Reoxygenation after hypoxia and glucose depletion causes reactive oxygen species production by mitochondria in HUVEC

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Although hypoxia alone can produce tissue injury, it is well known that exposure of hypoxic tissues to oxygen upon reperfusion can be an important cause of organ damage (16). In clinical practice, several situations may correspond to hypoxia, followed by a reoxygenation period. This is the case, for instance, in hemorrhagic shock, where local hypoxia (44) is present in several organs and followed by reoxygenation during the therapeutic process. 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). ROS generated by hypoxia or reoxygenation are now recognized to interact with physiological signal transducers rather than to behave like simple reagents that peroxidize membrane lipids, oxidize DNA, or denature enzyme proteins. The term ROS includes superoxide radical (O2−), hydrogen peroxide (H2O2), hydroxyl radical (·OH), singlet oxygen, ozone, lipid peroxides, nitric oxide (NO•), and peroxynitrite (ONO•). The quantity of O2− produced by reoxygenated cells depends on the duration of both anoxia or hypoxia and reoxygenation (37, 59). Because endothelial cells are ubiquitous and located at the blood barrier, they have been proposed as the initial site of tissue injury (37). Previous studies demonstrating that intravascularly administered radical-scaping enzymes prevent reoxygenation injury in a variety of tissues have led to the hypothesis that vascular endothelial cells could be the central source of the ROS burst that occurs in ischemic tissues on reoxygenation (58).

The reactive species-mediated mechanisms of cellular dysfunction in H/R have been studied by several authors using the experimental model of cells submitted to hypoxia (lack of oxygen relative to metabolic needs) followed by reoxygenation (reintroduction of oxygen in hypoxic tissue; see Refs. 37 and 59). It appeared that several enzymes can generate ROS, depending on the stimulus and the organ or cell type (51). Endothelial cells express a membrane NADPH oxidase similar to the membrane enzyme complex that produces the respiratory burst in granulocytes. The membrane oxidase complex is an important source of ROS in ischemic mouse lung (1) and probably in other reoxygenated tissues (23). Xanthine oxidase (XO) is capable of ROS generation, but its abundance varies greatly in different cell types, organs, and species (37). Eddy et al. (11) showed that, in human heart submitted to ischemia, xanthine dehydrogenase is rapidly converted to an oxidase that uses molecular O2 as the electron acceptor producing the free radical, superoxide, and hydrogen peroxide. NOS is an important source of vascular superoxide in cell types in which it is highly expressed, such as endothelial cells. The concentration of L-arginine is a factor limiting NO production by NOS and has been thought to increase superoxide production by NOS (5). The cyclooxygenase (COX) reaction has been

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The reactive species-mediated mechanisms of cellular dysfunction in H/R have been studied by several authors using the experimental model of cells submitted to hypoxia (lack of oxygen relative to metabolic needs) followed by reoxygenation (reintroduction of oxygen in hypoxic tissue; see Refs. 37 and 59). It appeared that several enzymes can generate ROS, depending on the stimulus and the organ or cell type (51). Endothelial cells express a membrane NADPH oxidase similar to the membrane enzyme complex that produces the respiratory burst in granulocytes. The membrane oxidase complex is an important source of ROS in ischemic mouse lung (1) and probably in other reoxygenated tissues (23). Xanthine oxidase (XO) is capable of ROS generation, but its abundance varies greatly in different cell types, organs, and species (37). Eddy et al. (11) showed that, in human heart submitted to ischemia, xanthine dehydrogenase is rapidly converted to an oxidase that uses molecular O2 as the electron acceptor producing the free radical, superoxide, and hydrogen peroxide. NOS is an important source of vascular superoxide in cell types in which it is highly expressed, such as endothelial cells. The concentration of L-arginine is a factor limiting NO production by NOS and has been thought to increase superoxide production by NOS (5). The cyclooxygenase (COX) reaction has been
shown to act as a significant source of vascular superoxide while simultaneously generating prostaglandins as a result of endothelial cell activation by stimuli such as bradykinin (20). In myocardial ischemia-reperfusion injury, recent evidence has implicated cytochrome P-450 monooxygenase as a significant factor of ROS production (14). Monoamine oxidase (MAO) located at the outer mitochondrial membrane has also been proposed as a potential source of ROS production (45, 54). Studies of isolated mitochondria from bovine heart have provided evidence that the electron transport chain produces superoxide at two sites, in the NADH dehydrogenase (complex I) and Q-cycle (complex III; see Ref. 6) region. The actions of inhibitors at specific mitochondrial sites in the electron transport chain upon oxygen sensing-related vascular signaling have suggested that these sites can be important participants in the generation of oxidant species. However, it should be noted that controversial results have been reported concerning the site of ROS production within the mitochondrial respiratory chain.

The present study was designed to identify the mechanism of ROS production in human umbilical vein endothelial cells (HUVEC) submitted to conditions close to those occurring during hemorrhagic shock. Indeed, it should be stressed that, when hypoxia is the result of hemodynamic perturbations, the supply of nutrients can also be severely diminished. Thus, in the present study, we associated hypoxia with glucose deprivation, and subsequent reoxygenation with glucose reintroduction in the perfusate.

In this experimental model, we used fluorescence microscopy to quantify ROS production after inhibiting potential sources of ROS, such as the cytoplasmic enzymes XO, NOS, COX, cytochrome P-450 monooxygenase, membrane-bound oxidases dependent on NADPH, MAO, and the four different complexes of the mitochondrial chain reaction. Our results provide evidence that, in endothelial cells, H/R-induced ROS production occurs in the complex III mitochondrial respiratory chain.

MATERIALS AND METHODS

Cells and culture. HUVEC were obtained from PromoCell Laboratories (Heidelberg, Netherlands) as cryopreserved cells. After being thawed, cells were plated (5,000–10,000 cells/cm²) in culture flasks precoated with rat tail type I collagen (5 g/cm²; Boehringer Mannheim, Mannheim, Germany) and cultured to confluence in nutritive medium containing endothelial cell growth factor and heparin, phenol red. The medium was supplemented with Supplement Mix/G10/H9262, 1.2 KH₂PO₄, 1.23 CaCl₂, 1 lactate, and 11.1 glucose and was equilibrated during stabilization and reperfusion in an atmosphere of 20% O₂-5% CO₂-75% N₂. In the perfusion chamber, PO₂ was ~120 mmHg, and the perfusate pH was 7.40. During hypoxia, the perfusate was equilibrated with 5% CO₂-95% N₂ (PO₂ ~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 fluorescence illumination and included a mercury lamp (80 watts), a 12-bit digital-cooled camera (model RTE/CCD-1317-K1; 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 x20 objective for fluorescence (Leica). Data were acquired and analyzed using Metamorph software (Universal Imaging).

Measurement of ROS and assessment of cell death. ROS generation in HUVEC was assessed using the 2',7'-dichlorofluorescein (DCF) probe (Sigma), which is very sensitive to H₂O₂. The reduced membrane-permeable form of the dye H₂DCF-DA was added to the perfusate at a final concentration of 5 μM. Within the cell, esterases cleave the acetate groups on H₂DCF, thus trapping the H₂DCF ester. ROS in the cells oxidize H₂DCF, yielding the fluorescent product DCFH (40). After 1 h, fluorescence was measured every 10 min using an excitation wavelength 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 H₂O₂ or hydroxyl radicals, but is relatively insensitive to superoxide (49).

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; it 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, and its excitation maximum is shifted ~30–40 nm into the red and its emission maximum ~15 nm into the blue. After 1 h of perfusion, fluorescence was measured every 30 min 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 Metamorph 3.5 over a region of interest that included the whole observational field. Intensity values are reported as percentage of initial values.

Experimental protocol. Cells plated on plastic coverslips were placed in the perfusion chamber, and flow was started. Two groups of at least six monolayers each were studied. 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 to block glycolysis in the perfusion chamber. Flow was then reduced 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% O₂-5% CO₂-75% N₂) with glucose and without H/R. During the 4 h of experimentation, one fluorescent image of H/R 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 identify the site of intracellular production of ROS in response to H/R, additional experiments were performed using the same design with different inhibitors, which were perfused for 3 h of H/R. Twelve groups of at least six monolayers each were studied.

To identify the site of ROS production in mitochondria, we used inhibitors of the mitochondrial respiratory chain complexes. Cells were exposed to H/R in the presence of rotenone (10 μM; see Ref. 46) and of thenoyltrifluoroacetone (TTFA, 10 μM; see Ref. 36). Rotenone

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is an inhibitor of complex I (NADH dehydrogenase) and inhibits electron transport from complex I to ubiquinone; TTF-A inhibits electron transport from complex II (succinate dehydrogenase) to ubiquinone. In another set of experiments, we used the complex III inhibitor antimycin A (AA), a quinone analog that binds to cytochrome (cyt) b_{562} and blocks electron transport to the ubiquinone in complex III. AA was added at the concentration of 10 μM (9). Involvement of complex III was also studied by using myxothiazol (2 μM; see Ref. 39) and stigmatellin (10 μM; see Ref. 3), which block electron transport to both the “Rieske” (2Fe-2S) iron sulfur protein (ISP) and heme b_{566} (21) by different mechanisms (3) and has been shown to be less toxic than myxothiazol (47). Complex IV was tested as a site of ROS production with the complex IV inhibitor, sodium azide (10 mM; see Ref. 9). To study the contribution to ROS production of membrane-bound NADPH-dependent oxidase, we ran sets of experiments in the presence of the NADPH oxidase inhibitor diphenylene iodonium (DPI, 10 μM; see Refs. 28 and 32) or of apocynin (10 μM; see Ref. 42), another NADPH oxidase inhibitor. DPI is also more generally a flavin inhibitor that can inhibit complex I of the electron transport chain (18, 28) and also cytochrome P-450 monooxygenase (43). Cytochrome P-450 is also inhibited by α-naphthoflavone (ANF, 1 μM; see Ref. 2). Pargyline (100 μM; see Ref. 19), an inhibitor of MAO A/B activity, was used to test MAO involvement in ROS production. To test the contribution of XO to ROS production, experiments were conducted in a Krebs-Henseleit buffer supplemented with the XO inhibitor allopurinol (10 μM; see Refs. 15 and 24).

Last, the respective contributions of COX and endothelial NOS to ROS generation were explored by simultaneous administration of the competitive COX inhibitor mefenamic acid (20 μM; see Refs. 10 and 13) and the L-arginine pathway inhibitor N^ω-nitro-L-arginine (L-NNA, 1 mM; see Refs. 10 and 31). All the above-mentioned inhibitors were administered during H/R, after uptake of the DCFH and PI dyes, and fluorescence equilibration.

Presentation of 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 Chemical, as specified above. Inhibitors were dissolved in DMSO. To avoid repeated freezing and thawing, working aliquots were prepared and stored at −20°C.

**Fig. 1.** Effect of hypoxia-reoxygenation (H/R) on 2',7'-dichlorofluorescein (DCF) fluorescence in human umbilical vein endothelial cells (HUVEC). Data are means ± SE and represent the difference between the last hypoxic point and the last reoxygenation point for the H/R group and the difference between the measurements made at the corresponding times in the control group. A significant rise in fluorescence was observed in cells submitted to H/R (*P < 0.0001 vs. H/R, n = 8 experiments) compared with the control (Ct) cells (n = 6).

**RESULTS**

**Effects of hypoxia on ROS production in HUVEC.** 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 143 ± 16% after 1 h of reoxygenation (Fig. 1). In contrast, this production was low (27 ± 4%) in control cells. See Fig. 2 for a typical experiment.

**Effect of cytosolic enzyme inhibitors on ROS production during reoxygenation.** The NADPH-dependent oxidase inhibitors DPI and apocynin did not significantly inhibit the rise in ROS production induced in HUVEC by 2 h of hypoxia and 1 h of reoxygenation, and neither did the XO inhibitor allopurinol, the NO and COX pathway inhibitors L-NNA and mefenamic acid, the cytochrome P-450 inhibitor ANF, the monoxygenase inhibitor DPI, or the MAO inhibitor pargyline (Fig. 3).

**Effect of mitochondrial respiratory chain inhibitors on ROS production during reoxygenation.** As mentioned above, there was no inhibition with DPI, a NADPH-dependent oxidase inhibitor that also blocks the flavin of complex I, or when the electron flows between mitochondrial complexes I and III, and between complexes II and III, were blocked by rotenone and TTF-A, respectively (in fact, in this case, we even observed an amplification of the increased DCF fluorescence associated with reoxygenation). In contrast, the inhibition was almost complete with antimycin A, which binds to complex III cyt b_{562} and blocks the transfer of electrons from cyt b_{562} to ubiquinone. In addition, we found a significant inhibition with the complex III inhibitor stigmatellin, which prevents electron transfer from ubiquinol to both ISP and heme b_{566}, but not with myxothiazol, which affects complex III but does not reduce the mobility of ISP (3, 52). Inhibition of complex IV by sodium azide did not reduce the increase in DCF fluorescence (Fig. 4).

**Cell death.** Images were taken every 30 min for four consecutive hours comprising 1 h of stabilization, 2 h of hypoxia, and 1 h of reoxygenation. PI fluorescence, which reflects cell death, did not vary significantly in HUVEC exposed to H/R or in control cells. Mitochondrial inhibitors did not change PI fluorescence, except in HUVEC treated with rotenone plus TTF-A, which induced a significant rise (20 ± 3%) compared with HUVEC exposed to H/R and not treated with inhibitors. The other cytosolic inhibitors did not change PI fluorescence either (Fig. 5).

**Fig. 2.** Real-time experiment comprising 2 h of hypoxia followed by 1 h of reoxygenation. Images were taken every 10 min and are represented by points. Note the large rise in reactive oxygen species (ROS) production during reperfusion. There was ROS increase of 51% after 10 min of reoxygenation and 115% after 1 h.
ROS is mediated by cyt b$_{562}$ in complex III, since blocking cyt b by a quinone analog inhibited intracellular ROS production. 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 (59). HUVEC were submitted to 2 h hypoxia, because Zweier et al. (59) showed that 90 min of anoxia is the minimal time required for large ROS production during reoxygenation. In addition, because severe shock in vivo can affect both O$_2$ and nutrients, we did not combine hypoxia and glucose depletion with acido- or hyperkalemia.

In our experiments, we observed a burst of ROS production during reoxygenation after hypoxia, and we postulated that the cells underwent specific changes in their enzyme activities, mitochondrial functions, cytoskeletal structure, membrane transport, and antioxidant defenses in response to hypoxia and glucose deprivation, factors that together predispose to reoxygenation injury (25). It is noteworthy that, unlike the results observed in cardiomyocytes by Vanden Hoek et al. (49), we did not observe a significant increase in ROS during the period of hypoxia. However, unlike these authors, in our experiments we did not combine hypoxia and glucose depletion with acidosis or hyperkalemia.

ROS generation by cytoplasmic enzymes has been reported in different cell types, including bovine pulmonary artery endothelial cells (55), mouse retinal endothelial cells (38), mouse coronary microvascular endothelial cells (27), and human aortic endothelial cells (57).

Potential alternative sources of endothelial ROS production in the cytosol or the cytoplasmic membrane include NADPH...
oxidase (22), XO (15), NOS (50), COX (13), cytochrome P-450 monoxygenase (14), and MAO (45). NADPH oxidase, originally characterized in neutrophils, is also present in endothelial cells (22) and is the principal source of superoxide production in several animal and human models of vascular diseases (17). However, in our experiments with HUVEC, the NADPH oxidase inhibitors DPI or apocynin at concentrations that inhibited ROS production in other cell types failed to inhibit this production significantly.

We also attempted to establish whether the inhibition of XO by allopurinol reduced the ROS production generated by hypoxia (i.e., without glucose) followed by reoxygenation with glucose, but the absence of inhibition makes it unlikely that XO plays an important role in the ROS production observed.

We also studied the role of the NOS and COX pathways by combined treatment of HUVEC with the NOS inhibitor l-NNA (31) and the COX inhibitor mafenamic acid (13). We chose this combined pharmacological approach because NOS and COX are potential sources of ROS (13, 50) and because strong interactions may exist between their respective pathways. Because inhibition by l-NNA and mafenamic acid did not prevent ROS production in our model, it is unlikely that these pathways are responsible for the production observed in our experiments.

Cytochrome P-450 has also been proposed as a potential source of ROS (14). This mechanism is unlikely to be involved in the ROS production observed in the present study, since α-ANF, an inhibitor of P-450 isoforms present in HUVEC (2), did not affect this production. In addition, if this source of ROS was important in the present H/R experiments, it is likely that an inhibition would have been also observed with DPI, since this inhibitor of flavoproteins has been shown to inhibit monooxygenase, which is part of the enzymatic complex of cytochrome P-450 (43).

MAO located at the outer mitochondrial membrane has also been proposed as a potential source of ROS production (45, 54). This was unlikely in the present experiments, since parargyline, an inhibitor of MAO A/B activity, did not affect ROS production.

Taken together, our results suggest that mitochondria were the main site of ROS production in endothelial cells submitted to H/R.

The involvement of mitochondrial electron transport in H/R-induced ROS production has indeed been demonstrated in several cell types, including chick cardiomyocytes (29), rabbit retinal cells (4), human lung epithelial cells (26), and Hep 3B cells (8).

Several mitochondrial enzymes have been proposed to affect the level of ROS after hypoxia, by interfering with ROS elimination or production.

In isolated mitochondria, the ROS generation stimulated by the substrates NADH, ubiquinone, and succinate has been shown to involve respiratory chain complexes I and III (6). A similar mechanism was also reported in hepatocytes (7) and cardiomyocytes (56) after reoxygenation following anoxia.

Several sites in complex I have been proposed to produce ROS, including the flavin-containing enzymes of complex I and the iron-sulfur cluster (12, 48). H/R-induced ROS production was not inhibited when cells were treated with rotenone, which inhibits electron transfer between the Fe-S cluster and ubiquinone (34), and the complex II inhibitor TFFA. On the contrary, we observed an increase in ROS production with these inhibitors, as reported in different studies of submitochondrial particles (12, 48). This effect is explained by Genova et al. (12), who proposed that this is because of the fact that rotenone blocks complex I downstream from the redox center responsible for oxygen radical formation, thus inducing an increase in ROS production. However, this effect was not specific to the cells submitted to H/R because the same phenomenon occurred in control cells; consequently, we cannot be sure of the precise role of complex I in H/R-induced ROS production. For this purpose, in additional experiments, we used the complex I inhibitor DPI, which reacts with flavoenzymes to form phenylated flavin adducts (33). DPI did not inhibit ROS production, thus suggesting that the site of ROS production affected by H/R is situated in a complex downstream from complex I and that, in the presence of DPI, electrons can enter the respiratory chain via complex II, which is not inhibited by DPI (18).

Indeed, the respiratory chain complex III has also been reported to play a key role in ROS generation in response to different stimuli. The electron transfer in this complex has been described in detail. In the Q-cycle, ubiquinol is oxidized at the quinol oxidase (Qo) site, which is formed by the interaction of the cyt b protein with ISP (53). Electron transfer from ubiquinol is diverted, so that the first electron is transferred to the “high-potential chain,” consisting of the ISP and the cyt c1 heme. The initial one-electron oxidation leaves an unstable semiquinone species at the Qo site that is oxidized by the “low-potential chain,” consisting of cyt bl and cyt bH, which are embedded in the cyt b subunit. The two protons from the quinol oxidized at the Qo site are released in the intermembrane space. After two turnovers of the Qo site, the two electrons sent to the low-potential chain reduce a ubiquinone molecule at the quinone reductase site to ubiquinol, with an uptake of two protons from the matrix (30).

In this complex, the ubisemiquinone site or reduced cyt b site has been proposed as the major site of ROS production (39, 41).

Here, we found that ROS production induced by H/R was inhibited by AA. This observation is similar to those by Sanchez-Alcazar et al. (39) in TNF-α/cycloheximide-induced cytotoxicity, and by Armstrong and Jones (3) in glutathione-depleted HL-60 cells in which Bel-2 was overexpressed.

The interpretation of the present results can be very similar to that proposed by Sanchez-Alcazar et al. (39). As reported with TNF-α/cycloheximide-induced cytotoxicity, they could be explained by the hypothesis that H/R can affect cyt c release. H/R may induce an accumulation of electrons in reduced cyt b that can be transferred to molecular oxygen, leading to generation of ROS.

AA may inhibit ROS production, since it is a quinone analog that binds to the quinone site of cyt b and induces a conformational change of the b-c1 complex, which blocks the transfer of the electrons in cyt b pathways and affects their redox potential. Stigmatellin, which prevents electron transfer from ubiquinol to both ISP and heme b566, also inhibited ROS production as was also found by Armstrong and Jones (3) in HL-60 cells. In contrast, myxothiazol, which acts on the same site, did not block ROS production. This result is similar to that observed in the experiments of Sanchez-Alcazar et al. (39) on TNF-α/cycloheximide-induced cytotoxicity and those of Armstrong and Jones (3) on HL-60 cells. Differences between these
two inhibitors of the Q0 site as regards ROS production have been noted by several authors (3, 21, 30, 52). This could be explained by the fact that stigmatellin reduces the motion of ISP, whereas myxothiazol might cause an increase in the motion of ISP (3, 52). Another possibility proposed by Muller et al. (30) and Iwata et al. (21) is that stigmatellin but not myxothiazol would interact with reduced ISP.

Because we also observed that the complex IV inhibitor sodium azide did not affect ROS production, it seems that we have been noted by several authors (3, 21, 30, 52). This could be because the duration of the experiment, which was very short (4 h), and because of the intensity of the stimulus (H711, 1987). For the present study demonstrated that, in endothelial cells submitted to hypoxia and glucose depletion followed by reoxygenation with glucose, the cyt b located in complex III is responsible for increasing ROS production.

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