Late steady increase in cytosolic Ca^{2+} preceding hypoxic injury in hepatocytes

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Upon initiation of hypoxia, the ATP content of hepatocytes in monolayer cultures fell within 5 min from 22 to 12 nmol of ATP/ 10^6 cells. This decrease in ATP was not followed by early alterations in the cytosolic Ca^{2+} concentration; for up to 60 min it remained around 100 nM. However, after that period cytosolic free Ca^{2+} steadily increased, up to 400 nM. This increase began around 60 min before the cells lost their viability, and primarily resulted from an influx of extracellular Ca^{2+} . Likewise, in experiments where the mitochondrial respiratory chain was blocked by KCN and glycolysis was blocked by iodoacetate, the ATP content fell within minutes to 10 nmol/ 10^6 cells, whereas the cytosolic Ca^{2+} concentration only began to increase 30 min later (up to 600 nM). However, also under these conditions of 'chemical hypoxia' this increase was clearly (about 10 min) earlier than the loss of viability.

INTRODUCTION

It is generally accepted that depletion of intracellular ATP is an early and decisive event in hypoxic cell injury. The subsequent steps leading to cell death are less clear. Much attention has been focused on disturbances of the cellular ion homeostasis. An increase in the cytosolic Ca^{2+} concentration, in particular, has been put forward as an essential later step in the development of hypoxic injury (Hochachka, 1986). In accordance with this hypothesis, increases in the cytosolic Ca^{2+} concentration, directly related to decreases in the energy (ATP) content of the cells, have been reported to occur under hypoxic conditions (Snowdowne *et al.*, 1985; Trump *et al.*, 1989; Diederichs *et al.*, 1990), as well as under conditions where both oxidative phosphorylation was blocked by KCN and glycolysis was blocked by iodoacetate ('chemical hypoxia') (Nicotera *et al.*, 1989; Trump *et al.*, 1989).

However, the crucial role played by increased cytosolic free Ca^{2+} in hypoxic cell injury is not generally accepted. In experiments with isolated hepatocytes, increases in cytosolic Ca^{2+} have not been observed before the onset of cell death, either under hypoxic conditions (Hayashi *et al.*, 1986; Berger *et al.*, 1989) or in the presence of KCN and iodoacetate (Lemasters *et al.*, 1987; Nieminen *et al.*, 1988).

In the present paper we report that no alteration in the cytosolic Ca^{2+} concentration occurred in hepatocytes in primary culture early after ATP depletion either owing to hypoxia or owing to the addition of KCN and iodoacetate. However, at a later time Ca^{2+} steadily increased under both conditions, beginning several minutes before the hepatocytes lost their viability.

METHODS

Materials

Collagenase (HEP plus), collagen (Type R), dexamethasone and gentamycin were purchased from Serva (Heidelberg, Germany). Hanks balanced salt solution (HBSS), Leibowitz L-15 medium and fetal-calf serum were from Gibco (Eggenstein, Germany), Synperonic was from Boehringer (Mannheim, Germany), propidium iodide was from Sigma (Deisenhofen, Germany) and ionomycin and fura-2/AM were from Calbiochem (Frankfurt, Germany).

Isolation and culture of hepatocytes

Hepatocytes were isolated by collagenase perfusion from fed male Wistar rats (200–240 g body wt.) as previously described (de Groot & Brecht, 1991). They were cultured in 25 cm² flasks (Falcon, Heidelberg, Germany) or, for fluorescence measurements, by seeding cells on glass coverslips in Petri dishes. Both flasks and coverslips were coated with collagen (0.2 %). Leibowitz L-15 medium supplemented with fetal-calf serum (5 %, v/v), L-glutamine (2 mM), glucose (8.3 mM), BSA (0.1 %), NaHCO_3 (14.3 mM), gentamycin (50 µg/l) and dexamethasone (1 µM) was used as culture medium. After incubation for 2 h in an atmosphere of CO_2 /air (1:19) at 37 °C, adherent cells were washed three times with HBSS, pH 7.4, to remove unattached and non-viable cells. Fresh culture medium was added, and the hepatocytes were further incubated for 15–16 h until the start of the experiments.

Incubation procedure

Before starting the experiments, the cells were washed three times with HBSS. Incubations were performed at 37 °C in Krebs–Henseleit (1932) bicarbonate buffer, pH 7.4, supplemented with Hepes (20 mM). Propidium iodide (5 µM) was added where indicated. In those experiments where the cytosolic Ca^{2+} concentration was determined, coverslips with attached hepatocytes were transferred into a chamber (Pentz chamber system; Heraeus, Hanau, Germany) which was subsequently mounted on the thermostatically regulated table of an inverted microscope (IM35; Zeiss, Oberkochen, Germany). Hypoxic conditions were initiated by closing the chamber with a rubber stopper equipped with two steel cannulae for gas inlet and outlet and subsequently flushing for the whole incubation period with a low gas flow rate of CO_2/N_2 (1:19; 1.3 ml/min). Control aerobic incubations were performed in the same way, except that the gas used was $\text{CO}_2/\text{O}_2/\text{N}_2$ (5:21:74). Experiments under 'chemical hypoxia' were initiated by adding 1 mM-KCN and 0.5 mM-iodoacetate. Incubations in Ca^{2+} -free Krebs–Henseleit bicarbonate buffer were carried out with or without 0.5 mM-EGTA. Experiments in tissue-culture flasks [determination of ATP content and lactate dehydrogenase (LDH) release] were performed essentially as described above and in detail in de Groot & Brecht (1991). ATP was determined as described by Dwyer & Brown (1988).

Abbreviations used: LDH, lactate dehydrogenase; HBSS, Hanks balanced salt solution.

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Cell viability

LDH leakage (Jauregui *et al.*, 1981), release of fura-2 (Niemenen *et al.*, 1988) and uptake of propidium iodide (Lemasters *et al.*, 1987) were used to indicate loss of cell viability. Intracellular fura-2 concentration was ascertained from the fluorescence at the Ca^{2+} -insensitive excitation wavelength of 360 nm (emission, 500–530 nm). Propidium iodide uptake was determined by the excitation of this fluorescent dye at 554 nm and counting the percentage of nuclei which emitted light at > 580 nm. All three parameters of cell viability provided essentially the same results, in line with previous observations where it was shown that nuclear labelling with propidium iodide and loss of cytosolic dyes occurs synchronously as hepatocytes lose viability (Herman *et al.*, 1988; Niemenen *et al.*, 1988).

Ca^{2+} measurements

Hepatocytes were loaded with fura-2 by incubation in Krebs–Henseleit bicarbonate buffer, pH 7.4, containing Hepes (20 mM), Synperonic (0.03 %) and fura-2/AM (5 μM), for 30 min at 37 °C. After loading, hepatocytes were washed three times with HBSS. Fura-2 fluorescence was measured at the single-cell level by using a video microscope system (ICMS, kindly provided for the experiments by Hamamatsu Photonics, Hamamatsu, Japan) at excitation wavelengths of 340/380 and 360/380 nm respectively. The emission wavelength was selected with a 500–530 nm filter. Calibration and calculation of free cytosolic Ca^{2+} were performed by the procedure described by Grynkiewicz *et al.* (1985). R_{max} (maximal fluorescence ratio) was obtained by adding ionomycin at a final concentration of 50 μM , and R_{min} (minimal fluorescence ratio) by adding EGTA at a final concentration of 10 mM. For background subtraction, 2 mM- MnCl_2 was added to quench any fura-2 fluorescence. Fura-2 fluorescence disappeared within seconds after addition of 20 μM -digitonin, indicating the predominant location of this fluorescent dye within the cytosol.

RESULTS

Authentic hypoxia

Areas of 20–30 hepatocytes were chosen under the inverted microscope, and hypoxia was achieved by blowing CO_2/N_2 (1:19) on to the surface of the incubation medium. Cell viability was estimated by counting the number of cells taking up the fluorescent dye propidium iodide, and intracellular Ca^{2+} was calculated from the ratio of 340/380 nm values of fura-2 fluorescence. At the start of the experiments viability was above 95 % and the cytosolic Ca^{2+} concentration around 100 nM. Hepatocytes slowly lost viability during the first 4 h of hypoxia (Fig. 1a). After that period viability rapidly decreased, and 1 h later almost all of the cells showed propidium iodide fluorescence. In these experiments no early increase in the cytosolic Ca^{2+} concentration was detectable (Fig. 1b). However, after about 2 h of hypoxia Ca^{2+} began to increase, first slowly and later more rapidly, and at the time when loss of viability was obvious cytosolic Ca^{2+} concentrations around 400 nM were measured. Later, for a very short period even higher Ca^{2+} concentrations were determined (up to 2 μM ; results not shown). However, this was at a time when most of the fura-2 had already left the cells and only very weak intracellular fluorescence was still detectable (about 10 % of the initial value). In control experiments performed under aerobic conditions, no measurable loss of viability and only slight increases in the cytosolic Ca^{2+} concentration of up to 120 nM were detectable.

Hypoxia-induced increases in cytosolic Ca^{2+} before the onset of loss of viability were especially pronounced in experiments

where cell injury was estimated by the release of fura-2, measured at an excitation wavelength of 360 nm (Fig. 2). In each cell the increase in cytosolic Ca^{2+} , calculated from the ratio of 360/380 nm values of fura-2 fluorescence, began at a time when the cell was still viable, and loss of viability was always indicated by a sudden decrease in fura-2 fluorescence.

In parallel experiments the ATP content of the hepatocytes was monitored (Fig. 1c). Under aerobic conditions, the initial ATP content of 22 nmol/ 10^6 cells decreased to 18 nmol/ 10^6 cells after 1 h of incubation, but then remained almost constant. In the hypoxic hepatocytes, 5 min after the onset of hypoxia the ATP content had already fallen to 12 nmol/ 10^6 cells. Within the next 2 h it further decreased to 8 nmol/ 10^6 cells, staying constant at that level for the residual incubation period.

In the absence of extracellular Ca^{2+} , with or without 0.5 mM-EGTA, hepatocytes already lost viability under normoxic conditions (Fig. 3a). Accordingly, in the absence of extracellular Ca^{2+} an actual increase in hypoxic injury was observed, rather than protection from such injury (Fig. 3b). In these experiments the cytosolic Ca^{2+} concentration initially decreased to 40 nM (results not shown). At the time when hypoxic cell injury became obvious, Ca^{2+} increased up to 100 nM. Once almost all of the fura-2 had left the cells, very high values for the cytosolic Ca^{2+} concentration were obtained in these experiments too.

Fig. 1. Loss of viability (a), increase in cytosolic free Ca^{2+} (b) and decrease in ATP (c) in hepatocytes under hypoxic conditions

Primary cultures of isolated hepatocytes from rat liver were incubated at 37 °C in an atmosphere of CO_2/N_2 (1:19). For control normoxic conditions, cells were maintained in an atmosphere of 21 % oxygen ($\text{CO}_2/\text{O}_2/\text{N}_2$, 5:21:74). Viability was estimated by propidium iodide exclusion, and cytosolic free Ca^{2+} was measured by fura-2 fluorescence (340/380 nm). Data presented are means $\pm$ S.E.M. for 8–26 separate incubations of 8–16 preparations.

Fig. 2. Increase in cytosolic free Ca^{2+} and release of fura-2 in single hepatocytes under hypoxic conditions

Cytosolic free Ca^{2+} was measured by ratio imaging of fura-2 fluorescence at 360/380 nm. Release of intracellular fura-2, determined by excitation at the Ca^{2+} -insensitive wavelength of 360 nm, was used to indicate loss of viability. Data presented are from one experiment showing typical time courses in three different (a, b and c) hepatocytes.

Fig. 3. Effect of Ca^{2+} -free medium, with or without EGTA, on the viability of hepatocytes under normoxic (a) and hypoxic (b) conditions

Hepatocytes were incubated with 2 mM- Ca^{2+} , without Ca^{2+} , and without Ca^{2+} plus 0.5 mM-EGTA. Viability was determined by LDH leakage. Values presented are means $\pm$ S.E.M. for four separate incubations of four preparations.

Chemical hypoxia

Results similar in principle to those described for authentic hypoxia were obtained when oxidative phosphorylation was blocked by KCN and glycolysis was blocked by iodoacetate. However, loss of cell viability and the increase in the cytosolic Ca^{2+} concentration occurred markedly earlier. ATP fell within 5 min to 10 nmol/ 10^6 cells, and then further decreased within 1 h to 5 nmol/ 10^6 cells (Fig. 4b). Rapid loss of cell viability, determined by either uptake of propidium iodide (results not shown) or fura-2 release (Fig. 4a), began 40–50 min after addition of 1 mM-KCN and 0.5 mM-iodoacetate. Only slight alterations in the intracellular Ca^{2+} concentration occurred during the first 30–40 min of incubation. About 10 min before cell injury became evident, Ca^{2+} suddenly increased from 100 nm to values close to 600 nm (Fig. 4a).

DISCUSSION

Increases in cytosolic free Ca^{2+} upon induction of hypoxia have been described for isolated kidney cells (Snowdowne *et al.*, 1985; Trump *et al.*, 1989), for isolated cardiomyocytes (Cheung *et al.*, 1986; Allshire *et al.*, 1987) and for the isolated perfused heart (Diederichs *et al.*, 1990). Whereas in the experiments with isolated kidney epithelium cells (Trump *et al.*, 1989) and with isolated perfused heart (Diederichs *et al.*, 1990) Ca^{2+} almost immediately rose after initiation of hypoxia and thus depletion of energy, in experiments with isolated cardiomyocytes Ca^{2+} began to increase only after rigor contraction, presumably owing to further depletion of remaining ATP by activation of myosin ATPase (Cheung *et al.*, 1986; Allshire *et al.*, 1987). In the hepatocytes ATP was already depleted within 5 min after induction of hypoxia, and subsequently only slightly decreased for

Fig. 4. Increase in cytosolic free Ca^{2+} and release of fura-2 (a) and decrease in ATP (b) in hepatocytes during inhibition of glycolysis and oxidative phosphorylation

Glycolysis was inhibited by 0.5 mM-iodoacetate and oxidative phosphorylation by 1 mM-KCN. Cytosolic free Ca^{2+} was measured by ratio imaging of fura-2 fluorescence at 360/380 nm. Release of intracellular fura-2, ascertained from the fluorescence at the excitation wavelength of 360 nm, was used to indicate loss of viability. Data presented are means $\pm$ S.E.M. for 8–17 separate incubations of 8–12 preparations.

the residual incubation period (Fig. 1c). An additional 60 min passed, however, before the increase in Ca^{2+} began (Figs. 1b and 2). Thus, evidently in hepatocytes no immediate response in cytosolic free Ca^{2+} occurs as a result of energy depletion. It appears to be more likely that in these cells, in addition to energy depletion, a certain time needs to elapse (implying the operation of processes so far unknown) before Ca^{2+} increases. The late beginning of hypoxia-dependent increase in cytosolic free Ca^{2+} may also explain why other authors (Hayashi *et al.*, 1986; Berger *et al.*, 1989) did not observe such an increase in experiments with isolated hepatocytes. In the experiments of Hayashi *et al.* (1986) the hepatocytes were reoxygenated after a hypoxic period of 90 min, and in the experiments of Berger *et al.* (1989) the intracellular Ca^{2+} was determined by X-ray microanalysis for up to 45 min.

The hypoxia-induced late increase in cytosolic free Ca^{2+} appears to result mainly from an influx of extracellular Ca^{2+} , since in the absence of extracellular free Ca^{2+} only slight increases in the cytosolic Ca^{2+} concentration occurred upon induction of hypoxic cell injury. However, absence of extracellular free Ca^{2+} , especially in the presence of EGTA, might also deplete intracellular Ca^{2+} stores. Thus at least part of the increased cytosolic Ca^{2+} may also originate from intracellular sources such as the mitochondria. As demonstrated by experiments with isolated liver mitochondria, these organelles are capable, in principle, of releasing Ca^{2+} under hypoxic conditions (Nishida *et al.*, 1989), and in hepatocytes a hypoxia-dependent decrease in mitochondrial Ca^{2+} has been described (Aw *et al.*, 1987).

The observation that the increase in cytosolic Ca^{2+} became substantial as early as about 60 min before the cell concerned lost its viability (Figs. 1a, 1b and 2) suggests its involvement in hypoxic cell injury. On the other hand, absence of extracellular free Ca^{2+} had no protective effect against hypoxic injury (Fig. 3b), in line with observations by Edmondson & Bang (1981) and Fariss *et al.* (1984). The missing protective effect of Ca^{2+} -free medium, however, may not necessarily mean non-involvement of Ca^{2+} in this injurious process, but may result from an additional injury owing to the absence of this ion, as indicated by the fact that even under aerobic conditions marked cell injury occurred in the absence of extracellular free Ca^{2+} (Fig. 3a). Hence, the question of the relevance of the late increase in cytosolic Ca^{2+} concentration in hypoxic injury of the hepatocytes remains to be answered.

A late increase in cytosolic free Ca^{2+} , occurring clearly (about 10 min) before the hepatocytes lost viability, was also observed in the experiments where the mitochondrial respiratory chain was blocked by KCN and glycolysis was blocked by iodoacetate ('chemical hypoxia') (Fig. 4a). This observation apparently contrasts with observations by Lemasters *et al.* (1987). Interestingly, however, in one of their papers (Niemenen *et al.*, 1988), they also described a late increase in the cytosolic Ca^{2+} concentration under 'chemical hypoxia', although they interpreted their results as indicating no rise in Ca^{2+} in advance of loss of cell viability. Increases in the cytosolic Ca^{2+} concentration under conditions of 'chemical hypoxia' have been reported for hepatoma 1c1c7 (Nicotera *et al.*, 1989), isolated heart (Hayashi *et al.*, 1990) and kidney epithelium cells (Trump *et al.*, 1989). However, fundamental differences in the pathomechanism may exist between cell injury induced by 'chemical hypoxia' and by authentic hypoxia. For instance, under 'chemical hypoxia' large amounts of reactive oxygen species are released, e.g. by the mitochondrial respiratory chain (Boveris & Chance, 1973; Boveris *et al.*, 1976; Forman & Boveris, 1982), and it is known that H_2O_2 -dependent enzymic oxidation of mitochondrial nicotin-

amide nucleotides via glutathione peroxidase and glutathione reductase may trigger Ca^{2+} release from mitochondria (Richter & Frei, 1988). On the other hand, omission of extracellular Ca^{2+} under aerobic conditions may also induce an increased formation of reactive oxygen species in hepatocytes, possibly owing to mitochondrial Ca^{2+} cycling, thus promoting cell injury (Thomas & Reed, 1988a,b). Differences between the two experimental models are also indicated by the marked difference in the time courses of cell injury under hypoxic conditions and under 'chemical hypoxia' (cf. Figs. 2a and 4a) and further suggested by the observation that the presence of 0.5 mM-iodoacetate alone, without KCN, was already enough for the hepatocytes to die rapidly within 60 min (M. Brecht & H. de Groot, unpublished work).

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