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SODIUM-MEDIATED CELL SWELLING IS ASSOCIATED WITH IRREVERSIBLE DAMAGE IN ISOLATED HEPATOCYTES EXPOSED TO HYPOXIA OR MITOCHONDRIAL TOXINS

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Incubation of isolated rat hepatocytes under hypoxic conditions or in the presence of inhibitors of mitochondrial functions such as KCN or carbonylcyanide m-chlorophenylhydrazone (CCCP) causes an increase of intracellular Na^+ content and cell swelling. Both these effects precede the apparence of irreversible damage as measured by trypan blue staining of non-vital hepatocytes. When the increase of cellular Na^+ is prevented by substitution of NaCl in the incubation medium with equimolar amount of choline chloride both cell swelling and loss of viability are greatly reduced. Thus, we propose that osmotic stress induced by an uncontrolled accumulation of Na^+ might be associated with the ultimate events precipitating irreversible membrane lesions in hepatocyte undergoing metabolic inhibition.

The molecular mechanisms leading to liver cell injury following hypoxia are still largely unknown (1). In isolated hepatocytes the impairement of mitochondrial respiration caused by the lack of oxygen is associated with the collapse of mitochondrial membrane potential, depletion of ATP, lowering of cell pH and rise of cytosolic levels of Ca^{2+}, Mg^{2+} and Na^+ (2-5). Although inhibition of ATP formation appears to be the crucial event in the progression of irreversible cell injury (6), the activation of phospholypase A_2 (7) and of non-lysosomal proteases (8) induced by the increase of Ca^{2+} levels have also been proposed to play a role in the processes leading to cell death. Morphological observations have demonstrated that either in intact tissues and in isolated cells an increase in cell volume precedes the loss of plasma membrane integrity and the leakage of the cytosolic constituents (9,10). In the recent years, a number of studies have shown that hepatocyte volume is largely influenced by osmolite fluxes across the plasma membrane (11,12). Since in hypoxic hepatocytes the function of Na^+/K^+-ATPase can be impaired by the loss of ATP, while cellular acidosis may trigger the activation of Na^+/H^+ exchanger and Na^+-HCO_3^- cotransporter leading to a Na^+ influx (13,14), we have investigated whether alterations of Na^+ homeostasis might be involved in the processes leading to cell swelling and death.

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Materials and Methods

Collagenase (Type I), N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid) (HEPES), KCN, rhodamine 123 and carbonylcyanide m-chlorophenylhydrazone (CCCP) were purchased from Sigma (St Louis, MO, USA). All the other chemicals were of analitical grade and were purchased from Merck (Darmstad, Germany). Male Wistar rats (180-250 g weigth) were obtained from Nossan (Corezzana, Italy) and allowed free access to water and food.

Isolated rat hepatocytes were prepared by liver perfusion with collagenase as previously described (15). For the experiments hepatocytes (10^6 cells/ml) were suspended in Krebs-Henseleit medium containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$ and 20 mM HEPES at pH 7.4 and incubated at 37° C in 50 ml glass bottles under continous fluxing of 5% CO_2 95% O_2 mixture. Experiments under hypoxic conditions were performed using Krebs-Henseleit medium equlibrated with 95% N_2 5% CO_2 and the hepatocytes were incubated in sealed bottles under 95% N_2 5% CO_2. When indicated, a Na^+ free medium containing 118 mM choline chloride instead of NaCl and 25 mM $KHCO_3$ replacing $NaHCO_3$ was employed.

Hepatocyte viability was expressed as the percentage of cells excluding Trypan blue.

Mitochondrial membrane potential was estimated by the uptake of the cationic fluorescent dye rhodamine 123 as previously reported (15).

The ATP content of hepatocytes was measured after protein precipitation with perchloric acid, using reverse phase HPLC as described previously (15).

Intracellular Na^+ content was measured by atomic absorbtion using a Varian AA-1475 atomic absorption spectrophotometer. Aliquots of cell suspensions were layered on the top of 3 ml Percoll (Pharmacia, Upsala, Sweden) solution (d = 1.06) in 0.25 M sucrose and spun 1 min at 1000 RPM on a beanch-top centrifuge in order to remove the incubation medium and damaged or dead cells. After centrifugation the Percoll solution was rapidly removed by aspiration and cell pellets were racted with 0.5 ml of 0.8 N perchloric acid. Na^+ was measured in aliquots of the protein-free acidic supernatants diluted 200 times with distilled water and the values corrected for the protein content of each pellet. Protein concentration was estimated by the Lowry method as modified by Peterson (16).

The changes in hepatocyte volume were estimated by cytofluorimetric analysis monitoring the light scattering of a laser beam. At each time point aliquots of the cell suspension were immediately analyzed with a FACScan analyzer (Becton Dickinson, Sunnyvair, CA, USA). The shift in the forward scatter of at least 10,000 cells was evaluated and the median values were used as an estimation of the relative changes in cellular dimensions.

Staristical analysis was performed with one-way ANOVA, with Bonferroni's correction for multiple comparisons.

Results and Discussion

The incubation of isolated rat hepatocytes under 95% N_2, 5% CO_2 atmosphere caused a rapid collapse of mitochondrial membrane potential, as estimated by rhodamine 123 uptake, and depleted intracellular ATP stores (Table 1). Such a metabolic impairement was followed by a progressive increase of intracellular Na^+ content (Fig. 1A). A similar behaviour was also observed when the effect of hypoxia on cellular energization was mimiked (Table 1) by blocking mitochondrial respiratory chain with 1 mM KCN or by using the uncoupler protonophore CCCP (25 μM) which collapses mitochondrial membrane potential (Fig. 1A). The increase in intracellular Na^+ caused by the absence of oxygen, KCN or CCCP was completely prevented when isolated hepatocytes were incubated in a medium where Na^+ was replaced by an equimolar amount of choline chloride (Fig. 1B), indicating that Na^+ increase was due to an influx of the ion from the extracellular space.

ATP depletion consequent to the collapse of mitochondrial membrane potential might be partially

Table 1

Rhodamine 123 uptake and ATP levels in isolated hepatocytes exposed to hypoxia or treated with mitochondrial inhibitors in the presence or in the absence of extracellular Na^+

	Rhodamine 123 uptake (%)		ATP (nmoles/10^6 cells)	
	+ Na^+	- Na^+	+ Na^+	- Na^+
Control	82 ± 3	85 ± 4	16 ± 5.3	14 ± 4.2
Hypoxia	44 ± 6	48 ± 5	6 ± 0.3	3 ± 2.1
KCN 1 mM	50 ± 4	56 ± 8	4 ± 2.5	5 ± 3.1
CCCP 25 μM	35 ± 3	37 ± 7	2 ± 0.3	1 ± 0.5

Hepatocytes were incubated 15 min under an atmosphere of 95% O_2, 5% CO_2 without any addition, with 1 mM KCN or 25 μM CCCP. Hypoxic conditions were obtained by fluxing the cell suspension with 95% N_2 5% CO_2. For the experiments performed in the the absence of Na^+, NaCl in the incubation medium was substituted with equimolar amount of choline chloride. The values are means of 3-4 different experiments ± S.D.

responsible for the elevation of cytosolic Na^+ by affecting the activity of Na^+/K^+ translocase in the plasma membranes. Furthermore, we have recently demonstrated that the activation of Na^+/K^+ exchanger and Na-HCO_3^- cotransporter as a result of intracellular acidification plays a major role in

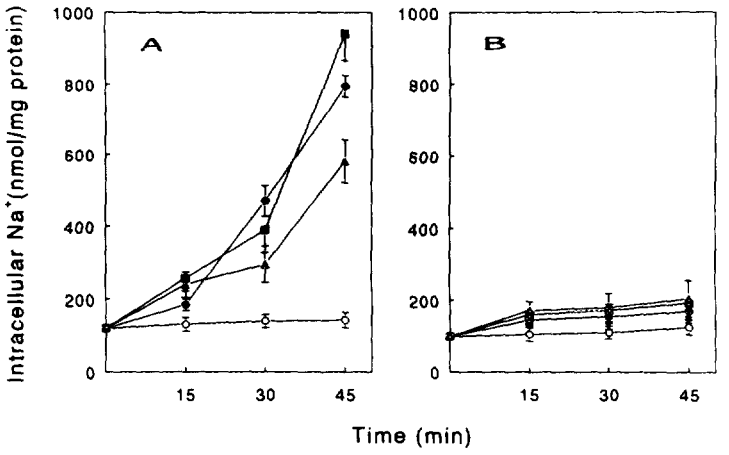

Figure 1. Changes in intracellular Na^+ content of isolated hepatocytes exposed to hypoxia or treated with mitochondrial inhibitors in the presence (Panel A) or in the absence (Panel B) of extracellular Na^+. Liver cells were incubated under an atmosphere of 95% O_2 5% CO_2 without any addition (○), with 1 mM KCN (■) or 25 μM CCCP (●). Hypoxic conditions were obtained by fluxing the cell suspension with 95% N_2 5% CO_2 (▲). For the experiments performed in the the absence of Na^+, NaCl in the incubation medium was substituted with equimolar amount of choline chloride. The values are means of 3-4 different experiments ± S.D.
The difference between control and hypoxic or KCN and CCCP-treated hepatocytes in panel A is statistically different (p value ranging from 0.01 to 0.001).

causing Na^+ accumulation in isolated hepatocytes where mitochondrial damage was induced by menadione (17).

Cytofluorimetric analysis of hepatocyte suspensions incubated for different times with KCN or CCCP revealed that the forward scatter parameter, indicative of cell volume, was only moderately shifted toward higher values after 15 min of incubation. (Fig. 2). However, 30-45 min from the addition of CCCP or KCN a large shift was evident (Fig. 2). Previous studies have shown that the inhibition of cell respiration by anoxia or KCN is followed by the formation of blebs on the plasma membrane due to alterations of cytoskeletron structure caused by the increase of cell Ca^{2+} and by the lack of ATP (9,18). However, the changes of hepatocyte shape due to plasma membrane blebbing were not the main responsibles for shift in the forward scatter of hepatocytes treated with KCN or CCCP, since no increase in cell volume was appreciable after 15 min of incubation when extensive blebbing was already present (Fig. 2). The replacement of NaCl with choline chloride in the incubation medium largely prevented the shift in forward scatter observed in isolated hepatocytes incubated with KCN or CCCP without influencing blebs formation (Fig. 2). This indicated that the increase in intracellular Na^+ was associated with an appreciable swelling of hepatocytes likely due to the osmotic effect of Na^+ overload. Consistently, Berger and coworkers have reported that a 2-4 fold elevation of cytosolic Na^+ levels occurs in hepatocyte exposed to anoxia and precedes an increase in the water content of the cells (5). We have also observed that the omission of Na^+ from the incubation medium significantly decreased cell death caused by hypoxia, KCN or CCCP (Table

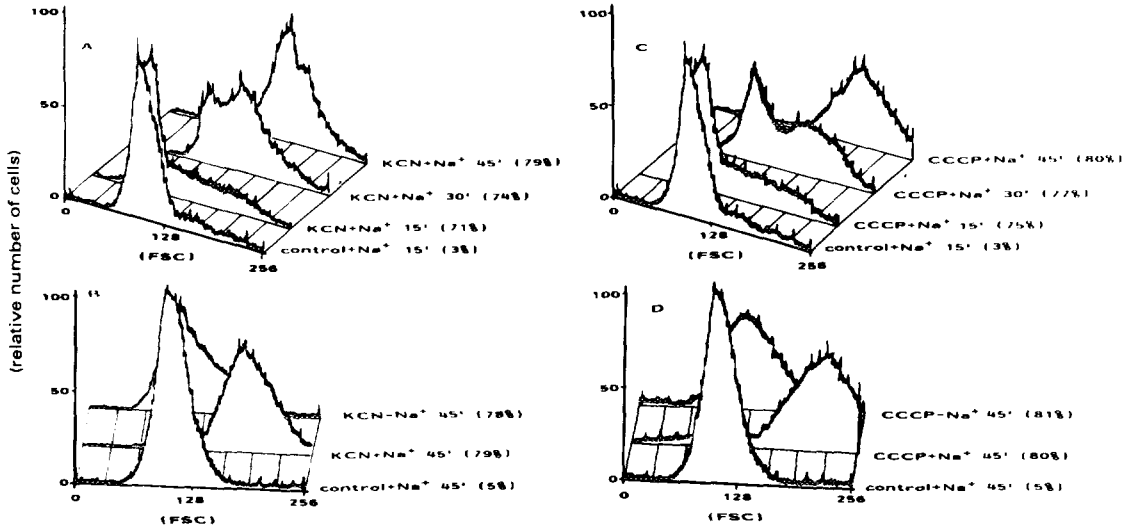

Figure 2. Changes in hepatocyte volume during the incubation with mitochondrial toxins in the presence or in the absence of extracellular Na^+. The variations in hepatocyte volume are expressed by the shift in the distribution frequency of forward light scatter (FSC) in control cells incubated without any additions or in hepatocytes exposed to 1 mM KCN (Panel A) or 25 μM CCCP (Panel C) in the a medium containing 118 mM NaCl. Panels B and D show the volume distribution frequency in isolated hepatocytes incubated 45 min with or without KCN or CCCP in, respectively, a standard Krebs-Henseleit medium or medium where NaCl was replaced by equimolar amounts of choline chloride. The values under parentheses represent the percentage of blebbed cells counted by microscopical observation in the same cell population. One experiment typical of three.

Table 2

Viability of isolated hepatocytes exposed to hypoxia or treated with mitochondrial inhibitors in the presence or in the absence of extracellular Na^+

	Percent of cells excluding Trypan blue	
	+ Na^+	- Na^+
Control	86 ± 7	84 ± 5
Hypoxia	3 ± 6	63 ± 11 *
KCN 1 mM	7 ± 4	69 ± 10 *
CCCP 25 μM	5 ± 3	70 ± 13 *

Hepatocytes were incubated 60 min under an atmosphere of 95% O_2, 5% CO_2 without any addition, with 1 mM KCN or 25 μM CCCP. Hypoxic conditions were obtained by fluxing the cell suspension with 95% N_2, 5% CO_2. For the experiments performed in the the absence of Na^+, NaCl in the incubation medium was substituted with equimolar amount of choline chloride. The values are means of 3-4 different experiments ± S.D.

* Difference statistically significant vs cell incubated in the presence of NaCl ($p < 0.001$).

2), without interfering with the loss of cellular energization (Table 1). Taken together, these results support morphological observations concering the swelling of hepatocytes undergoing metabolic inhibition (5,9) and suggest that, the osmotic stress induced by an uncontrolled accumulation of Na^+ might be associated to the events precipitating irreversible membrane lesions.

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