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Low O_2 and high CO_2 in LLC-PK1 cells culture mimics renal ischemia-induced apoptosis

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Ischemia, absence or loss of blood flow in organs always presents as a dual phenomenon: tissue oxygen deficit and CO_2 excess (hypercapnia). Commonly hypoxic cell culture models kept CO_2 at normal nonischemic values. We report a study of apoptosis in an *in vitro* model of renal hypoxia that mimics *in vivo* tissue gas atmosphere composition determined during experimental ischemia in rat kidney (low O_2 plus high CO_2). Renal tubular LLC-PK1 cell were transiently exposed to hypoxia, to hypercapnia or to both conditions (simulated ischemia). Exposure to simulated ischemic atmosphere, but not to low O_2 or high CO_2 alone, induced cell apoptosis *in vitro*. This suggests that ischemia-induced apoptosis *in vivo* would be dependent on the natural, joint action of hypoxia and hypercapnia. This should be taken into account in cell culture studies that would like to mimic *in vivo* ischemic conditions.
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Apoptosis or programmed cell death may play a significant role in the pathogenesis of relevant ischemic diseases.[1] This finding has been considered to be of great importance in pathology, including ischemic acute renal failure (ARF) due to tubular dysfunction;[2,3] partly, because, instead of rapid ischemic cell death by necrosis, attenuation of delayed cell death by apoptosis is potentially feasible, thus suggesting new approaches to therapy.[4,5]

In vitro cell culture models of ischemic ARF have become a basic tool to gain an insight into the mechanisms triggering and regulating tubular cell apoptosis.[3,6] Overall, *in vitro* models mimic some of the features of tissue ischemia that may be linked to the regulation of cellular pathways toward apoptosis, such as ATP depletion.[6] However, as *in vivo* tissues cannot be reproduced precisely, the results obtained should be transferred with caution to real-life physiological situations. Also, in *in vitro* models, one runs the risk of dismissing factors of physiological significance as regulatory stimuli influencing apoptosis. This is the case in the regulation of CO_2.

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When blood supply to an organ is blocked or impaired, leading to tissue hypoxia, low O_2 is always accompanied by CO_2 excess (hypercapnia).[7] CO_2 values increase as a result of bicarbonate buffering of anaerobic acid generation, coupled with CO_2 retention due to impaired perfusion.[7,8] Hypercapnia influences a number of cell features that have been closely linked to the apoptotic process, such as mitochondrial metabolism and intracellular pH.[9,10] However, until now, cell culture models of renal ischemia/reperfusion (I/R) injury have not considered the possible dependence of apoptosis on ischemic hypercapnia. Moreover, models of renal cell hypoxia/reperfusion injury submit cells to hypoxia or chemical anoxia, but unlike physiological ischemia, in general retain, CO_2 concentrations at normal nonischemic values during the cultured simulated ischemic period.[11,12] This implies that metabolically produced CO_2 is being continuously washed (open bicarbonate/CO_2 system), thus avoiding CO_2 accumulation and its potential action.

In the present study, we hypothesized that, during *in vitro* cell culture models of ischemia, not only hypoxia but also high CO_2 could influence cell apoptosis. We show here, using a renal tubular epithelial LLC-PK1 cell culture model of I/R, that a step toward well-established ischemic physiological conditions as represented to superimpose hypercapnia to hypoxic exposition, induces the appearance

of apoptotic cell death features. This suggests that tissue CO_2 levels may play a significant role in determining the mechanism of cell death associated to hypoxia.

Material and methods

In Vivo Study

Animals and protocol

The study on rats was conducted under the supervision of our institution's ethics commission and conformed to EU guidelines for the handling and care of laboratory animals. Briefly, 12 male Wistar rats weighing 250–300 g (Ifa Credo, Spain), six controls and six submitted to 30 min of unilateral left kidney ischemia were anesthetized with sodium pentobarbital (50 mg/kg). Polyethylene cannula (SPE-50, Clay Adams, Sparks, MD, USA) were inserted through the left carotid artery into the aorta for blood sampling. From arterial blood samples (0.15 ml) pre-ischemic pH, gas composition and plasma bicarbonate were determined (BMS3 MK2 Blood Microsystem and EML100 Electrolyte Metabolite Laboratory, Radiometer, Denmark). To induce 30 min kidney ischemia, laparotomy was performed and the left renal pedicle was dissected and occluded with a non-traumatic microvascular clamp.

Microelectrode pH measurement and pCO_2 calculation

Intrarenal pH was continuously measured, during the ischemic period, as previously described.[13] Briefly, miniaturized ISEs probes were inserted into the renal cortex after anteperitoneal exposure of the kidney. The monitoring assembly used, based on Microsystems Technology, was provided by the Centro Nacional de Microelectrónica (CNM) institute. Electrodes were calibrated with saline solutions and precision buffers, and the time responses of these electrodes were under 5 s in all circumstances. Cortical pCO_2 values were calculated from tissue pH measurements, assuming constant ischemic tissue bicarbonate during complete renal circulatory arrest, corresponding to preischemic arterial values. This is a common assumption in acid–base splanchnic physiology, and, although certainly arguable,[8] it should be considered that more precise CO_2 determinations would be needless for the purpose of our study. CO_2 values found were expressed as partial pressures in SI units (kPa). As gas mixtures used are also commonly expressed in %, it may be useful to recall that 1% dry gas is equivalent to 0.95 kPa (at 37°C, 1 atm, saturated with water vapor). So, kPa units are roughly equivalent to percent gas mixture.

In Vitro Study

Cell culture

Proximal tubular porcine LLC-PK1 cells (obtained from Professor Dr Bernhard Brüne) were cultured in Dulbecco modified Eagle medium (DMEM) with low glucose, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (Biochrom, Germany). Cells were transferred two times each week. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C.

Exposure to hypoxia/hypercapnia/simulated ischemia

Cultures were exposed transiently (7 h) either to low oxygen and/or high CO_2 gas atmospheres, followed by a return (48 h) to normal culture conditions (5% CO_2, air balance). Cell plates were incubated at 37°C in an open chamber, in which humidified-thermally equilibrated gas mixtures of controlled composition were continuously flushed. Six experimental groups were investigated:

Group I — Control group: 5% CO_2 in air

Group II — Hypoxic group (Hyp + 5%CO_2): 0.5% O_2–5% CO_2, N_2 balance.

Group III — Hypoxic + hypercapnic group (Hyp + 18%CO_2): 0.5% O_2–18% CO_2, N_2 balance.

Group IV — Hypoxic + hypercapnic group (Hyp + 30%CO_2): 0.5% O_2–30%, N_2 balance.

Group V — Hypercapnic group (CO_2 (18%)): 20% O_2—18% CO_2, N_2 balance.

Group VI — Hypercapnic group (CO_2 (30%)): 20% O_2–30% CO_2, N_2 balance.

Final setting gas values $\pm$1% within chamber was always attained within a 15 min time period.

Ternary (O_2, CO_2, N_2) precision gas mixtures from gas cylinders were used (AL Air Liquide España S.A.). Gas was conveyed to the environmental chamber through a valve controlled flowmeter system (Control Module 208-01, Instrumentation Laboratory Inc., Barcelona, Spain) and a humidifier (at 37°C). Oxygen and carbon dioxide partial pressures in the environmental chamber gas were continuously monitored by means of a flowthrough CO_2 electrode (Orion Research, Inc., Beverly, MA, USA) and a galvanic O_2 sensor (Engineered System & Designs, Newark, DE, USA), calibrated and frequently checked during the experiment with humidified precision gases. The experimental procedure also included a preliminary study to test if gas equilibrium was also attained inside cell plates placed in the environmental chamber. Continuous O_2 measurement within cell plates, using an oxygen microelectrode, (Microelectrodes, Londonderry, Inc., NH, USA), showed that hypoxic values below 1% O_2, were reached in less than 10 min when the initial gas flow was equal to or higher than 0.7 l/min^{-1}. Therefore, gas flow was initially set to this value for experimental procedures.

Assessment of Apoptotic Events

Caspase-3-like activity
Caspase 3-like activity was determined by measuring the proteolytic cleavage of the specific substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC; Biomol, Plymouth Meeting, PA, USA). Cells were lysed and sonicated in an assay buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% (w/v) CHAPS, 1 mM PMSF, 1 mM dithiothreitol). Cell supernatants (250 μg protein) were incubated in an assay buffer with 50 μM Ac-DEVD-AMC. The AMC released was quantified for 2 h at 37°C by fluoro-spectrophotometry using 380-nm excitation and 450-nm emission.

Diphenylamine assay
DNA fragmentation was determined by a modification of the method reported by Ikeda *et al*.[14] Briefly, cells were centrifuged, washed in PBS and harvested by centrifugation at 3000 r.p.m. for 5 min. Pellets were lysed by adding 400 μl of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8.0) containing 0.5% Triton X-100. Samples were allowed to lyse for 45 min at 4°C prior to centrifugation (15 000 r.p.m., 20 min) to separate intact chromatin (pellet) from DNA fragments (supernatant). The supernatant was placed in a separate microfuge tube and both fraction volumes were equalled and then precipitated and homogenized in 400 μl of 1 N perchloric acid for 30 min at 4°C. Precipitates were sedimented at 15 000 r.p.m. for 20 min. The supernatant was discarded and pellets resuspended with 100 μl of 1 N perchloric acid and diphenylamine reagent.[15] Samples were boiled for 5 min to hydrolyze DNA. The percentage of DNA fragmentation was defined as the ratio of the DNA content in the supernatant to the total DNA in the lysate.

DAPI staining
Nuclear changes such as chromatin condensation and fragmentation were analyzed by staining with DAPI, which stains nuclei (apoptotic or viable) blue under DAPI filter. Briefly, cells were fixed in 4% paraformaldehyde, washed with PBS, incubated with DAPI solution (0.2 μg/ml). for 5 min at room temperature. Cells were then rinsed twice in PBS, mounted and viewed by fluorescence microscopy.

TUNEL staining
Apoptosis was detected *in situ* by enzymatic labelling of DNA strand breaks using the Apotag Peroxidase *In situ* Apoptosis Detection Kit (Serologicals Corporation, CA, USA). Fixed cells were processed and incubated following the manufacturer's instructions. After that, samples were counterstained with hematoxylin, coverslipped and subsequent by visualized by light microscopy.

Western blot analysis
Cleavage of caspase 3 was detected in cell lysates by Western blot analysis. Following protein separation in 16% SDS-gel, proteins were transferred to nitrocellulose membranes, which were subsequently blocked with 5% nonfat dry milk in 0.06%. Tween-TBS for 1 h. Membranes were incubated with primary antibody (anti caspase-3 at 1:1000 dilution; Stressgen, Canada) overnight at 4°C. Membranes were washed five times with 0.06% Tween-TBS and then incubated for 1 h with a horseradish peroxidase conjugated anti-rabbit IgG antibody (dilution 1:5000) at room temperature. Membranes were washed five times with 0.06% Tween-TBS, followed by ECL-detection.

Statistical Analysis

Data are expressed as means $\pm$ s.e.m. The means of different groups were compared using one-way ANOVA. Student–Newman–Keuls test was used to evaluate significant differences between groups. Significant differences were assumed when $P<0.05$.

Results

In Vivo pCO_2

Figure 1 shows the evolution of cortical pCO_2 values during 30 min ischemia.

Renal vascular occlusion induced an immediate rapid rise in tissue pCO_2, reaching mean values over 15 kPa at 5 min of ischemia. A plateau phase, with a mean ischemic pCO_2 of in the order of 30 kPa, was reached after approximately 15 min of ischemia.

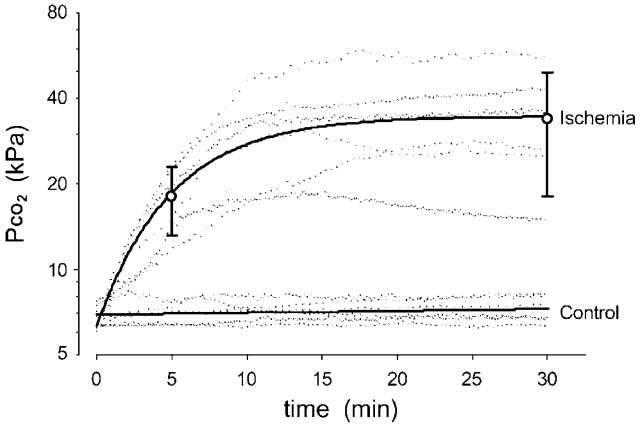

Figure 1 Effect of ischemia on intrarenal pCO_2. Exponential and lineal regression are fitted to ischemic and control semilogarithmic data plots showing individual experiments ($N=6$). pCO_2 mean values and 95% confidence intervals for early (5 min) and final (30 min) ischemic values are pointed up.

Apoptotic Indicators

Figure 2 shows caspase-3 activity during the recovery period. Significant variations were only seen in samples exposed to 'ischemic atmosphere' (hypoxia and hypercapnia together), either corresponding to short (18%CO_2) or long *in vivo* ischemia (30%CO_2). The large increase in the activity corresponding to hypercapnia of 18% CO_2 (Hyp + 18%CO_2) was note worthy. No changes were found in cultures submitted to low O_2 or high CO_2 exposition alone.

This enzymatic activity was concomitant with the protein profile as shown in the Western blot analysis of cleaved caspase 3 (Figure 3) corresponding to 17 kDa fragment. In the Hyp + 18%CO_2 and Hyp + 30%CO_2 groups, marked increases of cleaved caspase-3 expression were detected, but no increases were detected in the cultures exposed either to low O_2 or to high CO_2 independently.

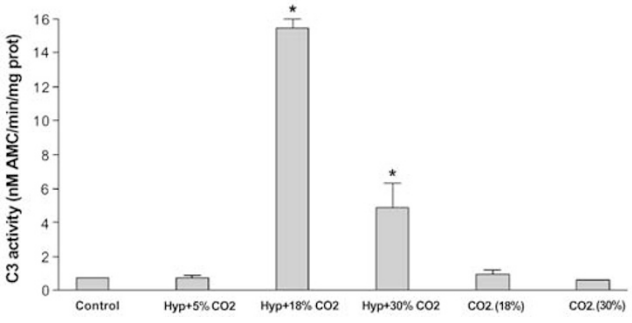

Figure 2 Caspase-3 activity (nmols AMC/mg protein/min) in cell lysates of the following groups: Control, Hyp + 5%CO_2, Hyp + 18%CO_2, Hyp + 30%CO_2, CO_2 (18%), CO_2 (30%). *$P<0.05$ *vs* Control.

Analysis of DNA fragmentation corroborated the previous results (Figure 4), showing that significant changes were restricted to groups with increases in caspase-3 activity.

With DAPI staining (Figure 5), viable cells that represent the large part of the total population showed homogeneous staining of their nuclei. In contrast, apoptotic cells showed irregular staining as a result of chromatin condensation and nuclear fragmentation (Figure 5b, c and e). This irregular staining was detected mainly in Hyp + 18%CO_2 (Figure 5b) and Hyp + 30%CO_2 (Figure 5c) groups.

In order to support DAPI staining and to discriminate better the apoptotic morphology, we also used TUNEL reaction (Figure 6) as one of the most commonly used techniques to detect apoptosis. With this technique, positive apoptotic cells showed a dark brown stain together with loss of the typical cell morphology. These cells were again detected mainly in Hyp + 18%CO_2 (Figure 6b) and Hyp + 30%CO_2 (Figure 6c) groups.

Discussion

Ischemia due to circulatory failure in organs has been defined as dual defects of O_2 deficits and CO_2 excesses, and it is likely that both factors impair tissue and organ function.[7] Hypercapnia modifies different cell issues linked to the apoptotic process, such as cytosolic pH and osmolality.[16] Taking these premises together, it could be expected that hypercapnia might play a shared role with hypoxia on

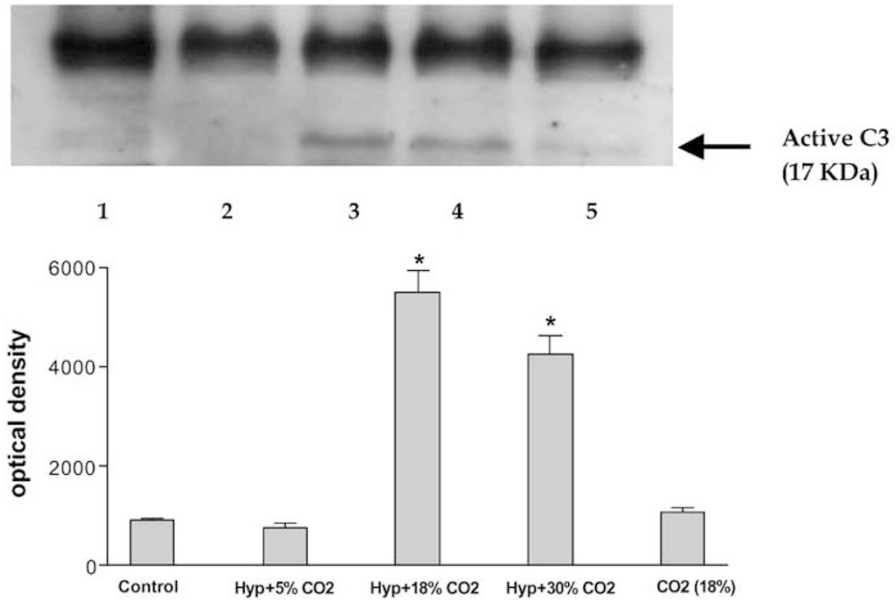

Figure 3 Cleaved caspase-3 identification by Western blot analysis. Blots illustrate representative experiments for control (lane 1), Hyp + 5%CO_2 (lane 2), Hyp + 18%CO_2 (lane 3), Hyp + 30%CO_2 (lane 4) and CO_2 (18%) (lane 5). Bottom panel: densitometric quantification.

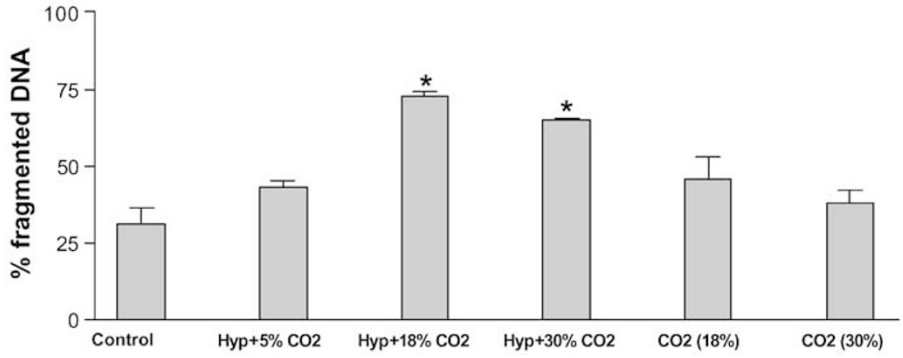

Figure 4 DNA fragmentation (%) in cell lysates of the following groups: Control, Hyp + 5%CO_2, Hyp + 18%CO_2, Hyp + 30%CO_2, CO_2 (18%), CO_2 (30%). *$P<0.05$ *vs* Control.

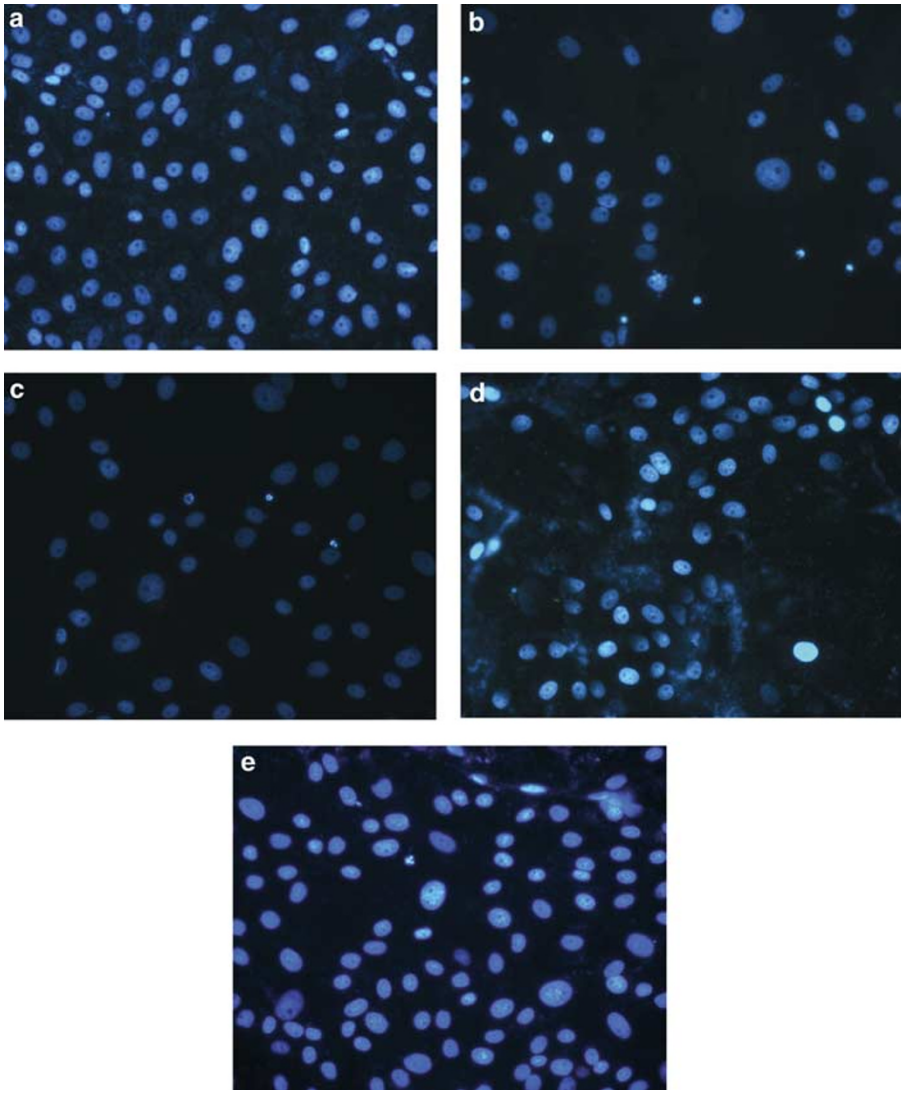

Figure 5 Representative DAPI staining showing homogeneous staining of the nucleus in viable cells and irregular staining with small and highlighted bodies as a result of chromatin condensation and nuclear fragmentation of the following groups. Control (**a**), Hyp + 18%CO_2 (**b**), Hyp + 30%CO_2 (**c**), CO_2 (18%) (**d**), CO_2 (30%) (**e**). Magnification $\times 40$.

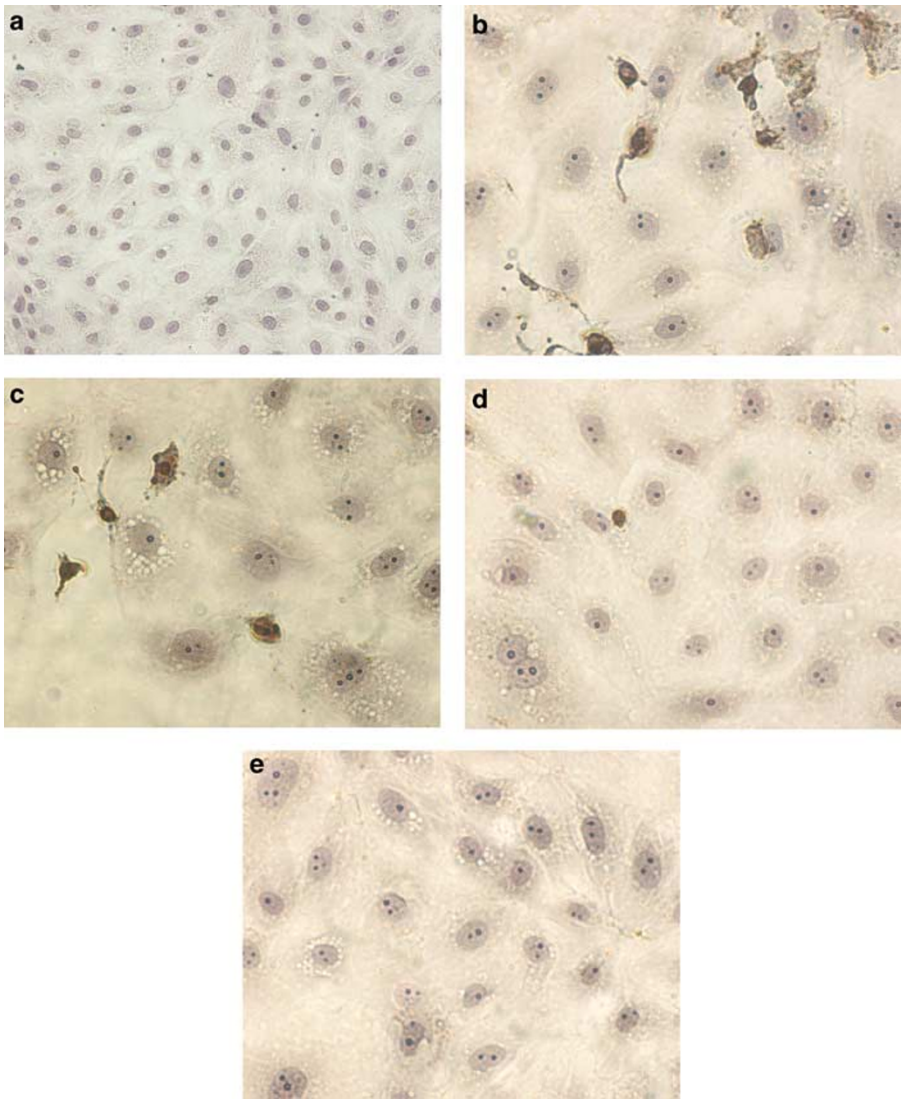

Figure 6 Representative TUNEL staining showing positive cells as a dark brown stain. Control (**a**). Magnification $\times 20$; Hyp + 18%CO_2 (**b**), Hyp + 30%CO_2 (**c**), CO_2 (18%) (**d**), CO_2 (30%) (**e**). Magnification $\times 40$.

ischemia-mediated cell death by apoptosis. However, the possible dependence of apoptosis on physiological hypercapnia in *in vitro* culture models of I/R injury has not been considered.

The current study reports as a new finding that, in a renal cell culture model of I/R, in which we reproduced tissue O_2 and CO_2 gas tensions found during experimental ischemia *in vivo*, the appearance of apoptotic signals is dependent not only on hypoxic but also on hypercapnic conditions.

In Vitro vs In Vivo 'Ischemic' Gas Atmosphere

Ischemic and critical renal oxygen tensions have been well established.[17–19] Taking into account these studies, we used a 0.5% O_2 gas atmosphere to keep the cell culture hypoxic below critical and in the range of oxygen pressures measured during acute renal arterial occlusion in experimental animals. We avoided the use of anoxia and, also, of commonly used gas mixtures over 1% O_2 for hypoxic cell culture; the use of anoxic gas induce the expression of cell transcription factors different from physiological hypoxic conditions.[20] By contrast, hypoxic O_2 gas mixtures over 1% may not be suited to reproduce *in vivo* ischemia conditions, as lower cortical oxygen pressures have been measured during acute renal arterial occlusion in experimental animals.[19]

Contrary to other organs, intrarenal CO_2 studies during ischemia are limited and discordant. To extend the *in vivo* frame of reference to set up *in vitro* simulated ischemic CO_2 conditions, we determined intrarenal CO_2 values during 30 min of experimental ischemia in rat kidney (Figure 1). This period of complete renal ischemia is based on previous studies that showed the presence of renal apoptotic signals.[21]

From the above, we decided to simulate ischemia *in vitro* by means of two different hypoxic–hypercapnic gas mixtures. The first mixture containing 18% CO_2 represents values found during early ischemia (5 min). The second with 30% CO_2 replicates mean stationary CO_2 values found during prolonged ischemia (30 min). Both 5 and 30 min of arterial clamping in the rat kidney have been reported to induce the presence of apoptotic signals after 24 and 48 h of reperfusion.[21]

Apoptotic LLC-PK1 Cell Features

Caspase-3 activation seems to be a defining feature of apoptotic LLC-PK1 cell death.[22,23] We found here large caspase-3 activity increases after exposure to hypercapnic hypoxia. Changes were not found in cultures submitted either to hypoxia or to hypercapnia independently (Figure 2). Western blot analysis of cleaved caspase 3 confirmed enzymatic activity assays (Figure 3). Additionally, analysis of DNA fragmentation showed the same tendency (Figure 4). All these results were supported by morphological criteria of apoptosis, as cells showing nuclear apoptotic morphology revealed by DAPI staining (Figure 5) and by positive TUNEL staining (Figure 6). Although we were unable to find any apoptotic signal in cells exposed to low O_2 alone, other similar hypoxic studies have shown apoptotic features in LLC-PK1 cells.[12,24,25] Disagreement with our finding may be due to the different model used, either chemically induced ATP or hypoxic gas exposure in our model. Interestingly, the work from Meldrum *et al*[26] supports our data. This model simulates tubular kidney ischemic conditions by immersing the monolayer cells in mineral oil. Then the oxygen is restricted but probably also CO_2 washout could be prevented, generating hypercapnic hypoxia. Nevertheless, this work does not discern whether there is a specific CO_2 effect on apoptosis.

Basis of CO_2 Action

Unlike O_2 deprivation, the hypercapnic influence on apoptosis has barely been considered. The question of which are the factors and the key steps in the apoptotic pathway that mediate the proapoptotic CO_2 effect opens multiple approaches for further research. High CO_2 influence on apoptosis could be indirect, mediated by factors such as intracellular hydrogen ion, or direct on cell metabolic pathways.

As an indirect mechanism, it should be mentioned that a lowering of intracellular pH-mediated CO_2 increase may be of special significance in LLC-PK_1 cell cultures, as it has been shown that in this cell line, intrinsic intracellular buffering (buffering power *vs* CO_2 changes) is the lowest reported for any cell.[27]

It is important to point out that the CO_2 acidifying effect differs essentially from extracellular acidification by the addition of exogenous acid, leading to different experimental models. As CO_2 diffuses easily through cell membranes, its pH influence would be immediate in all intracellular compartments, whereas intracellular acidification through extracellular acid load entails the contribution of cell membrane acid–base equivalent exchangers.

As direct actions (not pH mediated) of hypercapnia on cell metabolism, it was found that changing the environmental CO_2 concentration had a direct influence on kidney cell glucose metabolism and on mitochondrial ATPase activity in cortical renal tissue, whereas medullar structures contained cells that did not respond to CO_2 changes.[28,29] Changes in glycolytic ATP synthesis and mitochondrial ATPase activity have been shown to influence renal cell apoptosis.[30,9]

In summary, the essential new finding of this study is that CO_2 levels play a significant role in determining apoptosis during *in vitro* ischemia. Therefore, not only hypoxia but also hypercapnia must be considered when *in vitro* studies are used to try to reproduce *in vivo* ischemia.

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