dropped to nearly zero, which indicated its mitochondrial localization.

To check that the mitochondria were still intact after permeabilization, cells were loaded with rhodamine 123, which is a fluorescent indicator for the mitochondrial membrane potential (28, 31), before permeabilization. After treatment with digitonin-containing sucrose buffer, fluorescence of rhodamine was still observed. However, after treatment with digitonin and CCCP, fluorescence was lost due to permeabilization of the mitochondrial membrane.

**Determination of Intracellular Pₐ and ATP**—A 7-ml sample of the cell suspensions was taken, and the viable cells were separated from the medium and dead cells using a rapid centrifugation technique (32-34). In a tube 2 ml of dibutyl phthalate were layered onto 0.5 ml of 10% (v/v) aqueous HClO₄. The cell samples were layered on top of the dibutyl phthalate after which the tubes were centrifuged for 15 s at high speed (2500 × g). The viable cells were spun down into the HClO₄ fraction, leading to precipitation of cellular protein. Nonviable cells as well as the incubation medium itself remained on top of the dibutyl phthalate layer.

In a control experiment, cells were completely lysed with 150 μM digitonin (which caused permeabilization of the plasma membrane) and fractionated on the dibutyl phthalate/HClO₄ as described above. Lactate dehydrogenase was recovered in the upper fraction, and no precipitated protein was found in the HClO₄ fraction indicating that only viable cells move into the HClO₄.

Phosphate was assayed in the HClO₄ fractions using the molybdate assay (35), which was also used to measure phosphate in the medium. ATP was assayed by an optimized luciferin/luciferase method (36, 37).

**[³²P]PO₄ Uptake**—To determine uptake of [³²P]phosphate, cells were incubated in Hanks’ solution/HEPES buffer containing 1 μCi/ml [³²P]phosphate (specific activity 8500-9190 Ci/mmol). At various time points cell fractions were fractionated by the above described rapid centrifugation technique. A sample of the supernatant of the HClO₄ fraction was mixed with Emulsifier Sae (Packard), and the radioactivity was measured by liquid scintillation counting. In these experiments [³²P]phosphate uptake is expressed relative to the uptake determined after 1-min incubation of the cells with [³²P]phosphate.

**RESULTS**

**Dependence of ATP-induced Cytotoxicity on Extracellular Phosphate**—The dependence of cell death induced by extracellular ATP on extracellular Pₐ was studied by incubating the cells in the absence or presence of extracellular Pₐ. The hepatocytes were analyzed by flow cytometry after addition of 2 μM propidium iodide to the medium. After 3 h of exposure to ATP in the presence of 1.2 mM Pₐ, uptake of propidium iodide (indicating cell death) in a large number of cells was observed (Fig. 1). Release of lactate dehydrogenase from the cells into the incubation medium occurred parallel with uptake of propidium iodide (data not shown). When phosphate was omitted from the incubation medium a significant delay in cell death was measured. Increasing the concentrations of phosphate in the medium above 1.2 mM only slightly increased loss of viability (data not shown).

Extracellular ATP is hydrolyzed at the plasma membrane to generate inorganic phosphate (38), and therefore addition of ATP to a Pₐ-deficient medium will result in replenishment of the Pₐ. Indeed after addition of 0.4 mM ATP to the cells 0.9 mM phosphate was generated in the incubation medium linearly in 60 min (results not shown). This formation of Pₐ made it difficult to study the importance of extracellular Pₐ.

To study cytotoxic effects induced by high levels of [Ca²⁺]i, without generation of extracellular Pₐ, the nonhydrolyzable ATP analog ATPₐS was used (39–41). Exposure to 1 mM ATPₐS caused an immediate, sustained increase in [Ca²⁺]i (29), equivalent to the increase induced by 0.4 mM ATP (measured with Quin-2 fluorimetry (20, 42), results not shown). The presence or absence of extracellular Pₐ did not influence the induced rises in [Ca²⁺]i, by either ATP or ATPₐS. ATPₐS appeared to have similar cytotoxic effects as that of ATP: the loss of viability observed after 3 h of exposure to 1 mM ATPₐS was comparable to the extent of cell death observed after exposure to ATP. However, omission of phosphate from the

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**FIG. 1. Effect of omission of extracellular Pₐ on cell death induced by ATP.** The viability of cells (propidium iodide exclusion) at various time points is indicated. Control (◊) and 0.4 mM ATP (△) in the presence of 1.2 mM extracellular Pₐ; control (○) and 0.4 mM ATP (Δ) in the absence of extracellular Pₐ. Data are means ± S.E. of five separate isolations. * p < 0.05; † p < 0.01, compared with exposure to ATP in the absence of extracellular Pₐ (two-tailed Student's t test).
**P\textsubscript{r}-dependent Ca\textsuperscript{2+}-induced Cell Killing by ATP**

**Fig. 2.** Effect of omission of extracellular P\textsubscript{i} on cell death induced by ATP\textsubscript{y}S. Control (●) and 1 mM ATP\textsubscript{y}S (■) in the presence of extracellular P\textsubscript{i}; control (○) and 1 mM ATP\textsubscript{y}S (□) in the absence of extracellular P\textsubscript{i}. Data are means ± S.E. of four separate isolations. *p < 0.05; #p < 0.005, compared with exposure to ATP\textsubscript{y}S in the absence of extracellular P\textsubscript{i}.

**Fig. 3.** Cellular uptake of \[^{32}\text{P}\] after exposure to ATP or ATP\textsubscript{y}S. Cells were incubated in Hanks' solution/HEPES buffer containing 1 \(\mu\text{M}\) \[^{32}\text{P}\]. At various time points cellular radioactivity was determined. In viable cells uptake of radioactivity after 1 min at 4°C was taken as 100%. Control (■), 0.4 mM ATP (▲), and 1 mM ATP\textsubscript{y}S (△). Data are means ± S.E. of four separate isolations. *p < 0.02; #p < 0.01, compared with control.

incubation medium totally protected against ATP\textsubscript{y}S-induced cytotoxicity (Fig. 2).

The P\textsubscript{i} dependence of the effects could imply that a net uptake of phosphate by the cells was involved in ATP-induced cytotoxicity. To test this hypothesis the cells were exposed to ATP and ATP\textsubscript{y}S in the presence of \[^{32}\text{P}\]. As shown in Fig. 3, 0.4 mM ATP as well as 1 mM ATP\textsubscript{y}S caused a dramatic accumulation of \[^{32}\text{P}\] as compared to control values.

**Effects on Intracellular Phosphate—**Uptake of extracellular P\textsubscript{i} was expected to affect the intracellular P\textsubscript{i} content. Indeed, after addition of 0.4 mM ATP intracellular P\textsubscript{i} markedly increased (Fig. 4). Omission of extracellular P\textsubscript{i} reduced intracellular P\textsubscript{i} by 30% in the control and strongly delayed the increase in intracellular P\textsubscript{i} after exposure to ATP. To exclude intracellular ATP depletion as a cause of the measured increase in intracellular P\textsubscript{i}, [ATP], was also determined. As previously described (20), [ATP], greatly increased after exposure to extracellular ATP (Fig. 5). Omission of extracellular P\textsubscript{i} delayed the increase in [ATP].

**Determination of \([\text{Ca}^{2+}]_{\text{mito}}\)—**In isolated mitochondria phosphate has been shown to be directly involved in mitochondrial calcium handling (8). With use of a VIFM technique changes in mitochondrial calcium in individual cells were measured (28). Ten minutes after addition of ATP the cells were attached to glass coverslips and loaded with Fura-2/AM. [Ca\textsuperscript{2+}]\textsubscript{mito} was determined after 70 min of exposure to ATP just before the onset of cell death (see “Experimental Procedures”). Later measurements, when extensive loss of viability had occurred, could not be done because this interfered with the attachment of the cells to the coverslips. Most cells exhibited a highly increased level of [Ca\textsuperscript{2+}], and in addition a more than 2-fold increase in [Ca\textsuperscript{2+}]\textsubscript{mito} (Fig. 6) upon addition of ATP. Omission of extracellular P\textsubscript{i} prior to exposure to ATP, significantly reduced the number of cells with an increased [Ca\textsuperscript{2+}]\textsubscript{mito}, but had no effect on the measured [Ca\textsuperscript{2+}] levels.

**DISCUSSION**

Recently we reported that extracellular ATP induced a prolonged high increase of [Ca\textsuperscript{2+}] in isolated hepatocytes, which was associated with dissipation of the mitochondrial
P$_i$-dependent Ca$^{2+}$-induced Cell Killing by ATP

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Fig. 6. Effect on [Ca$^{2+}$]i and [Ca$^{2+}$]$_{mito}$ after exposure to ATP. After 10 min of exposure to ATP, cells were attached to polylysine glass coverslips and loaded with Fura-P/AM. Then the coverslips were mounted in the microscope. After 70 min of exposure images were taken and fluorescence intensities were measured to determine [Ca$^{2+}$]i. After permeabilization with digitonin images were taken for determination of [Ca$^{2+}$]$_{mito}$ (see “Experimental Procedures”). Per experiment 7-20 cells were examined. The percentage of cells which exhibited a more than 2- or 4-fold increase in [Ca$^{2+}$]i, compared to the averaged level of the control and the percentage exhibiting a more than 2-fold increase in [Ca$^{2+}$]$_{mito}$ are shown. Control (■): 0.4 mM ATP in the presence (narrow hash marks) or absence (wide hash marks) of extracellular Pi. In the control no cells were observed with a more than 4-fold increase in [Ca$^{2+}$]i. Omission of extracellular Pi alone without exposure to ATP had no effect. Data are the means ± S.E. of four to five separate isolations. *p < 0.005, compared with exposure to ATP in the presence of extracellular Pi.

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...lesser phosphate availability for synthesis caused by a lower uptake of phosphate from the medium. On the other hand lower cellular P$_i$ levels possibly reduce a Ca$^{2+}$-stimulated inhibition of mitochondrial respiration by interfering with mitochondrial Ca$^{2+}$ transport.

In isolated mitochondria phosphate facilitates Ca$^{2+}$ uptake causing high matrix Ca$^{2+}$ levels which induce permeability changes of the inner membrane. In cultured myocytes mitochondrial Ca$^{2+}$ levels were not very different from cytosolic Ca$^{2+}$ levels (28). In our freshly isolated hepatocytes higher calcium ratios (which reflected at least a two times higher free calcium concentration) were measured in mitochondria than in the whole cell. Nevertheless, in intact cells accumulation of free calcium in the mitochondria prior to cell death was measured after induction of high [Ca$^{2+}$]i, by extracellular ATP. This accumulation was reduced when an increase in intracellular P$_i$ content was less.

The data presented in this paper are in agreement with the hypothesis that mitochondrial dysfunction and cell death in intact hepatocytes observed after an ATP-induced, sustained increase in [Ca$^{2+}$]i, is associated with increased mitochondrial Ca$^{2+}$ levels. The mitochondrial uptake of Ca$^{2+}$ is dependent on intracellular P$_i$. After exposure to ATP the requirements for permeability changes of the mitochondrial inner membrane, i.e. high mitochondrial matrix calcium and the presence of a large amount of phosphate as inducing agent, are present. Permeabilization of the mitochondrial inner membrane is most likely involved in ATP-induced mitochondrial dysfunction and cell death. The exact mechanism of the permeability transition is not yet completely understood. Several processes, such as activation of Ca$^{2+}$-dependent phospholipases (43) or ADP ribosylation (1), have been proposed to be responsible for the nonspecific pore opening in the mitochondrial inner membrane.

It has been suggested that activation of Ca$^{2+}$-dependent proteases is involved in Ca$^{2+}$-induced cell killing (44). However, several groups could not reproduce any protective effect of protease inhibitors on cell death in hepatocytes in which calcium was elevated (15, 20, 45, 46). The present results and previous work from our laboratory strongly indicate that a Ca$^{2+}$-induced permeability transition of the mitochondrial inner membrane plays an important role in calcium-dependent cytotoxicity in hepatocytes.

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