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ORIGINAL ARTICLE

Preserved Coupling of Oxidative Phosphorylation But Decreased Mitochondrial Respiratory Capacity in IL-1β-Treated Human Peritoneal Mesothelial Cells

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Abstract

The peritoneal mesothelium acts as a regulator of serosal responses to injury, infection, and neoplastic diseases. After inflammation of the serosal surfaces, proinflammatory cytokines induce an "activated" mesothelial cell phenotype, the mitochondrial aspect of which has not previously been studied. After incubation of cultured human peritoneal mesothelial cells with interleukin (IL)-1β for 48 h, respiratory activity of suspended cells was analyzed by high-resolution respirometry. Citrate synthase (CS) and lactate dehydrogenase (LDH) activities were determined by spectrophotometry. Treatment with IL-1β resulted in a significant decline of respiratory capacity ($p < 0.05$). Respiratory control ratios (i.e., uncoupled respiration at optimum carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone concentration divided by oligomycin inhibited respiration measured in unpermeabilized cells) remained as high as 11, indicating well-coupled mitochondria and functional integrity of the inner mitochondrial membrane. Whereas respiratory capacities of the cells declined in proportion with decreased CS activity ($p < 0.05$), LDH activity increased ($p < 0.05$). Taken together, these results indicate that IL-1β exposure of peritoneal mesothelial cells does not lead to irreversible defects or inhibition of specific components of the respiratory chain, but is associated with a decrease of mitochondrial content of the cells that is correlated with an increase in LDH (and thus glycolytic) capacity.

Index Entries: Peritoneal mesothelial cells; interleukin-1β, mitochondria, respiration, citrate synthase, lactate dehydrogenase; cell viability.

INTRODUCTION

Inflammation is associated with exudation of the proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, which cause impairment of mitochondrial respiration and phosphorylation in various cell types. In hepatocytes, IL-1β inhibits mitochondrial adenosine triphosphate (ATP) synthesis rather than electron transport *(1)*. In enterocytes, however, reversible inhibition has been reported of cytochrome *c* oxidase by nitric oxide, and NAD+/NADH depletion occurs secondary to activation of poly-(ADP-ribose) polymerase by proinflammatory cytokines *(2)*. TNF-α and IFN-γ induce inhibition of complexes I and II of the respiratory chain in vascular smooth muscle cells *(3)*. Similarly, IL-1β stimulates nitric oxide production in cardiac myocytes, leading to reversible *(4)* or irreversible inhibition of mitochondrial activity and a decrease of cellular ATP levels *(5)*.

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Little is known about mitochondrial function under inflammatory conditions in mesothelial cells. Inflammation of the peritoneum is a common consequence of surgical trauma, chronic ambulatory peritoneal dialysis, peritoneal carcinosis, or systemic infection. Human peritoneal mesothelial cells (HPMC) that cover the entire surface of the peritoneal cavity serve as a mechanical barrier and bioactive cellular membrane *(6,7)*. Inflammatory cells or macrophages in the peritoneal tissue or effusions secrete proinflammatory cytokines, most importantly IL-1β, which regulate mesothelial responses to injury *(8,9)*. In HPMC, IL-1β stimulates the release of various growth factors and other cytokines that promote peritoneal healing, fibrosis, or adhesion formation *(10–15)*. In addition, mesothelial cells produce nitric oxide in response to cytokine exposure *(16)*.

In the present study, we examined the effects of IL-1β on respiratory capacity and mitochondrial integrity in human peritoneal mesothelial cells. In agreement with the general belief that impairment of oxidative phosphorylation is a result of exposure to proinflammatory cytokines, respiration per cell was reduced. Conclusive evidence, however, points to mitochondrial integrity being preserved, whereas the concomitant decline of citrate synthase and mitochondrial respiration indicated a reduction of mitochondrial density. Downregulation of mitochondrial content per cell thus provides an additional explanation of diminished cellular respiration, complementary to reversible or irreversible inhibition of mitochondrial respiration. A more detailed understanding of the mechanisms of mitochondrial injury in peritoneal inflammation might provide an important contribution to improve clinical diagnosis and intervention.

MATERIALS AND METHODS

Chemicals

Succinate, adenosine diphosphate (ADP), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), oligomycin, rotenone, and antimycin A were obtained from Sigma (Deisenhofen, Germany). Mitochondrial respiration medium (MiR05) contained 110 m*M* sucrose, 0.5 m*M* EGTA, 3.0 m*M* $MgCl_2$, 80 m*M* KCl, 60 m*M* K-lactobionate, 10 m*M* KH_2PO_4, 20 m*M* taurine, 20 m*M* HEPES, and 1.0 g/L bovine serum albumin (pH 7.1) *(17)*.

Isolation and Culture of Human Peritoneal Mesothelial Cells

HPMC were obtained from omental tissue of consenting patients undergoing elective abdominal surgery as described previously *(12)*. Briefly, small biopsies of omental tissue (~ 1–3 cm^3) from five different donors were rinsed in phosphate-buffered saline (Seromed, Biochrom, Berlin, Germany) at room temperature and incubated in a collagenase I solution (220 U/mL, 0.9–1.8 mg/mL; Seromed) for 1 h at 37°C. Subsequently, the collagenase solution containing the detached mesothelial cells was filtered through a 70-μm cell strainer and centrifuged at 500*g* for 10 min. The pellet was resuspended in RPMI-1640 medium (Seromed) supplemented with 10% fetal calf serum (Sebak, Suben, Austria), penicillin/streptomycin (each 200 U/mL, 200 μg/mL; Seromed), and L-glutamine (2 m*M*, Seromed). Cells were grown to confluence in 25 cm^2 tissue culture flasks (Greiner, Kremsmuenster, Austria) at 37°C in a humidified atmosphere of 5% CO_2 in air. HPMC were detached with trypsin (0.05%), washed once in culture medium, and seeded in 75 cm^2 falcon flasks in a volume of 15 mL. Confluent passage two cultures were then used for experiments. Primary cultures of HPMC were characterized by flow cytometry using monoclonal antibodies against cytokeratin subtypes 8 and 18 (MAb CAM 5.2, Becton Dickinson, Mountain View, CA) and vimentin (MAb V9, Dakopatts; Glostrup, Denmark) and the demonstration of a uniform coexpression of both intermediate filaments. A contamination by peritoneal phagocytes or endothelial cells was excluded using monoclonal antibodies against CD68 and CD34 (Becton Dickinson) as described previously *(12)*.

Treatment of HPMC With IL-1β

Confluent cultures were washed twice with fresh culture medium and incubated for 48 h in the absence or presence of recombinant IL-1β (1 ng/mL; R&D Systems, Minneapolis, MN). The present incubation regime was chosen on the basis of previous studies that showed that production of cytokines and growth factors, including IL-6 *(10)*, IL-8 *(11)*, and vascular endothelial growth factor (unpublished data), is maximized after incubation of HPMC with IL-1β at 1 ng/mL for 48 h. Incubation of mesothelial cells with IL-1β (0.01; 0.1; 1; 10 ng/mL) for up to 48 h did not have any significant effect on cell viability as shown by analysis with a CASY 1 Cell Counter and Analyser System (Schaerfe System, Reutlingen, Germany). This method combines an established particle measurement technique with the pulse area analysis signal-processing technique *(18)*. For measurement of cell number, 100-μL samples of suspended cells were diluted in 10 mL isotonic CASY solution. Then cells were introduced into the measuring system through a capillary of predefined geometry at a constant-stream velocity. Events in the range of 6–35 μm diameter were included in the calculation. For measurement of cell viability, 2×10^5 cells were incubated in 0.5 mL culture medium or ethanol (70%) for 10 min to separate the size range of viable (events in the range of 6–35 μm) and dead (events in the range from 0 to 5 μm) cells. Cell suspensions were then diluted in 9.8 mL CASYton (7.93

mg/mL NaCl, 0.38 mg/mL Na_2EDTA, 0.4 mg/mL KCl, 0.22 mg/mL $NaH_2PO_4 \cdot H_2O$, 2.45 mg/mL $Na_2HPO \cdot 2H_2O$, 0.3 mg/mL NaF; Schaerfe System) and cells analyzed using an aperture of 150 μm. When cells pass the capillary, which is filled with electrolytes with defined electrical resistance, the cells displace the electrolyte solution in proportion to the cell volume. The measuring signal is scanned by CASY1 at a frequency of 1 MHz. CASY1 captures the amplitude and width of the pulse and determines the integral of the measuring signal (pulse area analysis).

High-Resolution Respirometry

Passage two cells were washed by rinsing with phosphate-buffered saline and trypsinized (0.05% at 37°C for 2 min). Trypsin activity was stopped by RPMI-1640 at room temperature, centrifuged at 500*g* for 10 min at room temperature, and resuspended at 0.4×10^6 to 1.0×10^6 cells/mL in MiR05. The cell count was determined in the CASY 1 Analyser in 100-μL samples taken from the oxygraph chamber. Respiration rate of suspended mesothelial cells of five different donors was measured in two-channel titration injection respirometers (OROBOROS; Innsbruck, Austria) *(19)* in MiR05 at 37°C. Before each experiment, medium was equilibrated with air in the oxygraph chambers at 37°C until a stable signal was obtained for oxygen calibration. Medium was then replaced by the aerated cell suspensions, subsamples were removed, and the chambers were closed at a volume of 2 mL by insertion of the stoppers. Titrations were performed through the titanium injection port of stoppers using Hamilton syringes. The cell suspension was stirred continuously in the respirometer chamber at 460–600 rpm. Background controls were performed separately under experimental conditions without cells, and standard corrections were applied for obtaining cellular respiration *(19,20)*. Respiration rates were calculated as the time derivative of oxygen concentration measured in the closed respirometer and expressed per million viable cells (Fig. 1). In addition, two quantitative indices of cell membrane permeability were derived from these measurements (Fig. 2A). The respiratory effect of succinate and ADP is related to cell membrane permeabilization as (S-E)/(3u-E). S-E represents stimulation of respiration by succinate and ADP (S) over endogenous respiration (E) in permeable cells. These cells are depleted of substrates and adenylates, hence succinate + ADP stimulate mitochondrial respiration in permeabilized cells only. 3u-E expresses the total capacity over endogenous respiration in permeable and nonpermeable cells. All oligomycin-inhibited cells are stimulated by FCCP (3u), because intact cell membranes are permeable for the uncoupler and respiration is supported by endogenous substrates in these cells, whereas permeable cells respire on succinate. Progressive inhibition by rotenone and antimycin A is related to cell membrane permeabilization as (R-A)/(3u-A). R-A represents succinate-supported respiration (in the presence of the complex I inhibitor rotenone, R) over antimycin A–inhibited oxygen uptake (A). This effect is specific for permeable cells in the presence of succinate + FCCP. 3u-A expresses the total capacity over antimycin A–inhibited respiration in permeable and nonpermeable cells. These respiratory indices are based on the assumption that respiratory integrity of mitochondria is maintained after cell membrane permeabilization using a mitochondrial respiration medium (*see* Fig. 2).

Determination of Enzyme Activities and Protein Content

For determination of enzyme activities, 300 μL of mesothelial cell suspensions were pipetted from the stirred oxygraph chambers at the beginning of the experiment, frozen in liquid nitrogen, and applied in the assay. The activity of citrate synthase (CS) was measured spectrophotometrically at 412 nm and 30°C. One hundred microliters of cell suspension was added to 900 μL medium containing 0.1 m*M* 5,5-dithio-*bis*-(2-nitrobenzoic) acid, 0.5 m*M* oxaloacetate, 50 m*M* EDTA, 0.31 μ*M* acetyl coenzyme A, 5 m*M* triethanolamine hydrochloride, and 0.1 M Tris-HCl, pH 8.1 *(21,22)*. Lactate dehydrogenase (LDH) activity was measured at 340 nm and 30°C in a 0.1 M Tris-HCl buffer (Merck, West Point, PA) with 0.25% Triton X-100 (Serva, Vienna, Austria) at pH 7.1, with 10 m*M* pyruvate and 0.3 m*M* NADH (Fluka, St. Louis, MO) *(23)*. Lack of a decrease of LDH activity with increase in permeabilization indicates that LDH activity measured in the cells and medium reflects metabolic potential rather than LDH release (*see* Fig. 2B).

For protein measurements 2×10^6 cells were washed three times with phosphate-buffered saline (PAA Laboratories GmbH, Teddington Middlesex, UK), a final cell count was determined (CASY 1 Analyser), and the sample frozen in liquid nitrogen. Protein content of HPMC was measured in a Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard in the linear range of 5 to 0.31 μg bovine serum albumin/mL.

Data Analysis

Data analysis was performed with Datlab software (OROBOROS). All data are presented as mean ± SEM. The control and experimental group were compared using the nonparametric Wilcoxon signed-rank test for parallel samples. Significance was implied at $p < 0.05$.

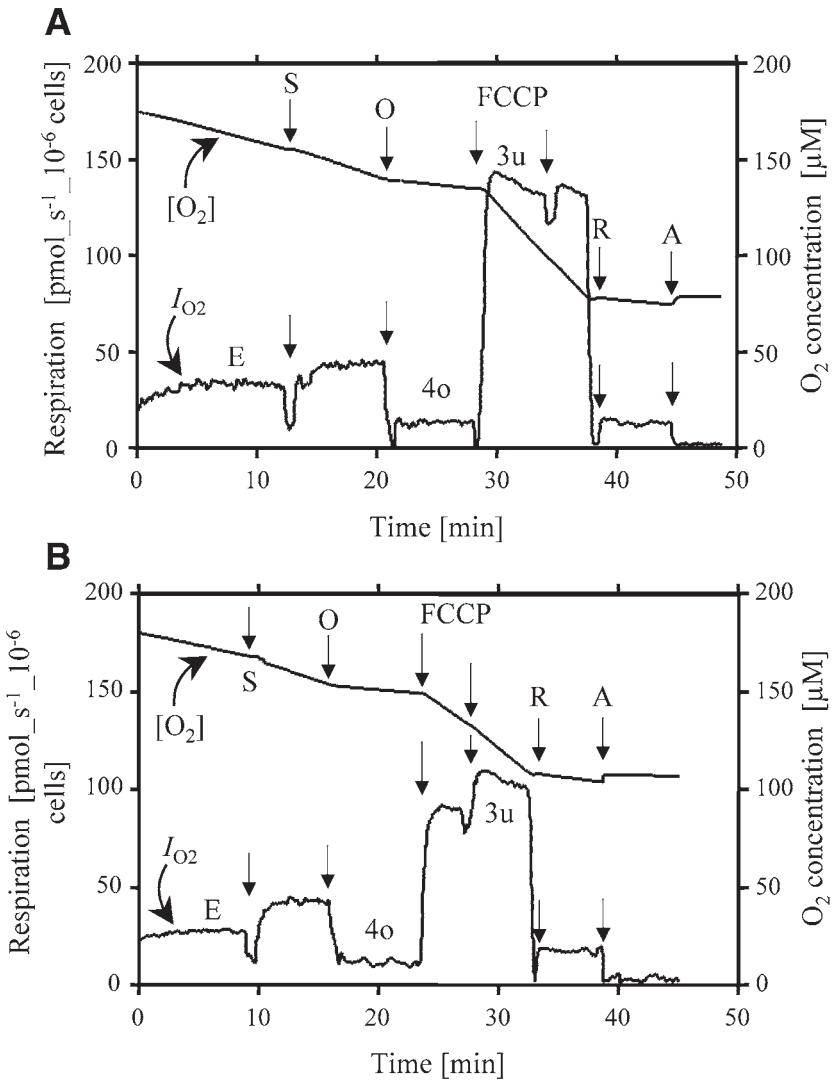

Fig. 1. Continuous traces of respiration in **(A)** controls and **(B)** interleukin (IL)-1β–activated human peritoneal mesothelial cells (HPMC). Traces show respiration, I_{O2} (pmol × s^{-1} × 10^{-6} cells), calculated as the time derivative of oxygen concentration [O_2]. Endogenous respiration without substrates (E), addition of 10 m*M* succinate and 5 m*M* ADP (S), 1 mg/mL oligomycin (O; state 4o), stepwise titration of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone up to 2 µ*M* (state 3u), and inhibition by 0.5 µ*M* rotenone (R) and 2.5 µ*M* antimycin A (A). Stability of respiratory activities within various respiratory states does not indicate a continuous loss of cell membrane integrity from vigorous stirring. Treatment of HPMC with IL-1β results in a significant decline of respiration per cell, related to loss of aerobic capacity.

RESULTS

Respiratory Activity of Human Peritoneal Mesothelial Cells

Respiration of controls in the absence of external substrates in MiR05 was 45.1 ± 6.4 pmol × s^{-1} × 10^{-6} cells. Respiration of cells in RPMI culture medium was lower, which could be explained by a high degree of plasma membrane permeabilization in suspended cells, as measured by trypan blue exclusion, and respiratory defects induced by exposure of mitochondria to high Ca^{2+} concentrations. Based on these preliminary results (data not shown), we selected MiR05, which protects mitochondria in permeabilized cells and yields stable endogenous respiration in unpermeabilized cells *(17,24)*. In combined tests for plasma membrane integrity and mitochondrial function, the respirometric titration regime included stepwise addition of succinate and ADP, oligomycin, FCCP, rotenone, and antimycin A (*see* Fig. 1). Oligomycin, FCCP, rotenone, and antimycin A

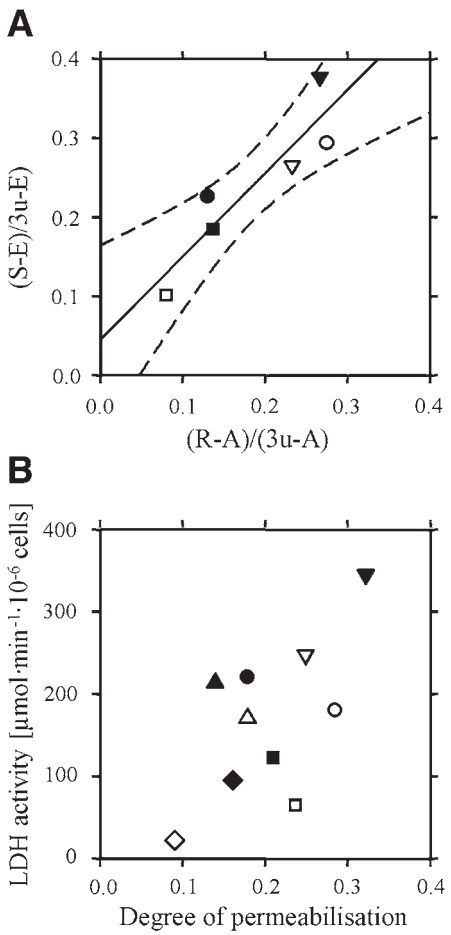

Fig. 2. **(A)** Respiratory indices for cell membrane permeabilization in human peritoneal mesothelial cells. *E*, endogenous respiration (in the absence of substrates in the medium); *S*, respiration after addition of succinate and ADP; *3u*, maximum flux after carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone titration; *R*, flux after addition of rotenone; *A*, flux after inhibition by antimycin A (*see* Fig. 1). The respiratory effect of succinate and ADP is related to cell membrane permeabilization as *(S-E)/(3u-E)*. Similarly, the effect of progressive inhibition by rotenone and antimycin A is related to cell membrane permeabilization as *(R-A)/(3u-A)*. Full line, linear regression; dashed lines, 95% confidence intervals. No difference between controls (open symbols) and interleukin-1β–activated cells (closed symbols; identical symbol types indicate matched pairs of controls and incubated cells). **(B)** Relation between lactate dehydrogenase (LDH) activity and degree of permeabilization for succinate (average for the two indexes in Fig. 2A). No significant relationship between LDH activity and degree of permeabilization.

are membrane-permeable and, therefore, exert their specific effects on mitochondrial respiration independent of cell membrane permeabilization. Succinate—a substrate that feeds electrons into complex II of the respiratory chain—and ADP are impermeable to the intact plasma membrane. Stimulation of endogenous respiration (in the absence of external substrates) after addition of succinate and ADP, therefore, indicated partial cell membrane permeabilization (*see* Fig. 1). The stimulatory effect was identical in controls (37.1% ± 4.0%) and HPMC treated with IL-1β (39.7% ± 3.9%).

ATP synthase was inhibited by addition of oligomycin to induce a state of minimum respiration (state 4o; 16.9 ± 3.1 pmol × s^{-1} × 10^{-6} cells). FCCP was titrated stepwise up to an optimum concentration of 2 µ*M* to obtain maximum uncoupled respiration (*see* Fig. 1; state 3u; 181.7 ± 25.8 pmol × s^{-1} × 10^{-6} cells). The respiratory adenylate control ratio (RCR) was calculated as the state 3u/4o ratio of respiration, and averaged 11.1 ± 0.6. This RCR remained unchanged after IL-1β treatment (Table 1), indicating tight coupling of oxidative phosphorylation and integrity of the inner mitochondrial membrane.

Residual oxygen uptake after addition of antimycin A (inhibitor of complex III) amounted to only 1% of state 3u. Less effective inhibition of respiration by rotenone (inhibitor of complex I), and subsequent full inhibition by antimycin A provided additional support for partial cell membrane permeabilization (*see* Fig. 1).

The quantitative indices of cell membrane permeability shown in Fig. 2 yield a viability in the range of 70–90%; indicate that viability did not decrease as a function of time in the respirometer, because rotenone inhibited respiration had not increased relative to endogenous respiration despite 40 min prolonged stirring (*see* Fig. 1); and show no difference between viability of controls (79% ± 3%) and IL-1β-exposed cells (80% ± 3%). Viabilities derived from respirometry were consistent with independent data from CASY analysis (Table 1).

Effect of IL-1β on Respiration and Enzyme Activities

Treatment of HPMC with IL-1β resulted in significantly reduced respiration in the coupled states (endogenous and 4o) and after uncoupling with FCCP (Fig. 3A). Similarly, the activity of CS, a marker for the mitochondrial matrix, decreased by 29.8% ± 8.4% compared with controls ($p < 0.05$; Fig. 4A). Respiration normalized for CS activity, therefore, was not different in IL-1β-activated HPMC and controls (Fig. 3B). This indicates that mitochondrial contents per cell were reduced after IL-1β treatment, whereas the specific respiratory chain activity (per unit mitochondrial matrix marker) remained unaffected. In contrast to the decreased respiration and CS activity, IL-1β treatment of HPMC resulted in a 38.1% ± 10.9% increase of LDH activity ($p < 0.05$; Fig. 4B).

Table 1.
Respiratory Parameters and Enzyme Activities of Cultured Human Peritoneal Mesothelial Cells in Controls and After Treatment With IL-1β for 48 h

Parameters	*n*	Controls	IL-1β
Viability (oxygraph) (%)	5	79.2 ± 3.4	79.8 ± 3.2
Viability (CASY1) (%)	10	86.6 ± 3.4	78.5 ± 2.1
Protein content (mg/10^6 cells)	5	0.82 ± 0.10	0.90 ± 0.12
Respiratory control ratio[a]	5	11.1 ± 0.6	10.5 ± 0.9
Lactate dehydrogenase/Citrate synthase	5	1832 ± 475	3830 ± 301[b]

[a]RCR is calculated as uncoupled respiration at optimum carbonyl cyanide *p* = trifluoromethoxy-phenylhydrazone concentration divided by oligomycin-inhibited respiration.
[b] $p < 0.05$, compared with controls.

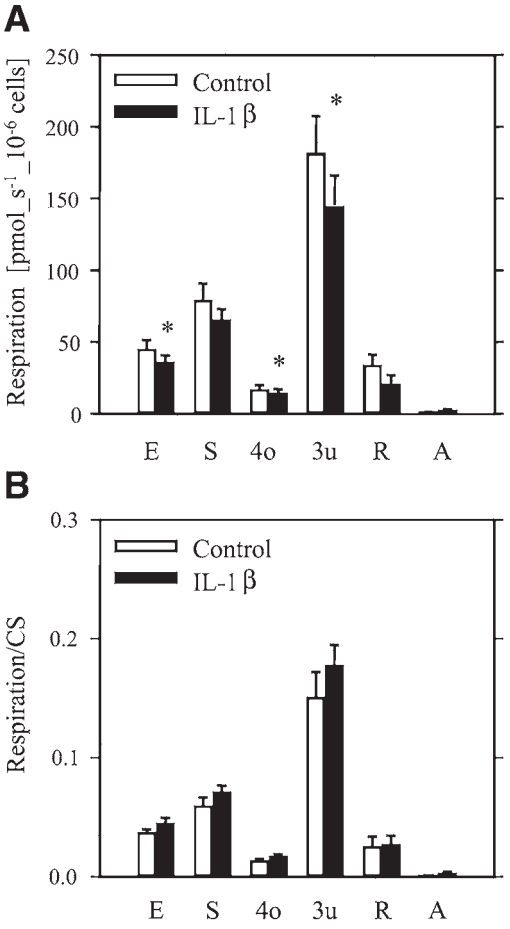

Fig. 3. Respiration in controls (open bars) and after interleukin-1β activation (closed bars) of human peritoneal mesothelial cells cultured from five different donors. (For explanation of respiratory states, *see* Fig. 1 legend.) **(A)** Respiration expressed as pmol × s^{-1} × 10^{-6} cells was significantly decreased (*$p < 0.05$) compared with controls. **(B)** Respiration normalized for citrate synthase activity was not different from controls.

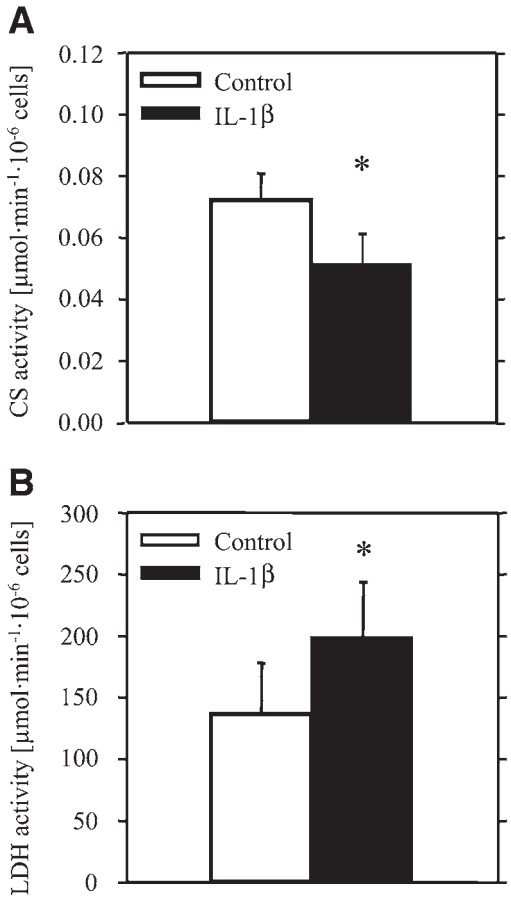

Fig. 4. Enzyme activities in controls (open bars) and after interleukin (IL)-1b activation (closed bars) of HPMC cultured from five different donors. Treatment with IL-1β resulted in a significant **(A)** decrease of citrate synthase and **(B)** increase of lactate dehydrogenase activity (*$p < 0.05$).

DISCUSSION

Proinflammatory cytokines cause inhibition of mitochondrial respiration in various cultured cells. In vivo, four different but not mutually exclusive mechanisms may lead to a decline of cellular respiration: (1) a lower level of mitochondrial activation by adenylates and substrates such as control by ADP, (2) reversible inhibition of respiratory chain complexes (e.g., of COX by NO), (3) irreversible defects of specific enzymes leading to lower respiratory capacity, and (4) reduction of mitochondrial density. Although endogenous nitric oxide exerts a stimulatory effect on mitochondrial biogenesis in a variety of cell types *(25)*, our study on IL-1β-exposed mesothelial cells indicates the importance of reduced mitochondrial content (per cell) and density (per cell protein). These changes, therefore, must be considered in addition to cytokine triggered derangements of mitochondrial function described previously for various cell types *(1–5,26,27)*.

This conclusion is based on the following evidence. (1) The activity of citrate synthase per cell and per cell protein was decreased. This marker enzyme of the mitochondrial matrix is comparatively stable *(22)* and reflects mitochondrial mass *(28)* under various pathophysiological conditions. The decline of CS activity at constant cell viability, therefore, suggests a reduction of mitochondrial content per cell *(24)*. (2) The respiratory capacity, 3u, as measured by uncoupler titration of cells declined to the same extent as CS activity. As a consequence, the 3u/CS ratio remained constant in IL-1β-treated cells compared with controls, corroborating the conclusion drawn from the result on CS activity. (3) Oligomycin-inhibited respiration, 4o, declined proportional to uncoupled respiration in IL-1β-treated HPMC and control cells, resulting in a constant index of respiratory coupling (ratio of 3u/4o respiration). The high RCR argues against impairment of respiration by insufficient availability of endogenous substrates in unpermeabilized cells. The RCR was greater than 10 in the present study, which is twice as high as RCR values reported for isolated mitochondria and permeabilized liver cells in the presence of succinate *(29)*. The high RCR obtained in mesothelial cells incubated in medium with external succinate, therefore, suggests the quantitative importance of internal complex I substrates, in line with the observed inhibition of respiration by rotenone. Any substrate limitation would result in a reduced RCR ratio, because respiration in the oligomycin-inhibited state would be less affected compared with the uncoupled state of maximum activation. In addition, the identical RCR in IL-1β-treated and control mesothelial cells indicates preserved integrity of the inner mitochondrial membrane. Taken together, these results suggest a downregulation of mitochondrial contents in general rather than a specific respiratory enzyme activity leading to a decreased level of oxidative phosphorylation. The pathophysiological consequence of the reduced mitochondrial content may be a lower cellular ATP production or compensation by either activation of mitochondrial respiration or aerobic glycolysis. The glycolytic potential is increased in activated mesothelial cells *(30)*, consistent with the finding of a significant stimulation of LDH activity in HPMC treated with IL-1β. This agrees with similar findings in various other cell types in which IL-1β treatment results in increased glucose consumption and lactate production, suggesting IL-1β as a potent regulator of energy supply during inflammatory processes *(5,31–35)*. Similar compensatory mechanisms appear to operate in human peritoneal mesothelial cells as a response to proinflammatory signals. In conclusion, activation of peritoneal mesothelial cells by IL-1β resulted in a decrease of oxidative phosphorylation most probably because of a downregulation of mitochondrial contents that was associated with an increase in glycolytic potential.

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