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EFFECTS OF COLD AND WARM ISCHEMIA ON THE MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION OF SWINE LUNG[1]

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Background. **The aim of the study was to investigate the consequence of warm and cold ischemia on lung mitochondria in order to define bioenergetic limits within lung could be suitable for pulmonary transplantation.**

Methods. **Twenty-two pigs underwent lung harvesting after lung flush with Euro-Collins solution. Mitochondria were isolated from fresh lungs, from lungs submitted to 24 or 48 hr of cold ischemia, to 30 or 45 min of warm ischemia, and to 30 min of warm ischemia followed by 24 or 48 hr of cold ischemia. Mitochondrial oxidative phosphorylation parameters were determined in isolated mitochondria by in vitro measurement of oxygen consumption.**

Results. **Relative to controls, mitochondria submitted to cold ischemia showed an alteration in the oxidoreductase activities of the respiratory chain but no membrane permeability alteration. After 48 hr of cold ischemia, there was a decrease in the yield of the oxidative phosphorylation. Thirty minutes of warm ischemia did not alter the mitochondrial respiratory parameters. However, lung submitted to 45 min of warm ischemia showed mitochondrial damage as a decrease in the oxidative phosphorylation efficiency and ADP availability but no change in the oxidoreductase activities. Relative to cold ischemia alone, 30 min of warm ischemia preceding cold ischemia promoted no significant change in the respiratory parameters.**

Conclusions. **On bioenergetic basis, lung submitted to warm ischemia could be suitable for transplantation if the warm ischemia duration does not exceed 30 min. This could be a major concern in lung procurement from non-heart beating donors.**

Pulmonary transplantation has remained limited by suitable organ donor shortage (*1*). A way to increase the pool of available grafts is the use of organs harvested from non-heart beating donors (NHBD*) (2). Many investigators reported successful use of pulmonary graft harvested from NHBD in experimental lung transplantation (*3–10*), and at least one clinical case has been described (*11*). However, the use of lung grafts harvested after warm ischemia (WI) seems controversial because, in clinical settings, lung grafts are very sensitive to ischemia/reperfusion injury. Primary lung graft dysfunction (PLGD) may occur even after brief cold ischemia (CI) of lung grafts harvested from brain-dead donors. PLGD significantly complicates the posttransplant course in 10–20% of lung transplant recipients, and increases the pulmonary transplantation morbidity and mortality (*1*). The cause of PGLD is poorly understood, but PLGD may be partially caused by an alteration of pulmonary cell structure and metabolism before, during, and after transplantation (*12*).

Mitochondria play a key role in the cellular energetic metabolism. They are responsible for cellular respiration, which is coupled to adenosine triphosphate (ATP) synthesis, so-called oxidative phosphorylation (OxPhos). During ischemia, the oxygen deficit induces a decrease in the cytosolic and mitochondrial ATP content (*13*, *14*). In prolonged ischemia, the ATP level becomes insufficient to insure the cellular energetic needs, and the cell ability to maintain the ionic homeostasis is compromised (*15*). Cytosolic sodium, calcium, and inorganic phosphate contents rise (*16*) and may induce mitochondrial dysfunctions as uncoupled respiration, permeability transition (*17–19*), and swelling (*20*). The mitochondrial transition pore opening may be involved in reperfusion injury (*17*). In most tissues, mitochondria may produce some oxygen radical at the level of respiratory chain (*21*, *22*). The increased radical production as a result of organ reoxygenation may overload the radical defense system (*23*). All these critical events occurring before or/and during reoxygenation can promote cell recovery or irreversible injuries leading to necrosis. If OxPhos damage secondary to ischemia/reperfusion is moderate, cellular recovery occurs. If the mitochondrial inner membrane becomes permeable, mitochondria are uncoupled and irreversibly lose their ability to synthesize ATP. In this case, cell death is unavoidable.

We have developed a model to study OxPhos in pulmonary swine mitochondria. We previously reported the use of this model to compare the efficacy of Euro-Collins (EC) or University of Wisconsin solutions on lung graft preservation (*24*). The aim of the present study was to investigate the consequences of CI and WI on the OxPhos of pulmonary mitochondria. We assessed the efficacy of the OxPhos of mitochondria which were isolated from fresh swine lungs, from swine lungs submitted to 24 or 48 hr CI, 30 or 45 min WI, and 30 min WI followed by 24 or 48 hr CI.

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* Abbreviations: BSA, bovine serum albumin; CI, cold ischemia; EC, Euro-Collins solution; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; NHBD, non-heart beating donors; OxPhos, oxidative phosphorylation; PLGD, primary lung graft dysfunction; WI, warm ischemia; WR, warm reperfusion.

TABLE 1. Mitochondrial oxidative phosphorylation data with ketoglutarate and pyruvate as oxidizable substrates[a]

Group	N	V_3[b] (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	V_{3s} (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	V_4 (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	RC
Control	7	5.90±0.13 (n=35)	6.45±0.31 (n=7)	1.62±0.03 (n=34)	3.66±0.07 (n=34)
30-min WI	5	5.14±0.08 (n=24)	5.95±0.27 (n=5)	1.57±0.05 (n=24)	3.31±0.06 (n=24)
45-min WI	5	4.12±0.17† (n=23)	5.18±0.26† (n=5)	1.60±0.07 (n=22)	2.69±0.13† (n=22)
24-hr CI	4	4.72±0.10† (n=20)	4.91±0.22† (n=4)	1.39±0.02 (n=20)	3.39±0.05 (n=20)
48-hr CI	5	3.63±0.12† (n=20)	3.86±0.13† (n=5)	1.44±0.04 (n=20)	2.53±0.04† (n=20)
WI+24-hr CI	5	4.65±0.12 (n=25)	5.29±0.51 (n=5)	1.43±0.03 (n=24)	3.24±0.05 (n=24)
WI+48-hr CI	5	3.63±0.16 (n=26)	4.11±0.42 (n=6)	1.34±0.06 (n=25)	2.73±0.08 (n=25)
24-hr CI+WR[c]	5	2.75±0.12†‡ (n=20)	3.06±0.27†‡ (n=5)	1.43±0.05 (n=19)	1.89±0.06†‡ (n=19)

[a] Data are expressed as mean±SEM. Abbreviations used in table: N, number of lungs used for mitochondrial preparation; n, number of measurements of mitochondrial function in the group; WI, warm ischemia; CI, cold ischemia; WR, warm reperfusion; RC, respiratory-control ratio; URC, uncoupled respiratory-control ratio; ADP/O: number of ADP phosphorylated moles per atom/g of oxygen consumed; †, $P<0.05$ vs. control group; ‡, $P<0.05$ vs. 24-hr cold ischemia group; *, $P<0.05$ vs. 48-hr cold ischemia group.

[b] V (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)=oxygen consumption, in nmol per min and per μl of mitochondria.

[c] 24-hr CI+WR, results from Detry et al. (24).

MATERIALS AND METHODS

Twenty-two Pietrain pigs (weight 20–30 kg) used in this study, received care in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institute of Health Publication 85-23, revised 1985). The protocol for the study was approved by the Animal Care Committee at the University of Liège, Liège, Belgium.

Lung harvest. The pigs were premedicated, placed on a heating operating table, anesthetized, and ventilated with room air according to our model described elsewhere (*24*). After median sternotomy, the pulmonary artery and both venae cavae were isolated and encircled with a ligature. Both pleurae were opened, and both azygos veins were ligated and divided. After systemic heparinization (300 U/kg), 500 μg of prostaglandin E_1 (Prostin, Upjohn, Puurs, Belgium) were injected intravenously.

Control and CI groups. Both venae cavae were ligated, and the pulmonary artery was immediately cannulated by a high flow tubing through the right ventricular outflow tract, and the ligature was tied around the pulmonary artery to secure the cannula. After opening of the left atrial appendage, the lungs were flushed by gravity at 50 cmH_2O with 2 L of cold (4°C) standard EC (Fresenius AG, Wilrijk, Belgium). The ventilation was continued during the flush, and topical pulmonary cooling was facilitated by the flow of the effluent in both pleura. Upon completion of the flush, the ventilation was discontinued, and the heart-lung block was excised. After one final mechanical ventilation, the trachea was clamped at end-inspiration and ligated. The heart-lung block was then stored inflated in cold EC. The mitochondria were isolated from the lungs immediately after the harvesting (control group, n=7), or after 24 hr or 48 hr of CI (4°C) (24-hr CI group, n=4; 48-hr CI group, n=5).

Warm ischemia group. After heparinization and prostaglandin E_1 injection, the pigs were killed with intravenous injection of 100 mg/kg sodium pentobarbital (Nembutal; Sanofi, Libourne, France). The ventilation was discontinued, and the tracheal tube was disconnected from the respirator. The start of the WI period was defined by the onset of ventricular fibrillation. The pig core body temperature was maintained between 35°C and 37°C with the heating operating table. After 30 or 45 min of WI (30-min WI group, n=5; 45-min WI group, n=5), the ventilation was restarted, and the lungs were flushed as described for the control or CI groups. The mitochondria were isolated immediately after the WI period.

Combined warm and cold ischemia groups. After 30 min of WI, the lungs were harvested and stored inflated with room air in cold EC. Mitochondria were then isolated after 24 or 48 hr (30-min WI + 24-hr CI group, n=5; 30-min WI and 48-hr WI group, n=5).

Combined cold ischemia and warm reperfusion group. The reperfusion method has been described elsewhere (*24*). In summary, after 24 hr of CI, the right lung block was submitted to 30 min of in vitro warm reperfusion (WR) with air ventilation. After ischemia, the heart-lung block was inserted in a humidified Plexiglas chamber at 37°C. The right lung was reventilated with room air. At the time of reventilation, the lung was reperfused in closed circuit through the pulmonary artery with a Krebs-Henseleit bicarbonated buffer solution (pH 7.3, 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L NaH_2PO_4, 25 mmol/L $NaHCO_3$, 2.5 mmol/L $CaCl_2$, 11.1 mmol/L glucose) at 37°C for 30 min. This solution was injected by a roller pump (type 914421; Jostra AB, Lund, Sweden) at a constant flow of 300 ml/min, perfused through the right lung, and drained by gravity through the aorta (24-hr CI + 30-min WR, n=5).

Mitochondrial isolation. The lungs were trimmed, and pieces were homogenized with a motor-driven hand-held homogenizer in the presence of SET solution (250 mmol/L sucrose, 2 mmol/L EDTA, 5 mmol/L Tris, 0.5% fatty acid-free bovine serum albumin [BSA]). After homogenization and filtration, the mitochondria were isolated from the solution with a standard technique of differential centrifugations with a Beckman J2-HC Centrifuge (Palo Alto, CA). Homogenate was centrifuged at 2000×*g* for 10 min and its supernatant at 17,500×*g* for 10 min. The pellet was resuspended with SET solution, and resedimented at 600×*g* for 10 min. The supernatant was centrifuged at 19,000×*g* for 10 min, and the surface of the pellet was rinsed with 5 ml of SET solution. This pellet was washed and centrifuged at 19,000×*g* for 10 min twice before obtaining final mitochondrial suspension.

Mitochondrial volume measurement. A sample of mitochondrial suspension was incubated with a radioactive medium ((15 mmol/L KCl, 2 mmol/L EDTA, 5 mmol/L $MgCl_2$, 50 mmol/L Tris, 3.3 μCi of tritiated water (^3H_2O)). The incubation mixture was centrifuged and the radioactivities of the mitochondrial pellets and of the supernatant solutions were determined as described elsewhere (*24*). The mitochondrial volume (volume of the mitochondrial pellet) was calculated according to the formula: *pellet volume* (μl) = (*pellet radioactivity*/*supernatant radioactivity*) × *supernatant volume* (μl). Respiratory rates were expressed per min per μl of mitochondria in the pellet.

Mitochondrial oxygen consumption measurement. Respiratory parameters were determined at 25°C in isolated mitochondria with a Gilson oxygraph (Gilson Medical Electronics, Middleton, WI) by in vitro measurement of oxygen consumption rates in a medium at pH 7.4 (15 mmol/L KCl, 2 mmol/L EDTA, 5 mmol/L $MgCl_2$, 50 mmol/L

TABLE 1. Continued

V_{Olig} (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	V_{FCCP} (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	URC	ADP/O
0.30±0.03 (n=7)	6.84±0.65 (n=6)	21.80±0.90 (n=7)	2.30±0.03 (n=34)
0.31±0.03 (n=5)	7.17±0.67 (n=5)	22.95±0.43 (n=5)	2.19±0.03 (n=24)
0.28±0.02 (n=5)	6.67±0.48 (n=5)	23.96±0.49 (n=5)	1.96±0.05† (n=22)
0.26±0.01 (n=4)	4.85±0.25† (n=4)	19.00±0.65 (n=4)	2.25±0.04 (n=20)
0.22±0.03 (n=5)	4.30±0.34† (n=5)	20.03±1.43 (n=5)	1.84±0.04† (n=20)
0.32±0.02 (n=5)	6.60±0.61‡ (n=5)	20.76±1.03 (n=5)	2.22±0.04 (n=25)
0.30±0.04 (n=6)	5.12±0.50 (n=6)	18.12±1.43 (n=6)	2.05±0.04* (n=25)
0.19±0.03† (n=5)	3.94±0.28†‡ (n=5)	22.64±2.89 (n=5)	1.54±0.04†‡ (n=19)

Tris), in the presence of 1% BSA, with 2.5 mmol/L phosphate (KH_2PO_4). Oxidizable substrates were 5 mmol/L ketoglutarate and 5 mmol/L pyruvate (NADH linked substrates) and 5 mmol/L succinate ($FADH_2$-linked substrate) in the presence of rotenone (complex I inhibitor). The concentration of added adenosine diphosphate (ADP), oligomycin, and uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) was 165 μmol/L, 16 μg/ml, and 15 μmol/L, respectively. The measured functional parameters of mitochondria were: the respiration rates in the presence of externally added ADP (V_3 during ADP pulse and V_{3s} with saturating ADP) or in its absence (V_4), which were used to calculate the respiratory control (RC) given by the ratio V_3/V_4; the respiration rates when ATP synthase is blocked by oligomycin (V_{Olig}) or in the presence of the uncoupler FCCP (V_{FCCP}); the uncoupled respiratory control (URC) given by the ratio V_{FCCP}/V_{Olig}. The yield of OxPhos, i.e., the number of moles of ADP phosphorylated by atom g of oxygen consumed (ADP/O) was also determined.

Chemicals. Ketoglutarate, pyruvate, BSA, and ADP were purchased from Roche Molecular Biochemicals (Mannheim, Germany), oligomycin, rotenone and succinic acid from Sigma Chemical (St. Louis, MO), FCCP from Du Pont de Nemours (Wilmington, DE), and ^3H_2O from Radio Chemical Center (Amersham, England). In the absence of specific indications, the other chemicals used in this study were purchased from Merck (Darmstadt, Germany).

Experimental design. The lungs were randomly assigned to one of the groups, and the same heart-lung block never gave both lungs to the same group. Each lung was used for only one mitochondrial isolation, but the same preparation may have been used for several mitochondrial oxygen consumption measurements.

Statistics. All data are presented as mean±SEM. One-way analysis of variance design and corresponding *F*-tests were used to analyze the mitochondrial oxygen comsumption data. Fisher test was used for comparison between two means. Results of the tests are expressed by their *P*-value, and *P*-values <0.05 were taken to be statistically significant.

RESULTS

The mitochondrial oxygen consumption data with ketoglutarate and pyruvate as oxidizable substrates are presented in Table 1. Respiration rates were measured in state 3 (V_3 and V_{3s}), in state 4 (V_4), and in the presence of oligomycin (V_{Olig}) or uncoupler FCCP (V_{FCCP}). The efficiency of the oxidative phosphorylation was also determined (ADP/O ratio). The respiratory parameters were measured on mitochondria isolated from fresh-flushed lungs (control) or from lungs submitted to WI, CI, WI+CI, and CI+WR.

Related to control lungs, 24 hr of CI induced significant decrease ($P<0.05$) in V_3, V_{3s}, V_{FCCP}, RCs, and URC. There was no difference in V_4, V_{Olig}, and ADP/O ratio. Forty-eight hours of CI, when compared to the 24-hr CI group, promoted no further changes in V_{FCCP}, V_4, V_{Olig}, and URC. Moreover RC underwent a significant decrease ($P<0.001$) as a result of V_3 decrease ($P<0.005$). ADP/O ratio also decreased ($P<0.001$).

Related to control, 30 min of WI induced no modification in the respiratory parameters. However, 45 min of WI, compared to control, promoted a highly significant decrease in V_3 ($P<0.005$), V_{3s}, RC, and ADP/O ($P<0.05$). V_{FCCP}, V_4, V_{Olig}, and URC were not modified. RC decrease ($P<0.05$) was caused by V_3 decrease and no change in V_4.

Related to 24 or 48 hr of CI alone, the combination of 30 min of WI and 24 or 48 hr of CI did not promote significant changes in the respiratory parameters except a significant improvement of V_{FCCP} ($P<0.05$) after 24 hr of CI and ADP/O after 48 hr as shown in Table 1.

Related to 24 hr of CI alone, the combination of 24 hr of CI followed by 30 min of WR promoted a significant decrease of V_3, V_{3s}, ADP/O ($P<0.001$), and V_{FCCP} ($P<0.05$).

The results described in Table 2 were obtained with succinate as oxidizable substrate in the presence of rotenone. Similar changes in the respiratory parameters were observed with some variations in statistical significance (see Table 2).

DISCUSSION

In this study, we investigated the OxPhos function in mitochondria isolated from swine lung after CI or WI. The measurements of the mitochondrial respiratory parameters allow to point out permanent OxPhos dysfunctions induced by lung ischemia and reperfusion. These mitochondrial damage are still perceptible after mitochondria isolation and may be partly responsible for cell death. Analysis of the respiratory parameters allows us to describe and to identify the location of the mitochondrial damage, i.e., phospholipid bilayer permeability, enzyme activity, and substrate or ADP availability. Indeed, according to the chemiosmotic coupling (*25*) between respiration and ATP synthesis, 10–12 protons (H^+) are extruded (*26*) from the mitochondria for each oxygen atom consumed by the respiratory chain and four H^+ are taken up to synthesize one ATP. The result of such proton stoichiometry rules is the existence of stoichiometric ratios H^+/O, H^+/ATP, and ADP/O that are fully respected in tightly coupled mitochondria because their inner membrane is almost impermeable to H^+ (no leak). In a tightly coupled mitochondria with very low H^+ membrane conductivity, 1) the O_2 consumption is tightly controlled by H^+ re-entry into the

TABLE 2. Mitochondrial oxidative phosphorylation data with succinate (in the presence of rotenone) as oxidizable substrate[a]

Group	N	V_3[b] (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	V_{3s} (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	V_4 (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	RC
Control	7	6.93±0.14 (n=31)	7.24±0.42 (n=6)	2.42±0.04 (n=29)	2.90±0.05 (n=29)
30-min WI	5	6.00±0.14 (n=24)	6.49±0.24 (n=5)	2.32±0.07 (n=24)	2.61±0.06 (n=24)
45-min WI	5	5.05±0.10† (n=21)	5.86±0.16† (n=5)	2.12±0.07 (n=22)	2.12±0.07† (n=22)
24-hr CI	4	5.02±0.10† (n=18)	5.27±0.12† (n=4)	2.06±0.02 (n=17)	2.45±0.05† (n=17)
48-hr CI	5	4.27±0.16† (n=19)	4.43±0.29† (n=5)	2.19±0.07 (n=19)	1.94±0.03† (n=19)
WI+24-hr CI	5	5.46±0.16 (n=23)	5.37±0.23 (n=4)	2.24±0.07 (n=22)	2.43±0.04 (n=22)
WI+48-hr CI	5	4.38±0.15 (n=23)	4.71±0.38 (n=5)	2.11±0.09 (n=22)	2.13±0.06 (n=22)
24-hr CI+WR	5	3.66±0.17†‡ (n=17)	4.03±0.32†‡ (n=4)	2.09±0.04 (n=15)	1.75±0.07†‡ (n=15)

[a] Data are expressed as mean±SEM. Abbreviations used in table: N, number of lungs used for mitochondrial preparation; n, number of measurements of mitochondrial function in the group; WI, warm ischemia; CI, cold ischemia; WR, warm reperfusion; RC, respiratory-control ratio; URC, uncoupled respiratory-control ratio; ADP/O: number of ADP phosphorylated moles per atom/g of oxygen consumed; †, $P<0.05$ vs. control group; ‡, $P<0.05$ vs. 24-hr cold ischemia group.

[b] V (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)=oxygen consumption, in nmol per min and per μl of mitochondria.

matrix; 2) the addition of protonophore (as FCCP), which permeabilizes the membrane to H^+, destroys this control and leads to maximal respiratory rate; 3) the full inhibition of ATP synthase by oligomycin reduces the oxygen consumption to a residual rate controlled by the H^+ leak.

According to their functional parameters, mitochondrial damage can be located at several levels. Oligomycin-resistant respiration rate that is sustained by H^+ leak informs on membrane damage increasing H^+ permeability; a V_{Olig} increase indicates an increase in H^+ leak through the membrane that alters the chemiosmotic coupling. If V_4 (non-phosphorylating respiration after complete consumption of externally added ADP) increases and V_{Olig} does not increase, the membrane lipid bilayer is not damaged, but the intrinsic stoichiometry of ATP synthase is impaired (H^+ slip, i.e., intrinsic uncoupling of ATP synthase). When both phosphorylating (V_3, V_{3s}) and uncoupled (V_{FCCP}) respiration rates similarly decrease, either the respiratory chain oxidoreductases are intrinsically damaged, or the upstream feeding of the respiratory chain with electrons is limiting (oxidizable substrate availability). However, if the phosphorylating respirations decrease without V_{FCCP} alteration, there is no oxidative enzyme impairment or substrate shortage, but a decrease in ADP availability or impairment of the ATP synthase. H^+ leak (membrane damage) and/or H^+ slip (modification of intrinsic H^+ stoichiometry of redox pump and/or ATP synthase) lead to a decrease in ADP/O ratio i.e. a decrease in OxPhos efficiency. RC and URC changes may only be interpreted according to the modified parameter, V_4 or V_3 and V_{Olig} or V_{FCCP}, respectively.

In this study, we applied the theoretical background described above to investigate the effects of ischemia on lung mitochondria. Two different electron sources were used to reduce oxygen through complex I or complex II. The same results were observed when we used NADH-linked substrates (ketoglutarate and pyruvate), electron providers at the level of respiratory chain complex I, and $FADH_2$-linked substrate (succinate) in the presence of complex I inhibitor (rotenone), electron donor for respiratory chain complex II. These results, in accordance with those previously reported for heart by Veitch (*27*), indicate that, in our study, complex I lesions were not more severe than complex II lesions, in contrast to some reported results in heart and liver (*28–30*), where ischemia led to significant complex I damage, but no alteration in complex II. The results described by Augustin (*31*) in liver and Almeida (*32*) in brain indicated that NADH-dependent respiration was more affected by hypoxia/reoxygenation than $FADH_2$-dependent respiration.

Mitochondria isolated from lungs submitted to 24 hr of CI exhibited a significant and similar decrease in the phosphorylating respiration rates (V_3 and V_{3s}) and in the uncoupled respiration rate (V_{FCCP}) but no change in V_4, V_{Olig}, and ADP/O. These results demonstrated that 24 hr of CI permanently altered either the oxidative enzymes or the oxidizable substrate availability. On the other hand, neither H^+ leak, H^+ slip, nor OxPhos efficiency drop were induced by 24 hr of CI. As the same substrate concentrations were provided in all measurements, a decrease in substrate availability, which could explain the respiratory rate decrease, could only be caused by partial inactivation of their translocators (ketoglutarate, pyruvate, and succinate) or their deshydrogenase. Forty-eight hours of CI led to an additional decrease in V_3 and V_{3s} without V_{FCCP} change, indicating, in addition to preceding damage, a decrease in ADP availability or impairment of the ATP synthase. As ADP was provided at the same concentrations, the decrease in ADP availability, if explanatory, is necessary linked to partial inactivation of the adenylic translocator. Moreover, the ADP/O ratio decreased after 48 hr of CI, without V_{Olig} and V_4 increase. These last results indicated that the second day of CI induced neither a H^+ leak nor a H^+ slip at the level of respiratory chain H^+ pumps but a H^+ slip at the level of the ATP synthase (change in H^+ stoichiometry) in addition to the decrease in its activity. The two impairments neutralized each other in V_4, which should increase in front of ATP synthase H^+ slip alone, and had no effect on V_{Olig}. Thus, 48 hr of CI induced a more severe impairment of the mitochondrial function with multiple targets.

The alterations in pulmonary mitochondrial respiratory function after CI were similar to the previously reported effects of CI in rabbit kidney (*33*) and different from CI effects in liver mitochondria (*34*). In rabbit kidney mitochondria, we

TABLE 2. Continued

V_{Olig} (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	V_{FCCP} (nmol $O_2 \cdot min^{-1} \cdot \mu l^{-1}$)	URC	ADP/O
0.78±0.07 (n=6)	6.56±0.32 (n=6)	8.59±0.46 (n=6)	1.31±0.03 (n=29)
0.79±0.04 (n=5)	6.80±0.33 (n=5)	8.61±0.08 (n=5)	1.23±0.02 (n=24)
0.72±0.03 (n=4)	6.17±0.23 (n=4)	8.55±0.38 (n=4)	1.03±0.03† (n=22)
0.67±0.01 (n=4)	5.07±0.12† (n=4)	7.54±0.22 (n=4)	1.19±0.02 (n=17)
0.59±0.04† (n=5)	4.60±0.37† (n=5)	7.75±0.33 (n=5)	0.93±0.02† (n=19)
0.80±0.05‡ (n=4)	5.60±0.23 (n=4)	7.65±0.09 (n=4)	1.20±0.02 (n=23)
0.68±0.07 (n=4)	4.65±0.49 (n=5)	6.25±0.22 (n=5)	1.10±0.02‡ (n=23)
0.52±0.04†‡ (n=5)	4.53±0.51† (n=4)	8.44±0.30‡ (n=4)	0.88±0.03†‡ (n=15)

observed that 24 hr of CI induced significant decreases in V_3, V_{3s}, and V_{FCCP} but no change in V_{Olig} and ADP/O ratio (unpublished data). After 48 hr of CI, ADP/O decreased and there was an additional decrease in V_3, V_{3s}, and V_{FCCP} (*33*). On the other hand, in liver mitochondria (*34*), CI induced an increase in the proton conductance (increased V_{Olig}) and no modification in the oxidoreductase activities (stable V_3 and V_{FCCP}).

Related to the control, 30 min of WI did not significantly modify the respiratory parameters of isolated lung mitochondria. However, 45 min of WI promoted several damages: 1) a decrease in phosphorylating respirations (V_3 and V_{3s}) without impairment of V_{FCCP} indicating a drop in ADP availability for the ATP synthesis or damage at the level of the enzyme ATP synthase; 2) a decrease in the ADP/O ratio linked to a H^+ slip at the level of ATP synthase as in 48-hr CI (no change in V_4 and V_{Olig}). The WI period exceeding 30 min seems to have a striking effect on the OxPhos affecting mainly, if not exclusively, the ATP synthase activity. The absence of V_{FCCP} decrease indicated that neither oxidoreductase nor substrate availability were impaired. Related to 24 or 48 hr of CI alone, the combination between 30 min of WI and 24 or 48 hr of CI promoted no significant alteration in the respiratory parameters of isolated lung mitochondria. Surprisingly, the prior WI could have a weak protective effect on V_{FCCP} with ketoglutarate and pyruvate after 24 hr of CI and on ADP/O ratio with succinate after 48 hr of CI. These observations could be a bioenergetic rationale of a short ischemic period use preceding long ischemia to improve organ resistance, so-called preconditioning (*35*).

Relative to lungs submitted to 24 hr of CI alone, mitochondria from lungs submitted to CI and subsequent warm reperfusion with air ventilation underwent a decrease in phosphorylating respirations (V_3 and V_{3s}), uncoupled respiration V_{FCCP}, and a drop in the ADP/O ratio, but there was no uncoupling (stable V_{Olig}) (*24*). These observations were mainly the consequence of a worsening in the ATP synthase activity that led to an acute alteration in the efficiency of OxPhos and an additional decrease in the oxidoreductase activities.

In this study, OxPhos function of mitochondria isolated after 30 min of WI did not show any evidence of dysfunction, compared to control mitochondria. However, after 45 min of WI, the mitochondria demonstrated significant alterations. The combination of 30 min of WI and CI did not induce further mitochondrial OxPhos dysfunction, compared to CI alone. These results could be a concern in the issue of the procurement and transplantation of lung grafts from NHBD. To date, lung transplantation is limited by several factors, including the scarcity of suitable organ donors and the lung sensibility to ischemia/reperfusion injury. Introducing organ procurement from NHBD could be a way to increase the donor pool, and has been successfully used in kidney transplantation (*2*). The use of NHBD as lung donors has been proposed, and some experimental data seem to indicate that a short period of WI could be tolerated by lung grafts (*3–5, 9, 36*). At least one lung from a NHBD was successfully used in clinical setting (*11*). Our results suggest that, on the basis of bioenergetic considerations, lungs submitted to 45 min of WI should not be suitable for organ transplantation. However, after 30 min of WI, mitochondrial function was similar to controls. Our model of WI does not fully copy the clinical situation of NHBD, because we choose to administrate heparin and prostaglandin E_1 to the animals before inducing cardiac arrest by injection of a lethal dose of barbiturate. In contrast, for ethical reasons, clinical NHBD should not receive any medication before cardiac arrest and some WI duration necessary to establish brain death. However, in this study, we wanted to compare the effects on in vivo WI to in vivo CI on pulmonary mitochondrial function, and therefore we submitted the lungs to the same treatment before inducing ischemia, because this treatment could have an effect (protective or deleterious) on mitochondrial function; for example, prostaglandin E_1, which is used in lung preservation as a potent dilator of the pulmonary and bronchial tree, may have a positive effect on mitochondrial stability (*37*). In part, our model of WI may correspond to the category 3 NHBD defined by Kootstra et al. (*38*), as donors dying in the intensive care unit or in the operative room from "withdrawal of support," so-called controlled NHBD. In this category, the WI duration may be kept very short, below 30 min, and this short WI might allow the use of these lungs for transplantation. In contrast to most of the solid organs that are very sensitive to WI, lung grafts could tolerate some period of normothermic cardiac arrest. This difference could be explained by the presence of oxygen in the airway, allowing some oxidative metabolism during cardiac arrest. This conservation of lung cellular metabolism could protect mitochondrial functions, at least partially. The ATP levels maintenance in ischemic lungs could be the consequence of this preserved cellular metabolism, and has been strongly related to presence of air or oxygen in the airway (*39, 40*). In these studies, cell viabil-

ity and membrane integrity was correlated to the ATP level maintenance during ischemia. In a model of deflated pulmonary WI in the rabbit, ischemic tolerance was limited to 1 hr, as assessed by physiologic measurements (*41*). In our model, 30 min of WI did not harm mitochondrial OxPhos. We hypothesized that this intact OxPhos function could allow post-reperfusion mitochondrial respiration and metabolism maintenance. This relative pulmonary tolerance to WI promoted by oxygen presence in the airway could be even enhanced by postmortem ventilation or room air pulmonary inflation (*42, 43*).

In conclusion, in this model, 48 hr of cold ischemia and 45 min of WI induce severe mitochondrial damage at the OxPhos level. However, 30 min of WI does not induce respiratory dysfunctions. This WI time-dependent impairment could allow, on bioenergetic considerations, lung procurement from NHBD if WI duration does not exceed 30 min.

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IN VITRO ANALYSIS OF VERAPAMIL-INDUCED IMMUNOSUPPRESSION

POTENT INHIBITION OF T CELL MOTILITY AND LYMPHOCYTIC TRANSMIGRATION THROUGH ALLOGENEIC ENDOTHELIAL CELLS[1]

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Background. **Cyclosporine A (CsA) and tacrolimus prevent proliferation but not transendothelial migration of alloreactive lymphocytes into donor organs. As a result, serious adverse effects, such as nephrotoxicity and neurotoxicity, have been observed under CsA/tacrolimus therapy. The incorporation of new drugs with infiltration blocking properties might enhance the efficacy of the current immunosuppressive protocol, allowing lower CsA/tacrolimus dosage. Because Ca^{2+} plays a critical role in cell-cell interaction, the Ca^{2+}-channel blocker verapamil might be a good cany.**

didate for supporting CsA/tacrolimus-based therap

Methods. **A T-cell endothelial cell coculture model or immobilized immunoglobulin G globulin chimeras were employed to investigate how S- and R- verapamil interfere with the lymphocytic infiltration process. The expression and arrangement of membranous adhesion receptors and cytoskeletal F-actin filaments were analyzed by fluorometric method in the presence of. verapamil.**

Results. **Both verapamil enantiomers strongly inhibited lymphocyte infiltration. $CD4^+$ and $CD8^+$ T-cells were influenced to a similar extent with regard to horizontal locomotion ($CD4^+$=$CD8^+$), but to a different extent with regard to adhesion and penetration ($CD4^+ > CD8^+$). Moreover, penetration was blocked to a higher extent than was adhesion. ID_{50}-values were 31 μM ($CD4^+$-adhesion) and 11 μM ($CD4^+$-penetration). Verapamil reduced P-selectin expression on endothelial cells and effectively down-regulated binding of T-cells to immobilized P-selectin immunoglobulin G globulins (ID_{50}=4.4 μM; $CD4^+$). A verapamil-induced reduction of intracellular F-actin in T-lymphocytes was proven to be mainly responsible for diminished cell locomotion.**

Conclusions. **The prevention of $CD4^+$ T-cell penetration by verapamil might argue for its use as an adjunct to CsA/tacrolimus-based immunosuppressive therapy.**

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