Transient Hyperoxic Reoxygenation Reduces Cytochrome c Oxidase Activity by Increasing Superoxide Dismutase and Nitric Oxide

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Oxygen therapies have been shown to be cytoprotective in a dose-dependent fashion. Previously, we have characterized the protective effects of moderate hyperoxia on cell viability of ischemic human cardiomyocytes and their mitochondrial membrane potential by transient addition of oxygenated perfluorocarbons to the cell medium. Now, we report that the activity and expression of cytochrome c oxidase (COX) after prolonged ischemia depend on the amount of oxygen delivered during reoxygenation. Transient hyperoxia during reoxygenation results in a decrease of COX activity by 62 ± 15% and COX expression by 67 ± 5%, when hyperoxic tensions of ≈300 mm Hg are reached in the cell medium. This decrease in COX expression is prevented by the inhibition of inducible nitric-oxide synthase (iNOS). Immunoblot analysis of ischemic human cardiomyocytes revealed that hyperoxic reoxygenation causes a 2-fold increase of iNOS, leading to a rise in nitric oxide production by 140 ± 45%. Hyperoxic reoxygenation is further responsible for a 2-fold activation of hydrogen peroxide production and an increase in cytosolic superoxide dismutase expression by 35 ± 10%. NADPH availability has no effect on the hyperoxia-induced decrease of superoxide. Overall, these results indicate that transient hyperoxic reoxygenation in optimal concentrations increases the level of nitric oxide by activation of iNOS and superoxide dismutase, thereby inducing respiratory arrest in mitochondria of ischemic cardiomyocytes.

Conversely, oxidative phosphorylation is a central site of reactive oxygen species production in the heart (5–7). Oxidative phosphorylation along with NADPH oxidase form the major sources of superoxide in myocytes (8) that may induce irreversible cellular damage and cell death (9). Nitric oxide (NO) can inhibit oxidative phosphorylation reversibly by blocking cytochrome c oxidase (COX) of the electron transport chain (ETC) (10). In this way, NO protects myocardial tissue from ischemia and reperfusion injury (11) possibly by defending the mitochondrion to maintain their membrane potential (12) and reducing cytotoxicity of reactive oxygen species.

Sandau et al. (13) have shown that NO production by inducible nitric-oxide synthase (iNOS) can mimic a hypoxic response under normoxia. We have shown previously that hypoxia-inducible factors 1α and 2α are stabilized after hyperoxic reoxygenation with oxygenated PFC and that their stabilization is protective for ischemic human cardiomyocytes (14). However, it is unclear how hyperoxia affects NO production in ischemic cardiomyocytes. Therefore, the aim of this study was to characterize the effects of hyperoxia on ischemic cardiomyocytes with regard to changes in oxidative phosphorylation and NO production and in particular by evaluating the role of iNOS, COX, and the production of reactive oxygen species during reoxygenation.

EXPERIMENTAL PROCEDURES

Materials—Aminoguanidine hemisulfate, anti-Cu,ZnSOD antibody, bisbenzimid Hoechst 33342, collagenase VII, diethylthiocarbamate, 2’,7’-dichlorofluorescein diacetate, 4’,5’-diaminofluorescein diacetate, HEPES, N-(3-[aminomethyl]-benzyl)acetamide (1400W), lucigenin, β-NADPH, phenylmethylsulfonyl fluoride, protease inhibitor mixture, Tris(1,10-phenanthroline)ruthenium(II)chloride hydrate (Ru(phen)₃²⁺), and Triton X-100 were purchased from Sigma. Acridine orange was from Difco-Becton Dickinson. Anti-iNOS antibody was bought from BD Transduction Laboratories (Lexington, KY). Perfluorooctyl bromide was purchased from ABCR (Karlsruhe, Germany). Propidium iodide was from PharMingen (Santiago, CA). Smooth muscle growth medium was purchased from PromoCell (Heidelberg, Germany). TRIzol was bought from Invitrogen.

Culture of Human Cardiomyocytes—Monolayer cultures of human cardiomyocytes were prepared by modifying the method of Merante et al. (15). Briefly, heart tissue from the right atrium was obtained from male, Caucasian patients undergoing cardiovascular bypass surgery. The tissue was care-
fully dissected and further softened by digestion with 0.1% collagenase VII. Tissue specimens were cultured in smooth muscle growth medium (PromoCell). Cultures exhibiting >95% purity were used for subsequent studies.

**Model of Hypoxia and Reoxygenation**—Ischemia was mimicked by exposing cardiomyocytes (seeding density, 3000 cells/cm²) to a low oxygen atmosphere (pO₂ < 40 mm Hg) as described previously (14). Hypoxia was maintained by continuous infusion of 95% N₂/5% CO₂. Pure PFC was ventilated with 100% carbogen (95% O₂, 5% CO₂), added to the cell medium, and removed after 10 min. Cells were incubated for an additional 3 h.

**Normobaric Hyperoxia Model**—The oxygen-carrying capacity of the perfluorooctyl bromide-supplemented cell medium obeys Henry’s law of partial pressures (Fig. 1) and was calculated as described previously (14). In brief, we added liquid oxygenated PFC to ischemic cardiomyocytes cultured in cell medium to induce hypoxic conditions around the cells. The increase of oxygen in the cell culture medium was verified with a GMH 3610 analyzer for dissolved oxygen from Greisinger Electronics (Regenstauf, Germany). Oxygen tensions were transiently elevated to hyperoxic values of 300 mm Hg for a period of 10 min. Cellular changes of ischemic cardiomyocytes were studied 3 h after reoxygenation.

**Cell Viability Staining**—Cell viability was analyzed by fluorescent staining as reported previously (16). Briefly, we used a triple dye combination of acidine orange, Hoechst 33342, and propidium iodide that visualizes all cell viability states simultaneously on the basis of nuclear and cytoplasmic signals. Healthy cells showed green colored cytoplasms; early apoptotic cells dark rust-yellow cytoplasms; necrotic cells showed orange cytoplasms. Axiovision® 3.1 and KS 300® 3.0 software, both from Zeiss (Goettingen, Germany), were used for quantification.

**Hydrogen Peroxide Fluorescence**—Hydrogen peroxide production was assessed by dichlorofluorescein, as described previously by Royall and Ischiropoulos (17). Cardiomyocytes were incubated in cell medium supplemented with 10 μM dichlorofluorescein for 45 min within the cell culture incubator, fixed in 3.7% formalin at 37°C for 15 min, and counterstained with DAPI.

**Nitric Oxide Production**—Cells were washed twice with phosphate-buffered saline, and 2.5 μM dianisofluorescein and 200 μM L-arginine with or without 1 μM aminoguanidine hemisulfate in 200 μl of phosphate-buffered saline were added. After incubation for further 2 h, the supernatants were transferred to black microplates, and the fluorescence was measured at 485 nm excitation and 538 nm emission using a SpectraMax Gemini fluorometer from Molecular Devices (Sunnyvale, CA).

**Western Blot Analysis**—Cardiomyocytes were lysed using the MEM-PER eukaryotic membrane protein extraction kit from Pierce. Protein concentration was determined by the Bio-Rad protein assay kit using bovine serum albumin as control. Protein aliquots were separated by electrophoresis on 7.5% SDS-polyacrylamide gel, blotted onto polyvinylidene fluoride membranes (Millipore), and immunoblotted overnight with the indicated primary antibody and secondary antibody. The CDP-Star Reagent from PerkinElmer Life Sciences was used for detection.

**Superoxide Chemiluminescence**—Superoxide production was analyzed by lucigenin chemiluminescence. Briefly, after incubating cardiomyocytes with 50 mM diethyldithiocarbamate for 1 h and fluorescence-labeled secondary antibody for 4 h, cells were analyzed in HEPES buffer containing increasing concentrations of NADPH after the addition of lucigenin that emits light on reduction of superoxide (19). Chemiluminescence was determined using a TD-20/20 luminometer from Turner Designs (Sunnyvale, CA).

**Immunohistochemistry**—Consecutively, cardiomyocytes were incubated in 1% formalin at 4°C overnight, in 0.2% Triton X-100 for 5 min, blocked for 1 h, and finally stained in 5% bovine serum albumin with the indicated primary antibody and secondary antibody. The CDP-Star Reagent from PerkinElmer Life Sciences was used for detection.

**COX Activity**—Mitochondria were extracted by homogenization and centrifugation with buffers of MITOISO® mitochondrial isolation kit from Sigma following the manufacturer’s protocol. 2 μg of mitochondria were used in a cytochrome c oxidase assay kit (Sigma) that measures the decrease in absorbance of ferrocyanochrome c by its oxidation with COX. Absorbance at 550 nm was determined using a SpectraMax Plus spectrophotometer from Molecular Devices. Vₐₘₙₐₓ was derived from the maximal change in absorbance/second and was calculated with SoftMax Pro software from Molecular Devices. The mean value of change in absorbance was 2 × 10⁻⁴ for normoxic and 2 × 10⁻⁵ for hypoxic reoxygenation.
**Hyperoxia Reduces Cytochrome c Oxidase Activity**

COX Expression—Hypoxic cardiomyocytes were reoxygenated during transient hyperoxia with and without iNOS inhibition by 1400W (5 μM). Cardiomyocytes were lysed in TRIzol, and RNA was extracted according to the manufacturer’s protocol. 1 μg of RNA was reverse transcribed with a cDNA synthesis kit from Roche Diagnostics. Transcripts for isoform 1 of COX4 subunit were amplified by real-time PCR using specific primers that have been described previously (20). PCR was performed using a LightCycler System and the corresponding FastStart DNA Master SYBR Green I kit (Roche Diagnostics).

**Visualization of Intracellular Oxygen**—Ru(phen₃)²⁺ is a fluorochrome that is used as an indicator of intracellular oxygen tension. Its fluorescence is inversely proportional to the cellular oxygen tension (21). After reoxygenation with oxygenated PFC, Ru(phen₃)²⁺ was added to the cell culture medium at a concentration of 0.1 mM. 3 h after loading of Ru(phen₃)²⁺, changes in the fluorescence of cardiomyocytes were documented with an Axioplan 2 imaging® fluorescence microscopy. Axiovision® 3.1 and KS 300® 3.0 software from Zeiss were used for densitometry analysis.

**Statistical Analysis**—Data are normalized for the amount of protein or number of cells engaged. All experiments were repeated at least four times. Data are given as percent from normoxic value, which was set at 100%, and are presented as mean ± S.E. Comparisons were made using unpaired Student’s t test or one-way ANOVA when appropriate. Results were considered to be significantly different when p < 0.05.

**RESULTS**

**Prolonged Ischemia and Not Moderate Hyperoxia Decreases Cell Viability**—We found that the cell viability of cardiomyocytes is decreased after 12 h of ischemia (by 29 ± 7%, p < 0.05; data not shown) compared with normoxic cells. Normoxic cells, not being exposed to ischemia and reoxygenation, do not behave differently from normoxic cells subjected to moderate hyperoxia (change in viability 3 ± 8%; data not shown).

**Transient Hyperoxic Reoxygenation Decreases COX Activity**—We investigated whether a brief treatment of ischemic cardiomyocytes inducing hyperoxic conditions in the cell medium during reoxygenation changes COX activity compared with normoxic conditions. After oxygen treatment, COX activity of ischemic cardiomyocytes showed a significant decrease (38 ± 15%, p < 0.05; data not shown). In addition, a significant decrease of pH was detectable (55 ± 17%, p < 0.05; data not shown).

**Consumption of Oxygen in Reoxygenated Cardiomyocytes**—Fig. 2, A and B, shows that reoxygenated cardiomyocytes have decreased their oxygen consumption after exposure to hyperoxia. This decrease in oxygen consumption was quantified as shown in Fig. 2C and reflects the decrease in COX activity (40 ± 9%, p < 0.05).

**Role of NADPH and Superoxide Production after Transient Hyperoxic Reoxygenation**—The role of NADPH in superoxide production was investigated by measuring superoxide chemiluminescence after exposing ischemic human cardiomyocytes to hyperoxic conditions during reoxygenation. Fig. 3 shows a decrease in superoxide production, after hyperoxia was applied. This decrease in superoxide production persisted throughout NADPH addition compared with superoxide levels of ischemic human cardiomyocytes after normoxic reoxygenation (75 ± 6%, p < 0.05).

**Hydrogen Peroxide Production Increases after Transient Hyperoxic Reoxygenation**—The contribution of hyperoxic conditions during reoxygenation on hydrogen peroxide (H₂O₂) production was tested by assessing the distribution pattern of the fluorochrome dichlorofluorescein in the cytosol of ischemic cardiomyocytes. Fig. 4B shows an ischemic cardiomyocyte with an increased cytosolic H₂O₂ production after reoxygenation under hyperoxic conditions compared with a cardiomyocyte after normoxic reoxygenation in Fig. 4A. In Fig. 4C, densitometry analysis confirmed that H₂O₂ production increased significantly (210 ± 37%, p < 0.05) after reoxygenation of ischemic cardiomyocytes.
Cytosolic Copper- and Zinc-containing SOD Is Activated by Transient Hyperoxic Reoxygenation

The cytosolic signal of copper- and zinc-containing superoxide dismutase (CuSOD) was detectable by immunhistochemistry in ischemic cardiomyocytes as soon as 1 h after reoxygenation applying the hyperoxic regime (H) in B. This signal is compared with the cellular fluorescence after reoxygenation under normoxic conditions (N) in A and quantified by densitometry analysis in C. Data are given as percent from normoxic value and represent the mean ± S.E. (error bars). *, significantly different from normoxic value (p < 0.05).

Decrease of COX Expression Depends on Nitric-oxide Synthase Expression and Production

To address the role of NO, the expression of nitric-oxide synthase isoforms was studied after applying hyperoxic conditions during reoxygenation of ischemic cardiomyocytes. Already 1 h after hyperoxia, the expression of iNOS increased significantly (196 ± 17%; p < 0.05), as shown in Fig. 6A. But when we performed Western blot analysis of cardiomyocytes after 12 h of ischemia, we were not able to find significant amounts of endothelial nitric-oxide synthase.
synthase or neuronal nitric-oxide synthase either with or without hyperoxic reoxygenation. NO production was determined by the fluorochrome diaminofluorescein, and the pattern of the NO production confirmed the results of iNOS expression (Fig. 6B). Subsequently, COX expression was decreased after transient hyperoxia as determined by real-time PCR in Fig. 6C (33 ± 5%, p < 0.05). When NO production was reduced by iNOS inhibitor 1400W, COX expression of ischemic cardiomyocytes showed a significant increase (194 ± 17%, p < 0.05), as shown in Fig. 6D.

DISCUSSION

We report here that the level of NO depends on the oxygen concentration during reoxygenation of ischemic cardiomyocytes. Transient hyperoxic reoxygenation increases the production of NO and the NO-protective expression of cytosolic SOD.

In our previous paper, we presented information about cell viability of ischemic cardiomyocytes (14). The rationale for using cardiomyocytes after prolonged ischemia in this model was to understand why patients with myocardial infarction subjected to normoxic reperfusion after 12 h often do not experience complete myocardial salvage. We showed that the viability of ischemic cardiomyocytes is significantly increased by reoxygenation using transient moderate hyperoxia.

Now, we report that transient increases in oxygen tensions to moderate hyperoxic values of ≈300 mm Hg during reoxygenation induce mitochondrial respiration arrest and cytoprotective mechanisms such as NO production whereas normoxic conditions do not induce these processes and lead to a shortening of lifespan of ischemic cardiomyocytes (Fig. 7). It has been shown that reoxygenation of ischemic cardiomyocytes is associated with an up-regulation of iNOS and that expression of iNOS protects ischemic tissue during reoxygenation (22). We show that transient hyperoxic conditions during reoxygenation increases iNOS expression and thereby may act cell protective by decreasing oxygen sensitivity of ischemic cardiomyocytes. Because this iNOS increase precedes all other effects we examined, we consider iNOS and its NO production to be the most important protective factors in our study.

It is established that NO inhibits oxygen consumption of mitochondria by ETC inhibition of complex IV (23, 24). We show that cytochrome c oxidase activity is decreased after reoxygenation of ischemic cardiomyocytes with oxygen supplementation and that this effect can be overcome by iNOS inhibition, confirming that NO inhibits oxygen consumption at the level of COX (10). The successive decrease of electron transport chain activity resulted in reduced oxidative phosphorylation and reduced proton consumption leading to a pH decrease after oxygen delivery. This result was consistent with previous findings after inhibition of oxidative phosphorylation (25). In addition, NO inhibition of ETC has been proven protective for the mitochondrial membrane potential (12), a phenomenon that we have shown previously for oxygen delivery (14). Thomas et al (26) demonstrated that NO inhibition of ETC might extend the zone of tissue oxygenation in vivo.

Reversible NO inhibition of ETC results in reduced cytotoxicity from superoxide (27) and may help mitochondria to cope with oxidative stress (28). We show that oxygen delivery results in a decrease in superoxide production that persists during stimulation of NADPH oxidase by NADPH, a major source of superoxide in myocytes (8). Increased oxygen tensions around ischemic cardiomyocytes during reoxygenation decreased superoxide production, which coincided with an augmented
expression of antioxidant cytosolic SOD, thereby protecting NO from inactivation by superoxide (29).

We have shown previously that the α-subunit of hypoxia-inducible factor 1 is stabilized after optimized oxygen treatment by moderate hyperoxia. Fukuda et al. (20) have demonstrated that the transcription factor hypoxia-inducible factor 1 regulates COX activity by switching from the normoxic isoform 1 to the hypoxic isoform 2 of COX subunit 4, thereby increasing the efficiency of mitochondrial respiration and reducing H$_2$O$_2$ production. We speculate that moderate hyperoxia of ≈300 mm Hg during reoxygenation may delay the shift of mitochondrial respiration from hypoxic back to normoxic ETC activity.

In summary, we have found that ETC activity after prolonged ischemia depends on the dose of oxygen supplementation during reoxygenation. Reoxygenation with elevated concentrations of oxygen results in decreased ETC activity and causes an increase of iNOS, leading to a rise in NO production. Reoxygenation using hyperoxic conditions is further responsible for an activation of H$_2$O$_2$ production and a decrease of superoxide generation using hyperoxic conditions is further responsible for increasing iNOS, leading to a rise in NO production. Reoxygenation results in decreased ETC activity and causes an increase of antioxidant cytosolic SOD, thereby protecting the efficiency of mitochondrial respiration and reducing H$_2$O$_2$ production. We speculate that moderate hyperoxia of ≈300 mm Hg during reoxygenation may delay the shift of mitochondrial respiration from hypoxic back to normoxic ETC activity.

In summary, we have found that ETC activity after prolonged ischemia depends on the dose of oxygen supplementation during reoxygenation. Reoxygenation with elevated concentrations of oxygen results in decreased ETC activity and causes an increase of iNOS, leading to a rise in NO production. Reoxygenation using hyperoxic conditions is further responsible for an activation of H$_2$O$_2$ production and a decrease of superoxide independently of NADPH availability by an increase in cytosolic SOD expression of antioxidant cytosolic SOD, thereby protecting NO from inactivation by superoxide (29).

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