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Hypoxia-Induced Increase in Intracellular Calcium Concentration in Endothelial Cells: Role of the Na^+-Glucose Cotransporter

Nancy Berna, Thierry Arnould, José Remacle, and Carine Michiels*

Laboratoire de Biochimie et Biologie Cellulaire, Facultés Universitaires Notre-Dame de la Paix, 61, rue de Bruxelles, B-5000 Namur, Belgium

Abstract Hypoxia is a common denominator of many vascular disorders, especially those associated with ischemia. To study the effect of oxygen depletion on endothelium, we developed an in vitro model of hypoxia on human umbilical vein endothelial cells (HUVEC). Hypoxia strongly activates HUVEC, which then synthesize large amounts of prostaglandins and platelet-activating factor. The first step of this activation is a decrease in ATP content of the cells, followed by an increase in the cytosolic calcium concentration ($[Ca^{2+}]_i$) which then activates the phospholipase A_2 (PLA_2). The link between the decrease in ATP and the increase in $[Ca^{2+}]_i$ was not known and is investigated in this work. We first showed that the presence of extracellular Na^+ was necessary to observe the hypoxia-induced increase in $[Ca^{2+}]_i$ and the activation of PLA_2. This increase was not due to the release of Ca^{2+} from intracellular stores, since thapsigargin did not inhibit this process. The Na^+/Ca^{2+} exchanger was involved since dichlorobenzamil inhibited the $[Ca^{2+}]_i$ and the PLA_2 activation. The glycolysis was activated, but the intracellular pH (pH_i) in hypoxic cells did not differ from control cells. Finally, the hypoxia-induced increase in $[Ca^{2+}]_i$ and PLA_2 activation were inhibited by phlorizin, an inhibitor of the Na^+-glucose cotransport. The proposed biochemical mechanism occurring under hypoxia is the following: glycolysis is first activated due to a requirement for ATP, leading to an influx of Na^+ through the activated Na^+-glucose cotransport followed by the activation of the Na^+/Ca^{2+} exchanger, resulting in a net influx of Ca^{2+}. J. Cell. Biochem. 84: 115–131, 2002.

Key words: hypoxia; endothelial cells; intracellular calcium concentration; Na^+-glucose transporter; Na^+/Ca^{2+} exchanger

Endothelium plays a central role in many physiological systems such as angiogenesis, vasoregulation, hemostasis, vascular permeability, and synthesis of biologically active factors. Given its location at the luminal vascular surface, endothelium must be able to respond to a wide range of environmental alterations. One of these is the hypoxic condition which is a commonly associated with many vascular disorders, especially those initiated by ischemia. The endothelial cell response to hypoxia is thus of considerable interest [Michiels et al., 2000].

Many of the biochemical changes occurring in endothelium under hypoxia have been observed using in vitro primary endothelial cell cultures. Indeed, endothelial cell monolayers can be maintained alive in hypoxic conditions, but the proliferation is suppressed and the permeability and cell surface procoagulant properties are increased [Ogawa et al., 1990]. Previous studies on hypoxic endothelial cells also demonstrated an increased production of platelet-activating factor (PAF), which is involved in the adherence of neutrophils to endothelial cells [Arnould et al., 1993], increased xanthine oxidase and xanthine dehydrogenase activities [Terada et al., 1992] and an alteration in cyclo-oxygenase metabolism [Farber and Barnett, 1991; Michiels et al., 1993].

To study the effect of oxygen depletion on endothelium, Michiels et al. [1992] developed an

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*Correspondence to: Carine Michiels, Laboratoire de Biochimie et Biologie Cellulaire, Facultés Universitaires Notre-Dame de la Paix, 61, rue de Bruxelles, B-5000 Namur, Belgium. E-mail: carine.michiels@fundp.ac.be

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in vitro model of hypoxia on human umbilical vein endothelial cells (HUVEC), where the cells are kept alive during 2 h of severe hypoxia. In this model, it was observed that hypoxia first leads to a decrease in the ATP content and then to an increase in the cytosolic calcium concentration ($[Ca^{2+}]_i$) [Arnould et al., 1992]. An increase in $[Ca^{2+}]_i$ was also observed in human aortic endothelial cells exposed to hypoxia [Hu and Ziegelstein, 2000]. Phospholipase A_2 (PLA_2) is then activated by calcium, leading to the synthesis of large amounts of prostaglandins [Michiels et al., 1993] and of PAF [Arnould et al., 1993]. A biochemical causal link between the decrease in ATP content and the increase in $[Ca^{2+}]_i$ was suggested since β-hydroxybutyrate, a ketone body which quickly supplies energy prevents the occurrence of both changes. Moreover, the decrease in ATP concentration preceded any change in $[Ca^{2+}]_i$. However, the relationship between these two parameters is still unclear. The purpose of this report was to investigate the relationship between the decrease in ATP content in HUVEC exposed to hypoxia and the subsequent increase in $[Ca^{2+}]_i$ which then leads to a cascade of cell activation.

In endothelial cells stimulated by vasoactive agents, an increase in $[Ca^{2+}]_i$ results from transient mobilization of Ca^{2+} from intracellular stores, followed by increased transmembrane Ca^{2+} influx from the extracellular space [Dolor et al., 1992; Inazu et al., 1995]. The entry of extracellular Ca^{2+} ions may occur via different routes. The Ca^{2+} entry across the plasma membrane is controlled either by receptor-mediated channels coupled to second messengers (phospholipase C pathway) [Clapham, 1995], by nonselective cation channels, by stretch-activated nonselective cation channels or by Na^+-Ca^{2+} exchangers [Adams et al., 1989; Himmel et al., 1993]. Furthermore, the controversial voltage-dependent Ca^{2+} channels of endothelial cells could also provide another Ca^{2+} entry route [Revest and Abbott, 1992].

In this report, we tested several hypotheses that could explain the hypoxia-induced increase in $[Ca^{2+}]_i$ in HUVEC. The first one is based on the observation that during hypoxia, glycolysis is activated to compensate for the decrease of ATP content, which is due to an inhibition of the mitochondrial oxidative phosphorylations. The production of lactic acid by glycolysis generates a large number of protons which could decrease the intracellular pH (pH_i). This decrease in pH_i could then stimulate the Na^+-H^+ exchange, causing an influx of Na^+. The influx of extracellular Ca^{2+} could be linked to the extrusion of Na^+ ions through the plasmalemmal Na^+-Ca^{2+} exchanger, which is a well-known major pathway for $[Ca^{2+}]_i$ increase. This mechanism was already demonstrated in cardiomyocytes [Satoh et al., 1995] and in whole hearts [Anderson et al., 1990]. A second possible mechanism linking changes in ATP content and $[Ca^{2+}]_i$ is based on the possible inhibition of the Na^+-K^+-ATPase pump due to a reduced ATP content. The accumulation of Na^+ ions in the cytosol could in turn lead to the activation of the Na^+-Ca^{2+} exchange, resulting in an increase in $[Ca^{2+}]_i$. That a rise in $[Na^+]_i$ can stimulate the Na^+-Ca^{2+} exchange has already been proposed for isolated myocytes exposed to hypoxia [Haigney et al., 1992]. A third possible mechanism is based on the higher glucose import during glycolysis compared to aerobic metabolism [Janssens et al., 1995]. Indeed, the ATP content is only reduced by two-fold during hypoxia, which suggests that glycolysis is strongly activated, and therefore that glucose consumption is strongly increased, since yield of ATP per molecule of glucose is much lower for glycolysis than for respiration. An increased influx of glucose into the cytosol through the Na^+-glucose cotransporter could lead to an accumulation of intracellular Na^+. This Na^+ loading could in turn activate the Na^+-Ca^{2+} exchanger, leading to increase in $[Ca^{2+}]_i$. These three hypotheses have been investigated using different channel blockers: the data support the third hypothesis which links the glucose entry and the Ca^{2+} influx in endothelial cells exposed to hypoxia.

MATERIAL AND METHODS

Chemicals

Modified Hank's balanced salt solution (140 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 4.3 mM $Na_2HPO_4 \cdot 2H_2O$, 0.4 mM KH_2PO_4, 5.5 mM glucose, pH 7.35) containing 1 mM $CaCl_2$ (HBSS) was prepared in our laboratory. 2, 4-dichlorobenzamil hydrochloride, 5-(N,N-hexamethylene) amiloride hydrochloride, 5-(N-ethyl-N-isopropyl) amiloride hydrochloride, Fura-2 acetoxymethyl ester (Fura-2 AM), and 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) were purchased from Molecular

Probes (Eugene, OR). Thapsigargin, nifedipine, ouabain, phlorizin, and ATP assay kit were purchased from Sigma (St Louis, CO). [3H]-arachidonic acid (specific activity = 212 Ci/mmol) and ^{45}Ca (specific activity 28 m Ci/mg) were from Dupont NEN (Boston, MA) and 2-deoxy-D-[2,6-3H]-glucose (specific activity = 52 Ci/mmol) were from Amersham Laboratories (Buckinghamshire, UK).

Human Umbilical Vein Endothelial Cell Isolation and Culture

HUVEC were isolated according to Jaffe et al. [1973]. Cords were stored at 4°C just after birth in stock solution (4 mM KCl, 140 mM NaCl, 10 mM Hepes, 1 mM glucose, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml fungizone, pH 7.3). Cords were rinsed with 30 ml phosphate buffer saline (PBS) containing antibiotics and fungizone at concentrations aforementioned. Umbilical veins were incubated 35 min at 37°C with 4 ml collagenase type II 0.05 % in M199. Collected cells were then seeded in M199 + 20 % fetal calf serum (Gibco, Praisley, Scotland), centrifuged 10 min at 1,000 rpm, and cultured in 0.2 % gelatine-coated culture dishes (Falcon Plastics, Oxnard, CA). The day after, cells were washed with medium in order to eliminate blood cell contamination. Only primary cultures were used for these studies. Confirmation of their identity as endothelial cells was obtained by detecting factor VIII antigen assessed by immunofluorescence staining.

In Vitro Model of Hypoxia

Ischemia was simulated by exposing cells to hypoxia (100% N2) at 37°C. HUVEC were seeded in gelatine-coated Petri dishes (diameter = 35 mm, Falcon Plastics, Oxnard, CA). For incubation, cells were rinsed twice with Hank's balanced salt solution containing 1 mM $CaCl_2$ (HBSS) and covered with 0.7 ml of HBSS in the presence or absence of the inhibitors. A 0.7 ml was chosen because more does not allow hypoxia to occur in the medium within 2h (the oxygen does not diffuse quickly enough) and less does not enough cover the cells. Hypoxia was produced with an atmosphere of 100% N_2 in an incubator gas chamber while the control cells were kept in normal atmosphere containing 20% O_2 in unhumidified chambers. Volume of the medium at the end of the 2 h incubation was measured and a decrease of less than 5% was observed. Normoxic controls were run in every experiment. PO_2 in the medium was 130 mm of Hg in normal conditions and dropped to 10 mm of Hg after 15 min of hypoxia as described here [Michiels et al., 1992]. Two hours of hypoxia was chosen because it is the maximal time of hypoxia that endothelial cells can sustain without loss of viability in these conditions. In all experiments performed which studied the effect of hypoxia, the assays were performed immediately after the hypoxia incubation (that means in less than 1 min for measurements of arachidonic acid release and just after 1 min for $[Ca^{2+}]_i$ and pH_i determinations).

Calcium Measurement

To measure $[Ca^{2+}]_i$, endothelial cells were seeded on a glass coverslip (13 × 27 mm, Belgolabo, Belgium) covered with gelatine 0.2% and placed on the bottom of a Petri dish (diameter = 35 mm). This coverslip fits perfectly in the cuvette of the fluorimeter. This allows the measurement of intracellular calcium concentration in intact cell monolayers [Wickham et al., 1988]. For loading with Fura-2 AM, cells were incubated during 1 h at 37°C in the dark with 1.5 ml of culture medium containing 10% fetal calf serum and 1.9 μl of pluronic acid (20 % in DMSO) and Fura-2 AM at a final concentration of 2 μM. Afterwards, cells were gently rinsed three times with HBSS and incubated under hypoxia as described above. For calcium assay, the coverslip was placed diagonally in a quartz cuvette filled with 3 ml of HBSS at room temperature. The cuvette was placed in a Kontron SFM-25 fluorimeter. Fluorescence was measured with emission signals detected at 510 nm and excitation wavelenght respectively at 340, 358, and 380 nm (= F). Thirty microliters of triton X100 10% in HBSS were then added to measure maximum fluorescence (Fmax), then 300 μl EDTA (50 mM in Tris 1 M pH 8.3) to measure minimum fluorescence (Fmin), and finally 3 μl of $MnCl_2$ 1 M to estimate the autofluorescence of the cells (AF). Calculations were performed according to Grynkiewicz et al. [1985] with a dissociation constant (Kd) for intracellular Fura-2 at 20°C of 135 nM.

Calcium Uptake

Seventy five thousand endothelial cells were seeded in Petri dishes (diameter = 35 mm). The day after, before incubation, cells were washed two times with 1 ml of HBSS, then 0.7 ml of

HBSS containing 2 μCi/ml ^{45}Ca was added and cells were incubated 30 min in normoxic or hypoxic conditions. After incubation, cells were rinsed three times with 1 ml 148 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.1 ml $LaCl_3$, 20 mM Hepes, pH 7.4 at 4°C and lysed with 0.7 ml NaOH 0.5 N [AbdAlla et al., 1996]. The radioactivity of the lysate was counted in a liquid scintillation counter after the addition of Aqualum (Lumac, Landgraaf, NL). Results are expressed in dpm.

[3H]-Arachidonic Acid Release

Fifty thousand endothelial cells were seeded in Petri dishes (diameter = 35 mm) and radiolabeled with 0.25 μCi/ml [3H]-arachidonic acid ([3H]-AA) during 18 h at 37°C. After this time, the cells were washed three times with 1 ml of HBSS, then 0.7 ml of HBSS was added and cells were incubated under hypoxia as described earlier. After incubation, the media were collected and counted for 3 min in a liquid scintillation counter after the addition of Aqualuma (Lumac, Landgraaf, NL). The total radioactivity of labeled cells was obtained by lysis with 0.7 ml of NaOH 0.5 N immediately after the three washings. The recovery of radioactivity was always higher than 95% when supernatant and lysate from incubated cells were counted separately. The percentage of arachidonic acid release was calculated as: 100 × number of dpm in the extracellular fluid/total radioactivity of labeled cells.

ATP Assay

ATP assay was performed using a bioluminescent ATP assay kit (FL-ASC; Sigma, St Louis) using luciferine and luciferase. Endothelial cells were grown to confluence in Petri dishes, exposed to hypoxia, rinsed with PBS, and permeabilized during a few seconds with 1 ml of the "somatic cell releasing reagent" also found in the kit. The lysates were collected at 4°C and the cells were recovered with NaOH 0.5 N for protein assay according to Lowry et al. [1951]. ATP assay was performed just as indicated by Sigma on a luminometer (Lumac Biocounter 2010, Switzerland). A standard curve was also performed and results were expressed as fmol ATP/μg proteins.

pH_i Measurements

For pH_i measurements, endothelial cells were seeded on a glass coverslip, as for calcium assays. For loading with BCECF-AM, cells were incubated during 45 min at 37°C in the dark with culture medium containing 10% fetal calf serum and BCECF-AM at a final concentration of 5 μM. Afterwards, cells were rinsed three times with HBSS and incubated under hypoxic conditions as described above. The coverslip was placed in the fluorimeter cuvette, and fluorescence was measured with emission signals detected at 535 nm and excitation signals at 505 nm (F505) and 439 nm (F439). pH_i was calculated from the F505/F439 ratio with a calibration curve. The high K^+-nigericin technique was used to obtain the calibration curve [Thomas et al., 1979]. Cells were exposed to solutions at different pH containing 105 mM KCl, 8 mM NaCl, 0.5 mM $MgSO_4$, 1 mM KH_2PO_4, 5 mM glucose, 10 mM Hepes, 1 mM $CaCl_2$, and 10 μM nigericin [Grynkiewicz et al., 1985]. After equilibration (5 min), the F505 and F439 were recorded. A plot of the F505/F439 ratio vs. pH yielded the calibration curve.

Glucose Transport Studies

Measurements of the rate of glucose transport were carried out during hypoxic or normoxic incubations using 2-deoxy-D-glucose, a nonmetabolizable glucose analog according to Janssens et al. [1995]. HUVEC seeded at confluence in Petri dishes were rinsed twice with HBSS and incubated under normoxia or hypoxia in 0.7 ml of HBSS containing 1 mM glucose and 1 μCi/ml of 2-deoxy-D-[2,6-3H]-glucose. The reaction was terminated by rapid removal of the media and by washing each dish three times with NaCl 0.15 M. The cell layer was let dry and then dissolved in 1 ml of NaOH 0.5 N overnight. The radioactivity of 200 μl aliquots neutralized with 200 μl of HCl 0.5 N was measured in 5 ml scintillation liquid (Aqualuma, Lumac, Landgraaf, the Netherlands). Two hundred microliter aliquots were also assayed for protein content according to Lowry et al. [1951]. The results are expressed in dpm/μg of proteins. They represent the amount of glucose incorporated in the cells at the end of the assay period.

Statistical Analysis

The data are presented as means ± 1 SD. Student's *t* tests were used for comparison of calcium measurements. Analysis of variance (ANOVA-1) was used for comparison within one experiment. When data from two experiments with different cell cultures from different

umbilical veins were combined, we took into account the inherent variability between the different primary cultures. The analysis of variance with two crossed factors (ANOVA-2), one which is random (the experiment number) and the other which is fixed (the type of treatment) was used to test for the equality of means. If the ANOVA-2 showed a similarity between means of each experiment, but a global difference between means of each treatment, the means for each treatment were compared one to each other by Scheffé's contrasts. One experiment in triplicate means that three different dishes were incubated and measured separately for each condition. All values of $P < 0.05$ were considered to be significant.

RESULTS

In order to investigate the origin of the hypoxia-induced increase in $[Ca^{2+}]_i$ in HUVEC, different channel blockers were tested. Due to the technical limits inherent to the $[Ca^{2+}]_i$ measurement and since changes in $[Ca^{2+}]_i$ directly regulate the PLA_2 activity in endothelial cells [Buckley et al., 1991], a wide concentration range of each channel blocker was first tested on the hypoxia-induced PLA_2 activation. Once the optimal concentrations were obtained, the effect of these inhibitors was then directly tested on the $[Ca^{2+}]_i$. All the incubations under hypoxia were performed within 120 min, where the mortality of the cells is very low but the hypoxia-induced increase in $[Ca^{2+}]_i$ maximal [Arnould et al., 1992].

Origin of Ca^{2+} Ions

To investigate the possible role of extracellular Na^+ in the hypoxia-induced increase in $[Ca^{2+}]_i$, the activation of HUVEC under hypoxia was followed in Na^+-containing and Na^+-free medium (NaCl was replaced by choline chloride and Na_2HPO_4 by K_2HPO_4) in normoxic and hypoxic conditions. After 120 min of hypoxia, the PLA_2 activity was two-fold higher than in normoxic conditions when Na^+ was present in the medium (Fig. 1A). In sodium-free medium, however, the hypoxia-induced PLA_2 activation was completely inhibited: there was no difference between normoxic and hypoxic cells. Figure 1B shows a marked increase in $[Ca^{2+}]_i$ during hypoxia. This increase was almost completely abolished in the absence of Na^+ in the incubation medium. These results demonstrate that extracellular Na^+ is necessary for the hypoxia-induced increase in $[Ca^{2+}]_i$ to occur and for the subsequent PLA_2 activation.

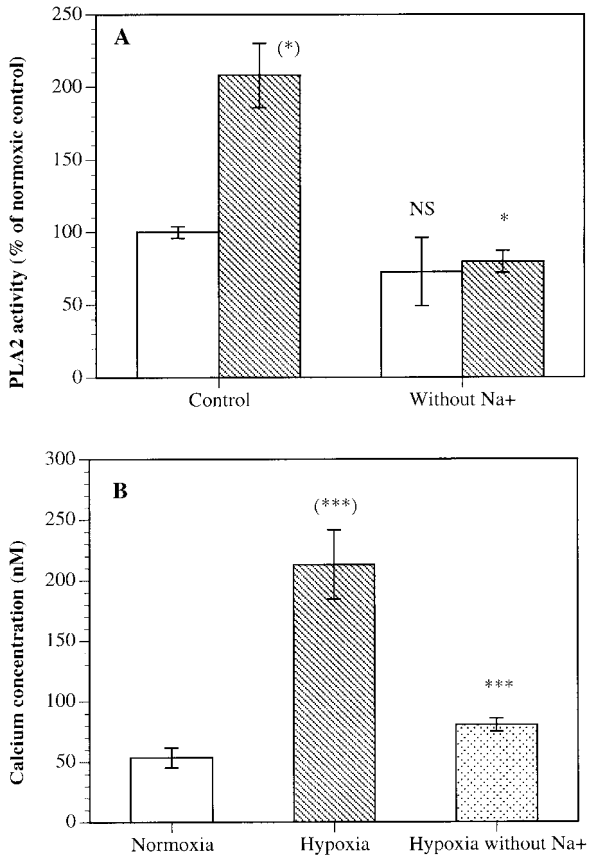

Fig. 1. A: Effect of sodium-free medium on the hypoxia-induced activation of PLA_2 HUVEC were incubated during 120 min under normoxic (□) or hypoxic (▨) conditions, in the presence (control) or in the absence of Na^+ in the medium. PLA_2 activity was estimated by the release of $[^3H]$-arachidonic acid in the medium during these incubations. Results are expressed as means ± 1 SD of percent of normoxic control for two experments performed in quadriplicated (n = 8). (*), significantly different from control cells incubated for 120 min under normoxia with $P < 0.05$. (NS), nonsignificantly different from control cells incubated for 120 min under normoxia. *, significantly different from control cells incubated for 120 min under hypoxia with $P < 0.05$. **B:** Effect of sodium-free medium on the hypoxia-induced increase in $[Ca^{2+}]_i$ HUVEC were incubated during 120 min under hypoxic conditions in the presence or in the absence of Na^+. Control cells were incubated during 120 min in normoxia. Results are expressed as means ± 1 SD for three separate experiments (n = 3). (***), significantly different from control cells incubated for 120 min in normoxia with $P<0.001$. ***, significantly different from control cells incubated for 120 min in hypoxia with $P < 0.001$ (Student's t test).

The increase in $[Ca^{2+}]_i$ can originate from several possible sources. There could be a Ca^{2+} influx from outside the cell through Na^+-Ca^{2+} exchangers or through voltage-gated channels. Calcium ions can also be released from

intracellular stores. Given the lack of $[Ca^{2+}]_i$ increase in the absence of extracellular Na^+, the Na^+-Ca^{2+} exchanger was the most obvious candidate.

Dichlorobenzamil, an analog of amiloride and well-described inhibitor of the Na^+-Ca^{2+} exchanger [Winquist et al., 1985], was able to inhibit in a dose-dependent way the PLA_2 activation induced by hypoxia (Fig. 2A). A significant effect (about 65% inhibition) was observed at 2 and 5 μM. At 10 μM, dichlorobenzamil was toxic for HUVEC as observed by phase contrast microscopy: cells rounded up but did not detach from the dish (data not shown). The effect of dichlorobenzamil at 2 μM was then examined on $[Ca^{2+}]_i$. As for arachidonic acid

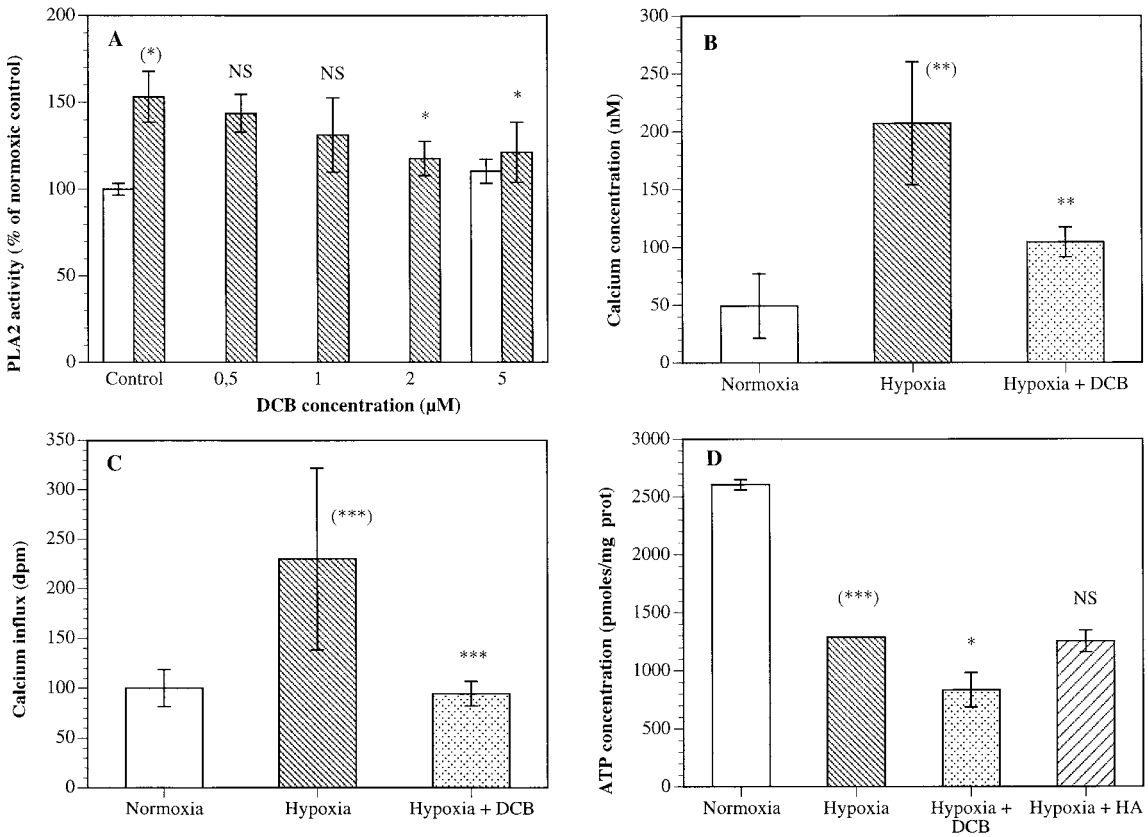

Fig. 2. **A:** Effect of dichlorobenzamil on the hypoxia-induced activation of PLA_2 HUVEC were incubated during 120 min under normoxic (□) or hypoxic (▨) conditions with different concentrations of dichlorbenzamil. Control cells were incubated without dichlorobenzamil. PLA_2 activity was estimated by the release of [^{3}H]-arachidonic acid in the medium during these incubations. Rsults are expressed as means ± 1 SD of percent of normoxic control for two experiments performed in triplicates (n = 6, except normoxia + 5 μM, n = 3). (**), significantly different from control cells incubated for 120 min under normoxia with $P < 0.01$. *, significantly different from control cells incubated for 120 min under hypoxia with $P < 0.05$. NS, nonsignificantly different from control cells incubated for 120 min under hypoxia. **B:** Effect of dichlorobenzamil on the hypoxia-induced increase in $[Ca^{2+}]_i$ HUVEC were incubated during 120 min under hypoxic conditions in the presence or in the absence of 2 μM of dichlorobenzamil. Control cells were incubated during 120 min in normoxia. Results are expressed as means ± 1 SD for n separate experiments (n = 3 for normoxia, n = 5 for hypoxia and n = 4 for hypoxia in the presence of DCB). (*), significantly different from control cells incubated for 120 min in normoxia with $P < 0.01$. ** significantly different from control cells incubated for 120 min in hypoxia with $P < 0.01$ (Student's t test). **C:** Effect of dichlorobenzamil on the hypoxia-induced influx in Ca^{2+}. HUVEC were incubated during 30 min under hypoxic conditions in the presence or in the absence of 2 μM of dichlorobenzamil. Control cells were incubated during 30 min in normoxia. The results are expressed as menas ± 1 SD of percent of normoxic control for three experiments performed in duplicates (n = 6). (***), significantly different from control cells incubated for 120 min in hypoxia with $P < 0.001$ (Student's *t* test). **D:** Effect of dichlorobenzamil (DCB) or hexamethylene amiloride (HA) on the hypoxia-induced decrease in ATP concentration. HUVEC were incubated during 120 min under hypoxia in the absence or in the presence of 2 μM of hexamethylene amiloride. Control cells were incubated during 120 min in normoxia. ATP concentration was assayed in HUVEC by the luciferine-luciferase system. Results are expressed in pmoles/mg proteins as means ± 1 SD (n = 3). (***), significantly different from control cells incubated for 120 min under normoxia with $P < 0.001$. **, significantly different from control cells incubated for 120 min under hypoxia with $P < 0.01$. NS, nonsignificantly different from control cells incubated for 120 min under hypoxia.

release, a significant inhibition (66%) was observed (Fig. 2B). Dichlorobenzamil also inhibited the hypoxia-induced Ca^{2+} influx as measured by ^{45}Ca labeling (Fig. 2C). These results strongly suggest that the Na^+-Ca^{2+} exchanger is involved in the hypoxia-induced increase in $[Ca^{2+}]_i$ and the subsequent PLA_2 activation.

However, we had to verify that dichlorobenzamil did not interfere with upperstream events of the HUVEC activation. For this purpose, we tested the effect of dichlorobenzamil on the ATP content of HUVEC. Dichlorobenzamil did not prevent the decrease in ATP content which could have explained the observed protection on PLA_2 activation (Fig. 2D). On the contrary, dichlorobenzamil even decreased further the hypoxia-decreased ATP content. This lower ATP content could be explained by the fact that by blocking the Na^+-Ca^{2+} exchanger, this inhibitor led to an accumulation of Na^+ ions in the cytosol, which could in turn stimulate the Na^+-K^+ ATPase pump, consuming more ATP molecules.

The inhibition of the Na^+-Ca^{2+} exchanger seemed to have a direct effect on the PLA_2 activation and on the increase in $[Ca^{2+}]_i$ but the inhibition was incomplete and other possible Ca^{2+} sources were investigated.

We first used thapsigargin, a sesquiterpene lactone recognized to selectively inhibit the Ca^{2+}-ATPase activity of the endoplasmic reticulum [Yamamoto et al., 1995], in order to evaluate the involvement of calcium intracellular stores. While PLA_2 activity was increased by 125% in hypoxic HUVEC compared to normoxic cells, thapsigargin (0.5 μM) was still able to increase further this activity by 58% in hypoxic HUVEC. This increment was the same as the one observed for normoxic HUVEC (+54%) (data not shown). Similar results were obtained in another independent experiment.

Although there is some controversy concerning the presence of voltage-gated Ca^{2+} channels in endothelial cells [Colden-Stanfield et al., 1987; Johns et al., 1987; Bossu et al., 1992], nifedipine, a specific inhibitor of these channels, was tested on HUVEC. Nifedipine had no effect on hypoxia-induced PLA_2 activation. Similarly, nifedipine had no effect on the hypoxia-induced increase in $[Ca^{2+}]_i$ (data not shown).

These results demonstrate that Na^+-Ca^{2+} exchanger is the major source responsible for the increase in $[Ca^{2+}]_i$ observed in HUVEC during hypoxia.

Origin of Na^+ Ions

The first results (Figs. 1 and 2) indicate that Na^+ is involved in the increase in $[Ca^{2+}]_i$ due to hypoxia, probably via a Na^+ influx. We thus investigated the possible Na^+ sources responsible for this influx.

During hypoxia, a decrease in the ATP content is observed. This decrease could induce an accumulation of intracellular Na^+ by lowering the activity of the Na^+-K^+ ATPase pump located in the plasma membrane. The involvement of this pump was tested using ouabain, a well-known specific inhibitor of the Na^+-K^+ ATPase pump. The inhibition of this pump is presumed to lead to a rise of $[Na^+]_i$, which could in turn induce the Na^+-Ca^{2+} exchanger to work in a reversed mode and thus induce an increase in $[Ca^{2+}]_i$ and in the PLA_2 activity. Figure 4 shows that between 20 and 200 μM, ouabain can effectively increase (by 26%) the PLA_2 activity in normoxic cells. If this pump is already inhibited by hypoxia, then no more effect of ouabain on PLA_2 activity is to be expected in hypoxic cells. This is not the case since ouabain at 100 μM was still able to increase by 37% the PLA_2 activity in hypoxic cells (Fig. 3). This effect

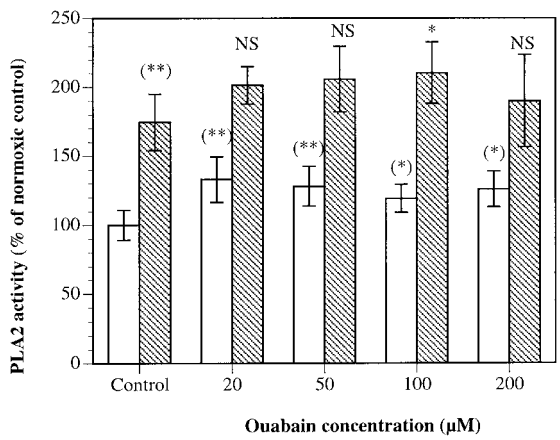

Fig. 3. Effect of ouabain on the hypoxia-induced activation of PLA_2. HUVEC were incubated during 120 min under normoxia (□) or hypoxia (▨) conditions with different concentrations of ouabain. Control cells were incubated without ouabain. PLA_2 activity was estimated by the release of [^{3}H]-arachidonic acid in the medium during these incubations. Results are expressed as means ± 1 SD of percent of normoxic control for three experiments performed in triplicates (n = 9). (*), (**), significantly different from control cells incubated for 120 min under normoxia with $P < 0.05$, 0.01. (NS), nonsignificantly different from control cells incubated for 120 min under normoxia. *, significantly different from control cells incubated for 120 min in hypoxia with $P < 0.05$. NS, nonsignificantly different from control cells incubated for 120 min under hypoxia.

is significant compared to hypoxia alone. The fact that the effect of ouabain and the effect of hypoxia are additive indicates that ouabain and hypoxia have independent effects on PLA_2 activity and that an inhibition of the Na^+-K^+ ATPase pump is not involved in the PLA_2 activation observed during hypoxia. This is in agreement with other data [Anderson et al., 1990], showing that higher ATP depletion that the one induced in the hypoxic conditions used here is necessary to inhibit the Na^+-K^+ ATPase pump.

A second possibility for Na^+ entry could be the result of the hypoxia-induced stimulation of glycolysis. Glycolysis produces lactic acid and generates a large amount of protons, which could lead to a decrease in pH_i. To regulate its pH_i, the cell can stimulate the Na^+-H^+ exchange resulting in an entry of Na^+ ions, which can then be followed by an activation of the Na^+-Ca^{2+} exchange. This hypothesis which involves the Na^+-H^+ exchanger can be tested using either hexamethylene amiloride or ethylisopropyl amiloride, two potent and specific inhibitors of this exchanger [Gerritsen et al., 1989]. These amiloride analogs were tested on the hypoxia-induced PLA_2 activation. They did not inhibit the arachidonic acid release (Fig. 4A,B nor did they inhibit the increase in $[Ca^{2+}]_i$ (Fig. 4C,D) or the Ca^{2+} influx (Fig. 4E) observed during hypoxia. It must be noted the highest concentrations of hexamethylene amiloride (2, 5, and 10 μM) and ethylisopropyl amiloride (5 μM) were toxic as detected by the observation under phase contrast microscope and by an abnormally high amount of arachidonic acid released in the medium.

Moreover, no difference in pH_i, measured with BCECF, could be detected in HUVEC incubated in hypoxic conditions in comparison to normoxic HUVEC: a similar decrease in pH_i was observed in both conditions (Fig. 5A). This decrease may be due to the fact that we changed the cells from complete medium to saline solution (HBSS) for the incubation in normoxia or hypoxia. One possibility to explain the fact that there is no difference between hypoxic and normoxic cells would be that the Na^+-H^+ exchanger rapidly extrudes the excess of H^+ generated by glycolysis, so that no decrease in pH_i is detected in hypoxic conditions. To test this hypothesis, ethylisopropyl amiloride (5 μM) was added to the cells during normoxia or hypoxia incubation, in order to block this pH_i regulating process, and the pH_i was measured. However, even in these conditions, no difference between normoxic and hypoxic conditions could be observed. It must be noted that the fluorescence values measured in the presence of ethylisopropyl amiloride are higher than in its absence, due to the fluorescent properties of this compound [Harootunian et al., 1989]. This led to an overestimation of the pH_i (Fig. 5B).

The facts that both amiloride analogs could not prevent the hypoxia-induced increase in $[Ca^{2+}]_i$ and that pH_i was not decreased in hypoxic HUVEC compared to normoxic HUVEC rule out the possible involvement of the Na^+-H^+ exchanger in the hypoxia-induced activation of HUVEC.

A third possible source for the entry of Na^+ ions into the cell is the Na^+-glucose cotransport. A direct consequence of the decrease of O_2 concentration in the medium during hypoxia is a decrease in mitochondrial oxidative phosphorylations. Previous results have shown that glycolysis is stimulated in HUVEC during the first hour of hypoxia [Janssens et al., 1995]. A large increase of glucose uptake is therefore expected during hypoxia. If the entry of glucose occurs through the Na^+-glucose cotransporter, the entry of glucose would concomitantly lead to an influx of Na^+ ions which could then be responsible for the entry of Ca^{2+} through the Na^+-Ca^{2+} exchanger. In order to evaluate the importance of the Na^+-glucose cotransport, the glucose entry was measured using the uptake of a radioactive non-metabolized glucose derivative, 2-deoxy-D-glucose. Figure 6A shows that glucose uptake was strongly activated in the HUVEC along with the hypoxia incubation. The time-dependency of the glucose uptake showed a significative difference between hypoxia and normoxia starting 60 min after the incubation. This kinetics closely paralleled to the one observed for the increase in $[Ca^{2+}]_i$, both occurring later than the decrease in the ATP content [Arnould et al., 1992]. After 120 min, the glucose uptake in hypoxic cells was 1.7-fold higher than in normoxic cells.

The involvement of the Na^+-glucose cotransporter in the hypoxia-induced increase in $[Ca^{2+}]_i$ was then tested using phlorizin, an inhibitor of the Sglt 1 transporter [Dickens et al., 1990]. The inhibitory effect of phlorizin on the Na^+-glucose cotransporter was checked by measuring the glucose uptake. In the presence of phlorizin, the glucose uptake in normoxic and

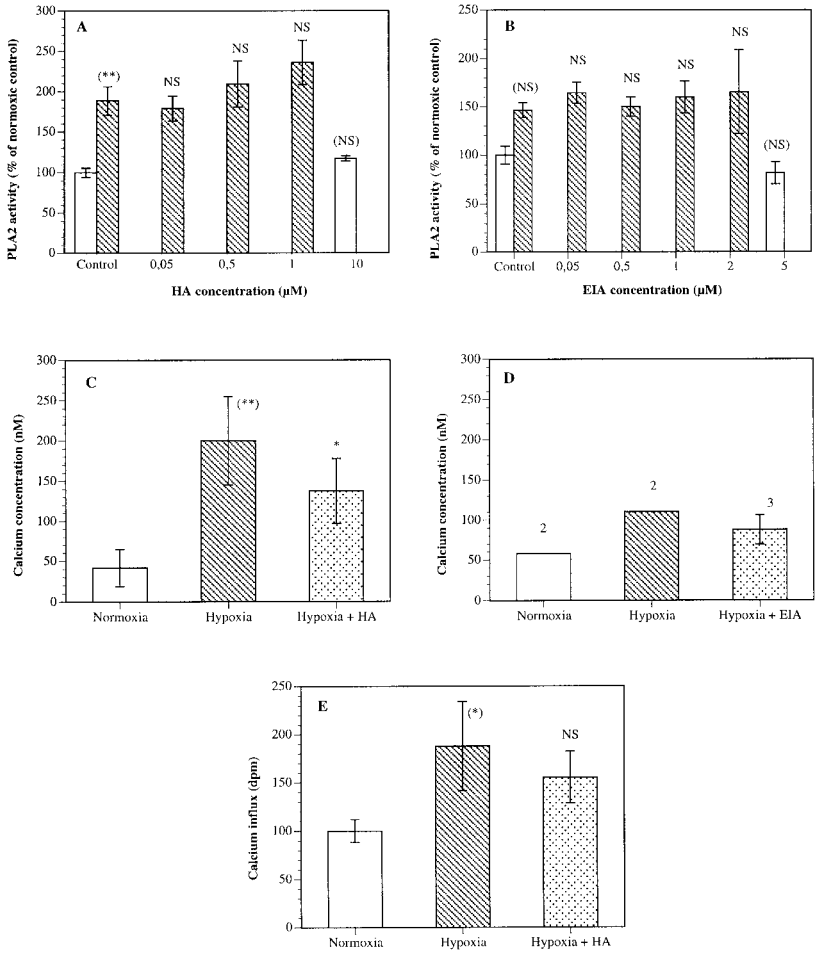

Fig. 4. **A:** Effect of hexamethylene amiloride on the hypoxia-induced activation of PLA_2 HUVEC were incubated during 120 min under normoxic (□) or hypoxic (▨) conditions with different concentrations of hexamethylene amiloride. Control cells were incubated without hexamethylene amiloride. PLA_2 activity was estimated by the release of [^{3}H]-arachidonic acid in the medium during these incubations. Results are expressed as means ± 1 SD of percent of normoxic control for one experiment performed in triplicates (n = 3). (**), significantly different from control cells incubated for 120 min under normoxia with $P < 0.01$. (NS), nonsignificantly different from control cells incubated for 120 min under normoxia. NS, nonsignificantly different from control cells incubated for 120 min under hypoxia. **B:** Effect of ethylisopropyl amiloride on the hypoxia-induced activation of PLA_2. HUVEC were incubated during 120 min under normoxic (□) or hypoxic (▨) conditions with different concentrations of ethylisoproply amiloride. Control cells were incubated without ethylisopropyl amiloride. PLA_2 activity was estimated by the release of [^{3}H]-arachidonic acid in the medium during these incubations. Results are expressed in percent of normoxic control as means ± 1 SD for two experiments performed in triplicates (n = 6). (NS), nonsignificantly different from control cells incubated for 120 min under normoxia. NS, nonsignificantly different from control cells incubated for 120 min under hypoxia. **C:** Effect of hexamethylene amiloride on the hypoxia-induced increase in $[Ca^{2+}]_i$ HUVEC were incubated during 120 min under hypoxic conditions in the presence or in the absence of 1 μM of hexamethylene amiloride. Control cells were incubated during 120 min in normoxia. Results are expressed as means ± 1 SD for n separate experiments (n = 3 for normoxia, n = 5 for hypoxia, and n = 4 for hypoxia in the presence of HA). (**), significantly different from control cells incubated for 120 min in normoxia with $P < 0.01$. *, significantly different from control cells incubated for 120 min in hypoxia with $P < 0.05$ (Student's *t* test). **D:** Effect of ethylisopropyl amiloride on the hypoxia-induced increase in $[Ca^{2+}]_i$ HUVEC were incubated during 120 min under hypoxic conditions in the presence or in the absence of 0.5 μM of ethylisopropyl amiloride. Control cells were incubated during 120 min in normoxia. The results are expressed as means (the number of replicate is indicated above the columns). **E:** Effect of hexamethylene amiloride on the hypoxia-induced influx in Ca^{2+}. HUVEC were incubated during 30 min under hypoxic conditions in the presence or in the absence of 1 μM of hexamethylene amiloride. Control cells were incubated during 30 min in normoxia. The results are expressed as means ± 1 SD of percent of normoxic control for two experiments performed on in duplicates and the other in triplicates (n = 5). (*), significantly different from control cells incubated for 120 min in normoxia with $P < 0.05$. NS, nonsignificantly different from control cells incubated for 120 min in hypoxia (Student's *t* test).

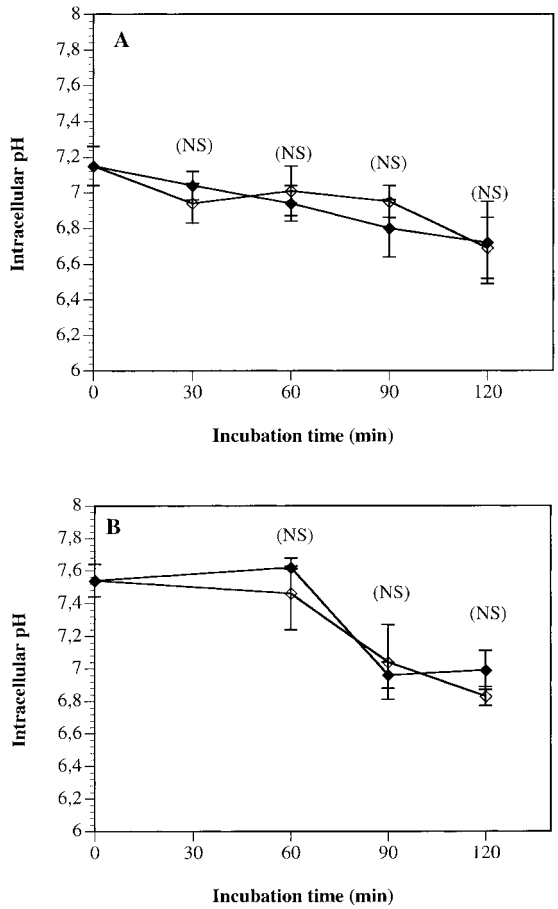

Fig. 5. **A:** Time course of pH_I under normoxic conditions (◇) or hypoxic conditions (◆). pH_i was estimated with the fluorescent probe BCECF-AM. Results are expressed as means ± 1 SD for three independent experiments (n = 6). (NS), nonsignificantly different from control cells incubated for 120 min under normoxia. **B:** Time course of pH_i under normoxic conditions (◇) or hypoxic conditions (◆) in the presence of 0.5 μM of ethylisopropyl amiloride. pH_i was estimated with the fluorescent probe BCECF-AM. Results are expressed as means ± 1 SD (n = 3). (NS), nonsignificantly different from control cells incubated for 120 min under normoxia.

hypoxic HUVEC was about the same and showed a strong inhibition compared to the control cells (Fig. 6B). The absence of activation in hypoxic conditions suggests that the Na^+-glucose cotransporter is actually involved in the hypoxia-induced activation of glucose uptake. In addition, the glucose uptake in both normoxia and hypoxia was inhibited and became identical when extracellular Na^+ was omitted. Both effects indicate that hypoxia-induced glycolysis activation leads to an activation of the Na^+-glucose cotransport, resulting in an influx of Na^+ within the cells.

The role of this glucose-induced Na^+ influx in the hypoxia-induced increase in $[Ca^{2+}]_i$ was investigated by testing the effect of phlorizin on hypoxia-induced PLA_2 activation and increase in $[Ca^{2+}]_i$. Figure 6C shows that phlorizin was able to inhibit in a dose-dependent way the hypoxia-induced PLA_2 activation reaching 60% of inhibition at 500 μM. This concentration had no effect on basal PLA_2 activity in normoxic conditions. Phlorizin at 500 μM also significantly inhibited the hypoxia-induced increase in $[Ca^{2+}]_i$ by75% (Fig. 6D) as well as in Ca^{2+} influx (Fig. 6E). These results suggest that the activation of the Na^+-glucose cotransport participates in the $[Ca^{2+}]_i$ increase in HUVEC under hypoxia.

Measurements of $[Na^+]_i$ were undertaken in normoxic and hypoxic HUVEC using the fluorescent probe SBFI, and preliminary results indicate that $[Na^+]_i$ in hypoxic HUVEC was higher than in normoxic HUVEC. However, despite nice calibration curves obtained with HUVEC maintained in culture medium, it was not possible to calculate the actual cytosolic Na^+ concentrations after the 2 h incubation under normoxia or hypoxia, due to the leakage of the probe during this incubation period, even in the presence of probenecid.

DISCUSSION

Studies on ischemic injury already showed that there is a link between the lack of oxygen, the energy deficiency and the disturbance of the Ca^{2+} homeostasis, leading to several biochemical tissue alterations [Piper, 1989]. An increase in $[Ca^{2+}]_i$ resulting from the energy deficiency has been proposed by several authors as one of the causes for cell mortality and tissue necrosis occurring during ischemia-reperfusion or hypoxia-reoxygenation. However, if the hypoxia and the incubation time are limited as in the model studied here, the increase in $[Ca^{2+}]_i$ is not high enough to induce cell death but is sufficient to lead to cell activation [Arnould et al., 1992]. Much work has been done to examine the relationship between the metabolic inhibition and the increase in $[Ca^{2+}]_i$ in hepatocytes, myocytes or perfused hearts, but little is known about endothelial cells. Aono et al. [2000] also demonstrated Ca^{2+} influx in HUVEC exposed to hypoxia. However, the nature of the transporter responsible for this process was not studied.

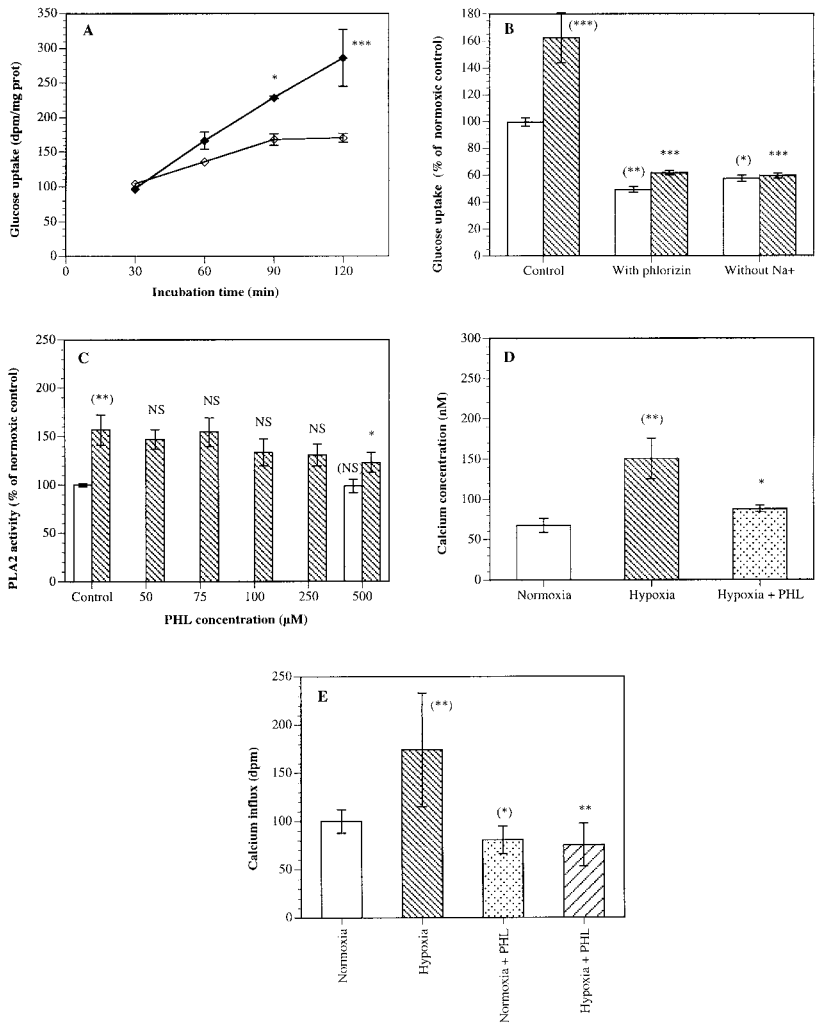

Fig. 6. **A:** Time course of glucose uptake under normoxic (◇) or hypoxic conditions (◆). Glucose uptake was measured by deoxy-5[^{3}H]-glucose incorporation. Results are expressed in dpm/μg of proteins as means ± 1 SD (n = 3). ***, significantly different from corresponding control cells incubated for 120 min under normoxia with $P < 0.001$. *, significantly different from corresponding control cells incubated for 90 min under normoxia with $P < 0.05$. NS, nonsignificantly different from corresponding control cells incubated for 30 or 60 min under normoxia. **B:** Effect of phlorizin or sodium-free medium on the hypoxia-induced glucose uptake. HUVEC were incubated during 120 min under normoxic (□) or hypoxic conditions (▨) in the presence of 500 μM of phlorizin or in the absence of sodium. Control cells were incubated during 120 min in the presence of sodium and without phlorizin. Glucose uptake was measured by deoxy-5-[^{3}H]-glucose incorporation. Results are expressed as means ± 1 SD of percent of normoxic control for one experiment with four dishes per condition (n = 4). (***), (**) or (*), significantly different from control cells incubated for 120 min under normoxia with $P < 0.001$, $P < 0.01$ or $P < 0.05$. ***, significantly different from control cells incubated for 120 min under hypoxia with $P < 0.001$. **C:** Effect of phlorizin on the hypoxia-induced activation of PLA_2. HUVEC were incubated during 120 min under normoxic (□) or hypoxic (▨) conditions with different concentrations of phlorizin. Control cells were incubated without phlorizin. PLA_2 activity was estimated by the release of [^{3}H]-arachidonic acid in the medium during these incubations. Results are expressed as means ± 1 SD of percent of control for 1 experiment performed in triplicates (n = 3). (**), significantly different from control cells incubated for 120 min under normoxia with $P < 0.01$. (NS), nonsignificantly different from control cells incubated for 120 min under normoxia. *, significantly different from control cells incubated 120 min under hypoxia with $P < 0.05$. NS, nonsignificantly different from control cells incubated for 120 min under hypoxia. **D:** Effect of phlorizin (PHL) on the hypoxia-induced increase in $[Ca^{2+}]_i$. HUVEC were incubated during 120 min under hypoxic conditions in the presence or in the absence of 500 μM of phlorizin. Control cells were incubated during 120 min in normoxia. The results are expressed as means ± 1 SD for two experiments performed in duplicates (n = 4, except normoxia, n = 3). (**), statistically significantly different from control cells incubated for 120 min under normoxia with $P < 0.01$. *, statistically significantly different from control cells incubated for 120 min under hypoxia with $P < 0.05$ using Student's *t* test. **E:** Effect of phlorizin (PHL) on the hypoxia-induced influx in Ca^{2+}. HUVEC were incubated during 30 min under hypoxic conditions in the presence or in the absence of 500 μM of phlorizin. Control cells were incubated during 30 min in normoxia. The results are expressed as means ± 1 SD of percent of normoxic control for two experiments performed in triplicates (n = 6). (*), (**), significantly different from control cells incubated for 120 min in normoxia with $P < 0.05$, $P < 0.01$. **, significantly different from control cells incubated for 120 min in hypoxia with $P < 0.01$ (Student's *t* test).

The purpose of this study was to investigate the biochemical mechanism linking the decrease in ATP content and the increase in $[Ca^{2+}]_i$ observed in HUVEC under hypoxia, which is the starting point of a cascade of reactions leading to the cell activation [Arnould et al., 1992]. To determine the regulatory mechanisms that may contribute to this hypoxia-induced increase in $[Ca^{2+}]_i$ in HUVEC, we studied HUVEC loaded with the calcium-sensitive fluorescent probe, Fura-2 AM, in the presence or in the absence of different inhibitors. Since the PLA_2 enzyme is directly regulated by changes in $[Ca^{2+}]_i$ [Michiels et al., 1993], its activity was used for a first screening of the activity of these inhibitors. The different pathways studied here and their possible involvement are schematically presented in Figure 7.

The first observations showed that the substitution of sodium ions by choline in the incubation medium almost completely prevented the hypoxia-induced PLA_2 activation and the $[Ca^{2+}]_i$ increase. These results indicate that extracellular Na^+ is necessary for the activation of hypoxic endothelial cells. The link between the extracellular Na^+ and the intracellular Ca^{2+} accumulation has to be found in the activity of the Na^+-Ca^{2+} exchanger. When we incubated HUVEC in hypoxic conditions in the presence of 2 μM of dichlorobenzamil, an inhibitor of the Na^+-Ca^{2+} exchanger, we observed an important inhibition of both PLA_2 activation and $[Ca^{2+}]_i$ increase. It must be noted that we do not know whether this exchanger was completely inhibited in the experimental conditions used here. If not, it would explain that the inhibition of PLA_2 and $[Ca^{2+}]_i$ increase in the presence of DCB was not complete. If yes, it suggests that another ion channel yet to be identified is involved. These results confirm the major involvement of the Na^+-Ca^{2+} exchanger in the hypoxia-induced increase in $[Ca^{2+}]_i$ in HUVEC. Indeed, the activation of the Na^+-Ca^{2+}

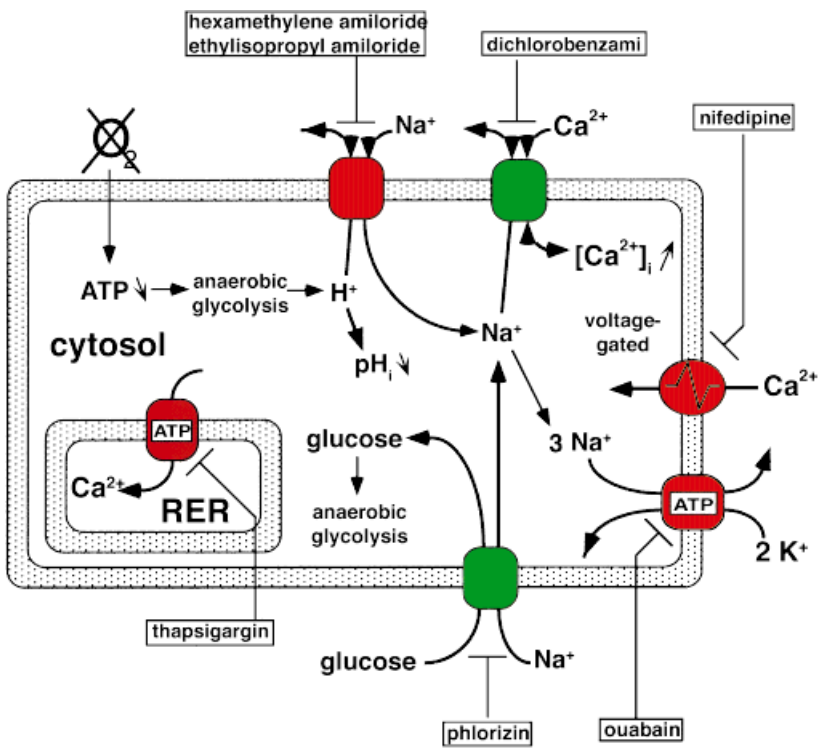

Fig. 7. Schematic representation of the different ion pathways investigated in the hypoxia-induced increase in $[Ca^{2+}]_i$ in HUVEC, using different specific inhibitors. The mechanisms involved are represented in green colour and the mechanisms noninvolved in red colour. Due to the inhibition of the mitochondrial oxidative phosphorylations, the cell requires high amounts of glucose for the anaerobic glycolysis, in order to supply for this energy deficiency. The glucose can enter through a Na^+-glucose-like cotransporter, leading to an accumulation of Na^+ in the cytosol. The Ca^{2+} can mostly enter by a Na^+-Ca^{2+} exchanger, which extrudes the accumulated intracellular Na^+ for extracellular Ca^{2+}. Others pumps such as the Na^+-H^+ exchanger, the Na^+-K^+ ATPase, the voltage-gated Ca^{2+} channel and the endoplasmic reticulum Ca^{2+} ATPase are not involved in the hypoxia-induced increase in $[Ca^{2+}]_i$ in HUVEC.

exchanger is a common pathway for Ca^{2+} entry and has been shown to occur in different cell types: endothelial cells [Hansen et al., 1991], in myocytes during a metabolic inhibition [Satoh et al., 1995] or during hypoxia [Haigney et al., 1992, 1994], in the release of endothelium-derived relaxing factor (EDRF) by isolated aorta stimulated with agonists [Winquist et al., 1985; Schoeffter and Miller, 1986] and in ischemic hearts [Karmazyn et al., 1993].

The reason for Na^+ accumulation in cells under hypoxia is linked to their higher requirement for glucose, given the low efficiency of glycolysis. Glucose enters mammalian cells by two types of catalyzed processes: facilitated diffusion and Na^+-dependent active transport. In the case of facilitated diffusion, glucose transport occurs through specialized transport proteins, Glut 1-5 and Glut 7. These transporters carry glucose down its concentration gradient in an energy-independent process [Mueckler, 1994]. Active transport is mediated by membrane transporters, Sglt 1 and Sglt 2. These are Na^+-glucose cotransporters which carry glucose coupled to Na^+ transport down its concentration gradient. This is an active process, which is usually associated with the activity of the Na^+-K^+ ATPase pump to uphold the Na^+ gradient across the membrane [Philpott et al., 1992; Wright, 1993]. However, in this case instead of extruding Na^+ by Na^+-K^+ ATPase pump, we observed that hypoxic HUVEC use the Na^+-Ca^{2+} exchanger. Na^+-Ca^{2+} exchanger does not use ATP. However, it must be noted that is activity is modulated by ATP concentration. Higher ATP concentrations stimulate its activity while ATP depletion (below 95%, which is much lower than in the conditions used here) decreases it [Condrescu et al., 1995]. On the other hand, the reversed mode of Na^+-Ca^{2+} exchanger has already been shown to be activated by ATP depletion in hepatocytes, and to be responsible for the elevation of cytosolic calcium levels [Carini et al., 1995]. The role of the Na^+-glucose transporter was confirmed by the following observations: first, we found that glucose uptake markedly increased in hypoxia. The kinetics was parallel to the one previously observed by Arnould et al. [1992] for the hypoxia-induced increase in $[Ca^{2+}]_i$. After 120 min, the glucose uptake in hypoxic conditions was 1.7-fold higher than in normoxic conditions. Secondly, we observed that glucose uptake was highly reduced in the absence of Na^+ or in the presence of phlorizin, an inhibitor of the Na^+-glucose cotransporter, but not of facilitative transporters. It must be noted that using a non-metabolizable form of glucose may underestimate glucose import. These results suggest that the Na^+-glucose cotransporter is for the most part responsible for this hypoxia-induced glucose uptake, since both the phlorizin inhibition and the presence of Na^+ are specific for Sglt glucose transporters. The presence of phlorizin or the absence of Na^+ was also found to have a 50% inhibitory effect on glucose uptake in normoxic HUVEC. In these cases, the glucose uptake was almost the same in normoxic and hypoxic cells. These pharmacological data suggest that in normal conditions about 50% of the basal glucose transport (phlorizin insensitive) is mediated by facilitative glucose-like transporter, probably Glut 1, constitutively present in the plasma membrane [Mueckler, 1994]. The other 50% will then be provided by the Na^+-glucose-like cotransporter [Nishizaki et al., 1995]. Under hypoxia, the activity of this Sglt-like transporter increased accounting for the higher glucose intake and the concomitant transport of Na^+. The relationship between the increase in Na^+-glucose cotransporter activity and the HUVEC activation was tested by using phlorizin during the hypoxia incubation. We oberved an important inhibitory effect both on PLA_2 activation (60% of inhibition) and $[Ca^{2+}]_i$ increase (75% of inhibition). The rather high phlorizin concentration used in this work is explained by the fact that phlorizin is a competitive inhibitor of the Na^+-glucose cotransporter [Toggenburger et al., 1982]. It was used in this work in the presence of 1 mM glucose (in experiments measuring glucose transport) or of 5 mM glucose (for PLA_2 activity and $[Ca^{2+}]_i$ measurement). Lower phlorizin concentrations (e.g., 10 μM) are used in the presence of lower glucose concentrations (0.1 mM) [Dickens et al., 1990] and higher (1 mM) in the presence of high glucose concentrations (10 mM) [Nishizaki et al., 1995].

All these data indicate a clear increase of the Sglt-like glucose transporter for the entry of Na^+, leading thereafter to an increase in $[Ca^{2+}]_i$ in HUVEC incubated under hypoxia. The possible release of Ca^{2+} from the intracellular stores was also investigated. Thapsigargin irreversibly inhibits the endoplasmic reticulum Ca^{2+}-ATPase and impedes the reabsorption of

Ca^{2+} [Yamamoto et al., 1995]. Thapsigargin was found to stimulate the PLA_2 activity in hypoxic cells as it did in normoxic cells which suggests that the hypoxia-induced increase in $[Ca^{2+}]_i$ was probably not due to the release of Ca^{2+} from intracellular stores. This is consistent with previous data which showed that thapsigargin has an effect on a much smaller time scale than here [Dolor et al., 1992; Salvaterra and Goldman, 1993]. In endothelial cells stimulated with agonists, it was clearly established that the Ca^{2+} of intracellular stores is only an transient source of Ca^{2+} and does not play a role in the sustained increase in $[Ca^{2+}]_i$ [Schoeffter and Miller, 1986]. Furthermore, Hallam et al. [1989] have shown that in agonist-stimulated HUVEC, there is a prolonged elevation of $[Ca^{2+}]_i$ in the presence, but not in the absence of extracellular Ca^{2+}, suggesting a stimulated influx of Ca^{2+} across the plasma membrane.

Other possible sources of Na^+ were also investigated in this study, but the results suggest that they do not seem to be involved. The first possibility was a higher activity of the Na^+-H^+ exchanger. Its participation has been found in hearts during ischemia and reperfusion [Karmazyn et al., 1993; Scholz and Albus, 1993] and in cardiomyocytes during metabolic inhibition [Satoh et al., 1995] or exposure to lactate [Cairns et al., 1993]. In these conditions, there was an intracellular acidosis due to a release of protons during the anaerobic glycolysis and the ATP hydrolysis. Acidosis due to stimulation of anion exchange was also described in cardiac myocytes exposed to hypoxia [Kawazaki et al., 2001]. The Na^+-H^+ exchange is activated in order to restore the intracellular pH value by extruding H^+ in the extracellular space which causes an influx of $[Na^+]_i$. The Na^+ may then be exchanged for Ca^{2+} via the Na^+-Ca^{2+} exchanger, leading to an increase in $[Ca^{2+}]_i$. To investigate if this mechanism plays a role in hypoxic HUVEC, we tested the effects of two amiloride analogs, hexamethylene amiloride and ethylisopropyl amiloride [Karmazyn et al., 1993]. These amiloride analogs did not significantly inhibit the PLA_2 activation nor did they inhibit the increase in $[Ca^{2+}]_i$ in hypoxic HUVEC. The hypothesis linking the Na^+-H^+ exchanger and the increase in $[Ca^{2+}]_i$ would also expect a decrease of pH_i, due to anaerobic glycolysis. However, no difference in pH_i could be detected in HUVEC incubated in hypoxic conditions in comparison to normoxic HUVEC. This rules out a relationship between pH_i and the hypoxia-induced increase in $[Ca^{2+}]_i$. Similarly, in rat cerebral and mesenteric arteries incubated under hypoxia [Aalkjaer and Lombard, 1995] and in anoxic rat liver sinusoidal endothelial cells [Fujii et al., 1994], there was no change in pH_i. The effect of intracellular acidification and the changes in $[Ca^{2+}]_i$ have been studied in other cell types and the results are highly variable. Whereas a decrease in pH_i resulted in an increase in $[Ca^{2+}]_i$ in rat aortic endothelial cells [Ziegelstein et al., 1993] or in rat ventricular myocytes [Hayashi et al., 1992; Cairns et al., 1993], the opposite relationship was observed in avian heart fibroblasts [Dickens et al., 1990]. It would appear, therefore, that the link between a decrease in pH_i and the regulation of $[Ca^{2+}]_i$ is either cell type specific or influenced by the experimental conditions. It must also be noted that hypoxia decreases Na^+-H^+ exchanger activity and expression in human pulmonary arterial endothelial cells exposed to hypoxia [Cutaia et al., 1998].

Another ATPase often proposed as being responsible for the increase in $[Na^+]_i$ and the following increase in $[Ca^{2+}]_i$ during hypoxia or metabolic inhibition [Grinwald, 1992; Satoh et al., 1995] is the Na^+-K^+ ATPase pump. The Na^+-K^+ ATPase activity is essential for the maintenance of the electrochemical gradients of Na^+ and K^+ ions. The inhibition of Na^+-K^+ ATPase, due to a decrease in ATP content in the cell, would lead to a rise of $[Na^+]_i$, which would in turn activate the Na^+-Ca^{2+} exchanger and thus induce an increase in $[Ca^{2+}]_i$. We tested this hypothesis using ouabain. The presence of ouabain already increased the PLA2 activity in normoxic conditions by 28%. Identical increase in PLA_2 activity was obtained in hypoxic conditions, which indicates that ouabain and hypoxia have independent effects on PLA_2 activity. Indeed, if the pump would have been inhibited by hypoxia, then no more effect of ouabain would be expected. Since this was not the case, we can exclude the involvement of the Na^+-K^+ ATPase pump in the hypoxia-induced activation of HUVEC. The above observation is consistent with previous reports which demonstrated the incomplete inhibition of the Na^+-K^+ ATPase pump during hypoxia/ischemia in guinea pig papillary muscles [Kleber, 1983] and canine ventricular muscles [Nakaya et al., 1985]. The Na^+-K^+ ATPase pump activity was

lowered in hypoxic or ischemic conditions only when a profound reduction of ATP was obtained. In this model, the ATP content of cells exposed 2 h to hypoxia reaches 60–40 % of the control cells, which is still sufficient for the activity of the ATPase pump. Anderson et al. [1990] also showed that during the first hour of ischemia in their model, the ATP level remained sufficient to fuel the Na^+-K^+ ATPase pump at elevated rates. In the isolated myocytes exposed to hypoxia, the major increase in $[Na^+]_i$ only occurs after full depletion of ATP when the Na^+-K^+ ATPase pump is inhibited [Haigney et al., 1992]. Other evidences for the fact that the Na^+-K^+ ATPase pump is still active is provided by the measurement of the ATP content during hypoxia in the presence of dichlorobenzamil, the inhibitor of Na^+-Ca^{2+} exchanger. Under hypoxic conditions, there was a decrease of 50% in the ATP content, but in the presence of dichlorobenzamil, we observed a fall in ATP content to 32% of control values. This suggests that when the Na^+-Ca^{2+} channel is inhibited, there is an accumulation of Na^+ ions in the cytosol and the Na^+-K^+ ATPase pump is able to extrude this Na^+ gain, consuming more ATP molecules. Finally, the Na^+-K^+ ATPase pump has been shown to use glycolytic ATP production preferentially [Glitsch and Tappe, 1993] and stimulation of Na^+-K^+ ATPase by epinephrine in skeletal muscle increases glycolysis activity so that there is a coupling between ATP production by glycolysis and ATP consumption by Na^+-K^+ ATPase activity [James et al., 1999]. The presence of glucose in our model, in addition to the hypoxia-induced increase in glycolysis activity, could produce adequate energy for the preservation of the Na^+-K^+ ATPase pump activity. Indeed, in aortic endothelial cells, cellular ATP levels have been shown to be minimally affected by inhibition of oxidative phosphorylation alone, modestly diminished by selective glycolytic inhibition, and rapidly and profoundly reduced by inhibition of both [Watanabe et al., 1991]. Consistent with our results, Oike et al. [1993] did not find changes in $[Ca^{2+}]_i$ when the Na^+-K^+ ATPase pump was blocked and reactivated in HUVEC.

In conclusion, several pathways were tested in this study to investigate the mechanism of $[Ca^{2+}]_i$ increase in HUVEC activated by hypoxia. The biochemical cell adaptation observed during 2 h hypoxia can be summarized in the following way. The first step is a decrease of about 50% in the ATP content, due to a decreased oxygen availability and an inhibition of the mitochondrial oxidative phosphorylations. In order to compensate for this energetic depletion, glycolysis is activated and glucose uptake by Na^+-glucose-like transporters increases, leading to an accumulation of intracellular Na^+. This Na^+ loading then activates the Na^+-Ca^{2+} exchanger, leading to an increase in $[Ca^{2+}]_i$. On the other hand, the Na^+-K^+ ATPase pump, the endoplasmic reticulum Ca^{2+} ATPase and the Na^+-H^+ exchanger, contrary to data obtained with other models using myocytes or whole hearts, do not seem to be involved. The link between the partial ATP depletion induced by hypoxia and the $[Ca^{2+}]_i$ increase is thus indirect and involves Na^+-glucose transporter and Na^+-Ca^{2+} exchanger. This link seems to be specific for the endothelial cells.

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