Reactive oxygen species production by mitochondria in endothelial cells exposed to reoxygenation after hypoxia and glucose depletion is mediated by ceramide

S. Therade-Matharan, 1 E. Laemmel, 1 S. Carpentier, 2 Y. Obata, 1 T. Levade, 2 J. Duranteau, 3 and E. Vicaut 1

1 Laboratoire d’Etude de la Microcirculation, Equipe d’accueil, Faculte de Medicine Paris, France; 2 Institut National de la Sante et de la Recherche Medicale, Laboratoire de Biochimie Centre Hospitalier Universitaire Rangueil, Toulouse, France; and 3 Equipe d’accueil, Laboratoire d’Anesthesie, Hopital de Bicetre, Kremlin Bicetre, France

Submitted 19 July 2004; accepted in final form 2 August 2005

Although hypoxia alone can produce tissue injury, different types of evidence suggest that a burst of reactive oxygen species (ROS) generation coincides with reoxygenation. The evidence that ROS are essential mediators of hypoxia/reoxygenation (H/R) injury is based on the observation that various chemical and enzymatic antioxidants appear to protect against the tissue damage seen with reperfusion (16, 17). ROS generated by hypoxia or reoxygenation are now recognized as interacting with physiological signal transducers rather than behaving like simple reactants that peroxidize membrane lipids, oxidize DNA, or denature enzyme proteins. Because endothelial cells are ubiquitous and located at the blood-brain barrier, they have been proposed as the central source of ROS burst that occurs in ischemic tissues on reoxygenation (47, 59).

In a previous study (50), we showed that the main site of production of ROS in human umbilical vein endothelial cells (HUEVC) after hypoxia/reoxygenation (H/R) was the cytochrome (cyt) b of the complex III of the mitochondrial electron transport chain. In several types of cells, ceramide is released from activation of a sphingomyelinase that cleaves membrane sphingomyelin (SM) has been demonstrated to be involved in tissue injury after ischemia or anoxia (22, 32, 46, 55, 58). In isolated mitochondria (17) or in L929 cells (43), ceramide has been shown to interfere with several mitochondrial mechanisms, including cyt c release, which compromises mitochondrial electron flow and triggers ROS formation. In addition, in a previous study (9), we showed that ceramide was involved in the mitochondrial ROS production by HUEVC exposed to TNF-α.

Interactions between ceramide and mitochondria have been proposed to be either direct (10, 14, 19) or indirect. Regarding the latter, several mechanisms have been reported, such as the activation of protein kinase (9, 31), the induction of the mitochondrial permeability transition (MPT) (38, 39), and the modulation of the phosphorylation of the antiapoptotic protein Bcl-2 (27, 42, 53).

The present study attempts to elucidate the role of ceramide in H/R-induced ROS formation. For this purpose, the following approach was used: 1) to demonstrate that sphingomyelinase that hydrolyzed sphingomyelin to form ceramide was involved in ROS production in endothelial cells submitted to H/R, 2) to demonstrate that ceramide can directly induce ROS production by a mechanism similar to that found after H/R, and 3) to test the hypothesis that the H/R-induced ROS formation was either related to the activation of a protein kinase, required the induction of MPT, or was sensitive to Bcl-2.

MATERIALS AND METHODS

Cells and culture. HUEVC were obtained from PromoCell Laboratories (Heidelberg, The Netherlands) as cryopreserved cells. After thawing, cells were plated (5,000 to 10,000 cells/cm²) in culture flasks precoated with rat-tail type I collagen (5 μg/cm², Böhringer-Mannheim, Mannheim, Germany) and cultured to confluence in endothelial cell growth medium containing 2% fetal calf serum, 0.1 ng/ml human recombinant epidermal growth factor, 1.0 ng/ml human recombinant basic fibroblast growth factor, 1.0 μg/ml synthetic hydrocortisone, 50

Address for reprint requests and other correspondence: E. Vicaut, Laboratoire d’Etude de la Microcirculation, Universite Paris 7 10, avenue de Verdun, 75010 Paris, France (e-mail: eric.vicaut@lrb.aphp.fr).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
μg/ml gentamicin sulfate, 50 ng/ml amphotericin B, and 0.62 ng/ml phenol red. Medium was supplemented with Supplement Mix (PromoCell) containing endothelial cell growth factor and heparin and with 1% penicillin-streptomycin solution (Sigma, St. Louis, MO). A cell suspension in nutritive medium was plated onto plastic Thermofax coverslips 25-mm diameter (Poly Labo, Strasbourg, France) to obtain a seeding density of ~5 × 10^5 cells/cm^2. The medium was replaced every 48 h, and cells were maintained in a humidified air with 5% CO_2 atmosphere at 37°C until confluence was reached. All cells were studied at the third passage.

**Perfusion system.** Endothelial cell monolayers on plastic coverslips were placed in a stainless steel flow-through chamber (volume, 1 ml; Penn Century, Philadelphia, PA). The chamber was sealed using thin wafer gaskets and mounted in a heated (37°C) water serpentine on an inverted microscope (Leica SA, Rueil-Malmaison, France). The perfusate consisted of Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) containing (in mM): 117.3 NaCl, 4.7 KCl, 25 NaHCO_3, 1.3 MgSO_4, 1.2 KH_2PO_4, 1.23 CaCl_2, 1 lactate, and 11.1 glucose; the buffer was equilibrated during stabilization and reperfusion in an atmosphere of 20% O_2-5% CO_2-75% N_2. In the perfusion chamber, PO_2 was ~120 mmHg and the perfusate pH was 7.40. During hypoxia, the perfusate was equilibrated with 5% CO_2-95% N_2 (PO_2, ~20 mmHg in the chamber and pH 7.38). The perfusion system was a nonrecirculating system with a constant flow rate of 0.5 ml/min.

**Fluorescence and light microscopy.** An inverted microscope (model DM-IRB; Leica) was equipped for epifluorescent illumination and included a mercury light source (50 W), a 12-bit digital cooled camera (model RTE/CCD-1317-K;1: Princeton Instruments, Trenton, NJ), and a shutter (model D-122; Uniblitz, Rochester, NY) under computer control (Metamorph Imaging System 3.5, Universal Imaging, West Chester, PA), with appropriate excitation and emission cubes. Fluorescent cell images were obtained using a ×20 objective for fluorescence (Leica). Data were acquired and analyzed using Meta
morph software (Universal Imaging).

**Measurement of ROS and assessment of cell death.** ROS generation in HUVEC was assessed using the 2',7'-dichlorodihydrofluorescein (DCF) probe (Sigma, St. Louis, MO), which is very sensitive to ROS (H_2O_2 and OH-) (51). The reduced membrane-permeable form of the dye H_2DCF-DA was added to the perfusate at a final concentration of 5 μM. Within the cell, esterases cleave the acetate groups on H_2DCF, thus trapping the H_2DCF intracellularly. ROS in the cells oxidizes H_2DCF, yielding the fluorescent product DCFH (33, 44). After 1 h of perfusion, fluorescence was measured every 10 min using an excitation bandwidth of 480 ± 20 nm, a dichroic 505-nm-long pass, and an emitter bandpass of 527 ± 15 nm. Previous studies of the behavior of DCFH revealed that the DCFH probe is readily oxidized by H_2O_2 or hydroxyl radicals but is relatively insensitive to superoxide (40, 51).

To evaluate cell viability, we used the fluorescent probe propidium iodide (PI; Sigma), which was added to the perfusate at a final concentration of 5 μM. This probe is a red fluorescent, cell-impermeant dye that is widely used to detect dead or dying cells and only penetrates into the cell when the membrane is injured and binds to the DNA. Once this dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold, its excitation maximum shifts ~15 nm into the red, and its emission maximum ~15 nm into the blue. After 1 h of perfusion, fluorescence was measured every 30 min by using an excitation wavelength of 515–560 nm, a dichroic 580-nm long pass, and an emitter bandpass of 590 nm. Fluorescence intensity was assessed by the imaging system Meta
morph 3.5 over a region of interest that included the whole observational field. Intensity values are reported as percentage changes of initial values.

**Experimental protocol.** Cells plated onto plastic coverslips were placed in the perfusion chamber and flow was started. HUVEC cells subjected to H/R underwent 1 h of stabilization in Krebs-Henseleit buffer under normoxic conditions with glucose, and were then exposed for 2 h to hypoxia without glucose and with deoxy-d-glucose blocking glycolysis in the perfusion chamber. Perfusion was then returned for 1 h under normoxic conditions with glucose. Control HUVEC underwent 1 h of stabilization, followed by exposure for 3 h to the same Krebs-Henseleit buffer (20% O_2-5% CO_2-75% N_2) with glucose but without H/R. HUVEC cells subjected to ceramide (C2) underwent 1 h of stabilization in Krebs-Henseleit buffer under normoxic conditions and was then exposed for 1 h to ceramide in the perfusion chamber. Control HUVEC underwent 1 h of stabilization, followed by exposure for 1 h to the same Krebs-Henseleit buffer (20% O_2-5% CO_2-75% N_2) without ceramide. During these experiments, one fluorescent image of H/R or ceramide and control cells was acquired every 10 min and analyzed to assess ROS production, and one image was acquired every 30 min and analyzed to assess cell death.

To test the involvement of the acidic sphingomyelinase (ASMase), which hydrolyzed sphingomyelin, cell responses to H/R were studied in the presence of the ASMase inhibitor desipramine (5 μM) (9, 52).

Then, to ascertain more directly the effect of ceramide, we studied ROS production in cells subjected to superfusion of Krebs + ceramide (N-acylsphingosine, C2, 20 μM) (17) during 1 h. To test the hypothesis that the effect of ceramide on ROS production in cells involved complex III, as previously shown for H/R (50), the same experiments were carried out in the presence of two different complex III inhibitors; antimycin A (AA; 10 μM) (8), a quinone analog that binds to cyt b_562 and blocks electron transport to the ubiquinone in complex III, and stigmastatin (stig; 10 μM) (1), which blocks electron transport to both the “Rieske” [2Fe-2S] iron sulfur protein and heme b_566 (18, 25). Both inhibitors were administered 30 min before and during ceramide administration.

To elucidate the mechanisms by which H/R ceramide-dependent pathway may affect mitochondrial ROS production, we studied the response to H/R in the presence of either the ceramide-activated protein kinase (CAPK) inhibitor dimethylaminopurine (DMAP; 1 mM) (31), or the mitochondrial permeability transition (MPT) inhibitor, cyclosporin A (CsA; 2 μM) (34) or the antiapoptotic protein Bcl-2 (40 nM) (17), which is known to be located at mitochondrial outer membrane and to inhibit cyt c release (6, 17, 27, 53).

In a complementary series of experiments, we checked that the effect we observed with Bcl-2 on H/R-induced ROS production was also found on ceramide-induced ROS production. For this purpose, cells were submitted to superfusion of Krebs + ceramide (N-acylsphingosine, C2, 20 μM) in the presence of Bcl-2 (40 nM) during 1 h. The inhibitor was administered 30 min before and during ceramide administration.

Finally, using dosages of ceramide we confirmed directly that ceramide levels increased during H/R. For this purpose, we submitted HUVEC to a sequence of H/R similar to that used for ROS measurements (n = 7 at each time) and compared it to control HUVEC under normoxic conditions for the same period of time (n = 4 at each time). For measurements, we used the methods as described by Bielawska et al. (3) and Malagarie-Cazenave et al. (30). The lipids were extracted from lipids with chloroform/methanol from the HUVEC lysates prepared for DEVDase assay. The ceramide content was determined using *Escherichia coli* diacylglycerol kinase (kindly provided by Drs. D. Perry and Y. A. Hannun, Charleston, SC) and [-32P]ATP (6,000 Ci/mmol; PerkinElmer Life Sciences).

**Presentation of the results and statistical analysis.** Differences between groups were analyzed using one-way or two-way ANOVA (within factor time between factor experimental vs. control). Statistical significance was set at the 0.05 level. Values are reported as means ± SE.

**Reagents.** All products, reagents, and inhibitors were purchased from Sigma-Aldrich, as specified above. Bcl-2 is from Sigma-Aldrich; Esch-
RESULTS

Effect of sphingomyelinase inhibitor on ROS production during exposure to H/R. After 2 h of hypoxia, during which the rise in ROS production was nil, reoxygenation induced a rapid production of ROS in HUVEC. This was evidenced by the significant increase in DCF fluorescence, which reached 126 ± 7% after 1 h of reoxygenation (Fig. 1). In contrast, this production was low (14 ± 2%) in control cells (P < 0.0001).

As shown in Fig. 1, a 3-h incubation of HUVEC during H/R with ASMase inhibitor desipramine (Desip; 5 μM) inhibited ROS production by ~62%. Indeed, the value of ROS increase fell to 48 ± 12% in the presence of desipramine (P < 0.05 compared with H/R group, n = 7).

Effect of H/R on ceramide content. As shown in Fig. 2, we confirmed the involvement of ceramide in the effect of H/R, because the dosage of ceramide content clearly showed that an increase in ceramide was induced during H/R (P < 0.05).

Note, however, that the increase in ceramide began during the hypoxic phase of the experiments.

ROS production induced by ceramide and the effect of complex III inhibitors. As shown in Fig. 3, when HUVEC were incubated with ceramide (C2), ROS production increased to 65 ± 3% after 1 h of exposure. When HUVEC were incubated with C2 and the complex III inhibitors AA or Stig, ROS production after a 1-h exposure was significantly inhibited (24 ± 3%, P < 0.0001, and 31 ± 2%, P < 0.0001, respectively, n = 6 in each case). In control experiments, we have checked that these inhibitors did not modify fluorescence by an unspecific manner.

Effect of different inhibitors of signaling pathway on ROS production. As shown in Fig. 4, inhibition of CAPK by DMAP did not inhibit H/R-induced ROS production. Similarly inhibition of mitochondrial permeability transition by CsA did not inhibit H/R-induced ROS production. In contrast, a significant inhibition was found with Bcl-2 (82 ± 8%) (P < 0.05, n = 6).

In addition, as shown in Fig. 5, we also found in complementary experiments (n = 3) that Bcl-2 also inhibited ceramide-induced ROS production (65 ± 3% vs. 41 ± 4%, P < 0.0001).

Cell death. PI fluorescence, which reflects cell death, did not vary significantly in HUVEC exposed to H/R or in control cells. When studying the different inhibitors that have been
previously tested for their possible effect on ROS production, we did not find any significant change in PI fluorescence compared with H/R exposed HUVEC not treated with inhibitors (Fig. 6A).

We also found that ceramide + complex III inhibitors did not significantly change PI fluorescence either compared with ceramide alone or to control HUVEC (Fig. 6B).

DISCUSSION

The present study was designed to identify the signaling pathway of ROS production in endothelial cells submitted to conditions close to those occurring during hemorrhagic shock.

Because endothelial cells are an important source of ROS, we used HUVEC, which are commonly used as an in vitro model to study hypoxia-reoxygenation (48, 60). HUVEC were submitted to 2 h of hypoxia, because Zweier et al. (60) showed that 90 min of anoxia is the minimal time required for large ROS production during reoxygenation.

In addition, it should be stressed that when hypoxia is due to hemodynamic perturbations, the nutrient supply can also be severely diminished. Thus, in the present study, we associated...
CERAMIDE AND RADICAL OXYGEN SPECIES AFTER HYPOXIA/REOXYGENATION

hypoxia at a level very close to that found in terminal vessels during hemorrhagic shock (35, 48) and glucose depletion, which induce a relevant additional stress (29, 36), and we associated the subsequent reoxygenation with glucose reinsertion into the perfusate. Note, however, that in additional control experiments (data not shown), we checked that glucose depletion was not the determinant for explaining our finding, as we observed similar H/R-induced increases in ROS even in the presence of normal glucose. In this experimental model, we used fluorescence microscopy to quantify ROS production. Recent results in this model provided evidence that in endothelial cells, a major site of H/R-induced ROS production is the cytochrome b (or cytochrome b) of complex III of mitochondrial respiratory chain (50). However, the signaling pathways of H/R-induced ROS production have not yet been described. The present study was carried out to know whether ceramide is a mediator in this ROS production and what are the mechanisms by which it can interfere with mitochondria to activate ROS production.

Several cellular pathways have been demonstrated to be activated by hypoxia reoxygenation (12, 21). A relationship between ceramide level and ischemia or ischemia reperfusion has been shown in different models. During acute renal failure in mice, ischemia causes decreases of ceramide, whereas during reperfusion, ceramide increases (55). In the hippocampus of gerbils, transient forebrain ischemia, induced an increase in ceramide level (32). An ischemic insult accelerates the degradation of gangliosides and causes an accumulation of ceramide in the ischemic human brain (28). The relationship between increased ceramide production and apoptosis in the intact heart submitted to ischemia/reperfusion has also been demonstrated (4). Ceramide has also been shown as a second messenger of hypoxia/reoxygenation in different cell types, even if different ceramide-related mechanisms have been proposed for explaining their role in hypoxia/reperfusion-induced cellular changes in kidney (55), in brain (32), in HUVEC (58), or in cardiac myocytes (22, 46). Early activation of a sphingomyelinase (SMase) that cleaves membrane sphingomyelin (SM) resulting in the formation of ceramide has been found by Schütze and coworkers (45), who demonstrated that in Jukak T lymphocytes, binding of TNF-α to its receptor is followed by a rapid activation of an ASMase, with subsequent SM hydrolysis and ceramide production within 3 min. In HUVEC exposed to TNF-α, ROS production has also been shown to be ceramide dependent (9).

Several results in the present study demonstrated the important role of the ceramide pathway in H/R-induced ROS production. First, we inhibited ROS production by desipramine, an inhibitor of SMase, which is responsible for ceramide production from SM. We also confirmed by direct dosage that H/R induced an increase in ceramide. Interestingly, this increase began in the hypoxic phase of the experiment. This is in line with the studies previously cited, showing that hypoxia can be responsible for the increase in ceramide. Note, however, that no ROS formation has been found during this period of the experiment. Further studies will be necessary to know whether this implies that either ROS formation is largely delayed from ceramide formation or whether it required the oxygen, which is provided during the reoxygenation period. We also confirmed the role of ceramides by demonstrating that exposure of HUVEC to ceramide (N-acylsphingosine) induced a ROS production. This result is similar to that found by others in neuronally differentiated PC12 cells (40) or in human myeloid leukemia U937 cells (13). In addition, we found that the effect of ceramide was inhibited by antymycin A and stigmatellin, which inhibit the mitochondrial respiratory chain complex III, which have been recently found to inhibit H/R-induced ROS production in HUVEC (50). It can be noted that ROS generated at the complex III of the mitochondrial respiratory chain has also been proposed to be an early major mediator in ceramide-induced apoptosis in human myeloid leukemia U937 cells (40).

Different mechanisms by which ceramide can increase ROS production have been proposed. In rat heart mitochondria, ceramide can directly inhibit mitochondrial respiratory chain function and generate ROS by decreasing the activity of the complex III. Gudz et al. (19) hypothesized that ceramide bound to complex III affects cyt b reoxidation in the Q-cycle with subsequent generation of oxygen radicals. In another way, Garcia-Ruiz et al. (14) hypothesized that in rat liver mitochondria, ceramides interact with components of the complex III of the electron transport chain, favoring the production of ROS. Alternatively, in HL60 cells, the effect of ceramide has been shown to involve the activation of a MAPK (41). Such a mechanism has also been reported by Corda et al. (9), showing that TNF-α induced mitochondrial ROS production in HUVEC occurred via a CAPK. However, in the present experiments DMAP did not inhibit the H/R-induced ROS production, thus making it unlikely that this effect was due to a CAPK.

Other mechanisms by which ceramide can indirectly interfere with mitochondrial ROS production have been proposed. The MPT caused by the opening of a large proteinaceous pore across the inner and outer membrane of mitochondria has been shown to be a causative event in cell injury by a variety of conditions, including ischemia (11, 24, 38). In the L929 line of mouse fibroblasts, MPT occurs as a consequence of the formation of ceramide, which acts to promote induction of the MPT (39). The possibility that H/R-induced mitochondrial ROS production could involve MPT has been explored in the present study. Indeed, no inhibition of H/R-induced ROS production has been found when cyclosporin A, a potent inhibitor of the inner membrane permeability transition, has been used (5). This result is in line with the observation made by Garcia-Ruiz and coworkers (14) who found that CsA did not affect the ROS formation in the presence of ceramide and concluded that the induction of H₂O₂ induced by ceramide is not the consequence of increased membrane permeability. The present results are also in line with those of several authors suggesting that MPT might be a consequence rather than a cause of ROS production (20, 37, 54, 56).

In the present study, we showed that the antiapoptotic protein Bcl-2 inhibited the H/R-induced ROS production. It should be stressed that a limitation of the present study is that we used external Bcl-2 rather than cells overexpressing Bcl-2. However, our observation is in line with several previous studies. Indeed, different authors have suggested a Bcl-2-mediated reduction of ROS production (26, 56). It has been proposed that its apparent antioxidant function (23, 26) was not due to a direct electron-scavenging or superoxide-metabolizing mechanism has also been reported by Corda et al. (9), showing that TNF-α induced mitochondrial ROS production in HUVEC occurred via a CAPK. However, in the present experiments DMAP did not inhibit the H/R-induced ROS production, thus making it unlikely that this effect was due to a CAPK.

In the present study, we showed that the antiapoptotic protein Bcl-2 inhibited the H/R-induced ROS production. It should be stressed that a limitation of the present study is that we used external Bcl-2 rather than cells overexpressing Bcl-2. However, our observation is in line with several previous studies. Indeed, different authors have suggested a Bcl-2-mediated reduction of ROS production (26, 56). It has been proposed that its apparent antioxidant function (23, 26) was not due to a direct electron-scavenging or superoxide-metabolizing mechanism has also been reported by Corda et al. (9), showing that TNF-α induced mitochondrial ROS production in HUVEC occurred via a CAPK. However, in the present experiments DMAP did not inhibit the H/R-induced ROS production, thus making it unlikely that this effect was due to a CAPK.

Several results in the present study demonstrated the important role of the ceramide pathway in H/R-induced ROS production. First, we inhibited ROS production by desipramine, an inhibitor of SMase, which is responsible for ceramide production from SM. We also confirmed by direct dosage that H/R induced an increase in ceramide. Interestingly, this increase began in the hypoxic phase of the experiment. This is in line with the studies previously cited, showing that hypoxia can be responsible for the increase in ceramide. Note, however, that no ROS formation has been found during this period of the experiment. Further studies will be necessary to know whether this implies that either ROS formation is largely delayed from ceramide formation or whether it required the oxygen, which is provided during the reoxygenation period. We also confirmed the role of ceramides by demonstrating that exposure of HUVEC to ceramide (N-acylsphingosine) induced a ROS production. This result is similar to that found by others in neuronally differentiated PC12 cells (40) or in human myeloid leukemia U937 cells (13). In addition, we found that the effect of ceramide was inhibited by antymycin A and stigmatellin, which inhibited the mitochondrial respiratory chain complex III, which have been recently found to inhibit H/R-induced ROS production in HUVEC (50). It can be noted that ROS generated at the complex III of the mitochondrial respiratory chain has also been proposed to be an early major mediator in ceramide-induced apoptosis in human myeloid leukemia U937 cells (40).

Different mechanisms by which ceramide can increase ROS production have been proposed. In rat heart mitochondria, ceramide can directly inhibit mitochondrial respiratory chain function and generate ROS by decreasing the activity of the complex III. Gudz et al. (19) hypothesized that ceramide bound to complex III affects cyt b reoxidation in the Q-cycle with subsequent generation of oxygen radicals. In another way, Garcia-Ruiz et al. (14) hypothesized that in rat liver mitochondria, ceramides interact with components of the complex III of the electron transport chain, favoring the production of ROS. Alternatively, in HL60 cells, the effect of ceramide has been shown to involve the activation of a MAPK (41). Such a mechanism has also been reported by Corda et al. (9), showing that TNF-α induced mitochondrial ROS production in HUVEC occurred via a CAPK. However, in the present experiments DMAP did not inhibit the H/R-induced ROS production, thus making it unlikely that this effect was due to a CAPK.

Other mechanisms by which ceramide can indirectly interfere with mitochondrial ROS production have been proposed. The MPT caused by the opening of a large proteinaceous pore across the inner and outer membrane of mitochondria has been shown to be a causative event in cell injury by a variety of conditions, including ischemia (11, 24, 38). In the L929 line of mouse fibroblasts, MPT occurs as a consequence of the formation of ceramide, which acts to promote induction of the MPT (39). The possibility that H/R-induced mitochondrial ROS production could involve MPT has been explored in the present study. Indeed, no inhibition of H/R-induced ROS production has been found when cyclosporin A, a potent inhibitor of the inner membrane permeability transition, has been used (5). This result is in line with the observation made by Garcia-Ruiz and coworkers (14) who found that CsA did not affect the ROS formation in the presence of ceramide and concluded that the induction of H₂O₂ induced by ceramide is not the consequence of increased membrane permeability. The present results are also in line with those of several authors suggesting that MPT might be a consequence rather than a cause of ROS production (20, 37, 54, 56).

In the present study, we showed that the antiapoptotic protein Bcl-2 inhibited the H/R-induced ROS production. It should be stressed that a limitation of the present study is that we used external Bcl-2 rather than cells overexpressing Bcl-2. However, our observation is in line with several previous studies. Indeed, different authors have suggested a Bcl-2-mediated reduction of ROS production (26, 56). It has been proposed that its apparent antioxidant function (23, 26) was not due to a direct electron-scavenging or superoxide-metabolizing activity of Bcl-2 itself but rather to the prevention of O₂⁻ production (6). Indeed, the effect of Bcl-2 in H/R-induced ROS production observed in the present study might be explained when considering the following: 1) Ceramides can induce cyt c release, as shown in cell cultures (7, 49, 56) and isolated
mitochondria (2, 10, 17). 2) Release of cyt c from mitochondria produces a gap in the hierarchically arranged mitochondrial respiratory complex III and IV, and therefore a site for electron leakage (17). In line with the study by Cai and Jones (6), Ghaforifar et al. (17) hypothesized that superoxide formation by mitochondria is a consequence, and not the cause, of cyt c loss. 3) Cyt c release was preventable by preincubation with or overexpression of the antideath protein, Bcl-2 (15, 17, 57).

In conclusion, the present study demonstrated that in endothelial cells submitted to hypoxia and glucose depletion followed by reoxygenation with glucose, the pathway implicated in mitochondrial complex III ROS production is ceramide-dependent and inhibited by the antiapoptotic protein Bcl-2.

REFERENCES


