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Cigarette smoke-induced blockade of the mitochondrial respiratory chain switches lung epithelial cell apoptosis into necrosis

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van der Toorn M, Slebos D-J, de Bruin HG, Leuvenink HG, Bakker SJ, Gans RO, Koëter GH, van Oosterhout AJ, Kauffman HF. Cigarette smoke-induced blockade of the mitochondrial respiratory chain switches lung epithelial cell apoptosis into necrosis. *Am J Physiol Lung Cell Mol Physiol* 292: L1211–L1218, 2007. First published January 5, 2007; doi:10.1152/ajplung.00291.2006.—Increased lung cell apoptosis and necrosis occur in patients with chronic obstructive pulmonary disease (COPD). Mitochondria are crucially involved in the regulation of these cell death processes. Cigarette smoke is the main risk factor for development of COPD. We hypothesized that cigarette smoke disturbs mitochondrial function, thereby decreasing the capacity of mitochondria for ATP synthesis, leading to cellular necrosis. This hypothesis was tested in both human bronchial epithelial cells and isolated mitochondria. Cigarette smoke extract exposure resulted in a dose-dependent inhibition of complex I and II activities. This inhibition was accompanied by decreases in mitochondrial membrane potential, mitochondrial oxygen consumption, and production of ATP. Cigarette smoke extract abolished the staurosporin-induced caspase-3 and -7 activities and induced a switch from epithelial cell apoptosis into necrosis. Cigarette smoke induced mitochondrial dysfunction, with compounds of cigarette smoke acting as blocking agents of the mitochondrial respiratory chain; loss of ATP generation leading to cellular necrosis instead of apoptosis is a new pathophysiological concept of COPD development.

chronic obstructive pulmonary disease; mitochondria

OXIDATIVE STRESS CAUSED BY cigarette smoke resulting in airway inflammation is assumed to be directly involved in tissue injury and cell death in chronic obstructive pulmonary disease (COPD) (5, 30). In patients with COPD, tissue injury and cell death have been related to the presence of both increased apoptosis and necrosis in lung and airway epithelial cells (16, 18, 21, 27, 43). The airway epithelium is the barrier between inhaled air, with toxic compounds like cigarette smoke present, and the underlying lung tissue. To maintain this barrier, continuous cell replacement and repair of the epithelium are of crucial importance. Cell death through apoptosis is essential for eliminating damaged cells during development, tissue remodeling, and inflammation (22, 41). Disturbance of this physiological process can, however, result in necrosis or excessive apoptosis with disruption of the barrier function of the epithelium, leading to lung disease (15, 19, 41). Cigarette smoke models have shown the induction of both apoptosis and necrosis in epithelial cells and fibroblasts in vitro. As recently demonstrated by our group (38), this death process depends on the amount and duration of cigarette smoke exposure and the type of cell line used. However, the regulatory mechanisms involved in these in vitro models, as well as in humans in vivo, remain speculative (10, 20, 27, 42).

Mitochondria play a crucial role in the regulation of apoptosis by the release of proapoptotic mediators (e.g., cytochrome *c*, apoptosis-inducing factor) in response to specific stimuli (14). Cytochrome *c*, released from mitochondria, together with apoptotic protease activating factor-1 and pro-caspase-9 combine to the apoptosome. This ATP-dependent apoptosome formation results in the activation of caspase-9, leading to caspase-3 activation and subsequent apoptotic cell death (14). Another crucial role for mitochondria in facilitating apoptosis is the production of ATP, a process linked to the action of the electron transfer chain (ETC) (34). Where apoptosis is a highly energy-consuming process, necrosis will occur even after an apoptotic stimulus when no energy is available (17). Therefore, alteration of cellular energy (ATP) levels is known to play a crucial role in the routing of cells to die by apoptosis or necrosis (11, 26, 31).

It has been known for decades that cigarette smoke contains many lipophilic compounds like phenolic structures, aldehydes, and aromatic compounds that are able to accumulate in mitochondria and may disturb the function of the mitochondrial respiratory chain, thereby affecting the cellular ATP production (13, 25, 29, 32, 39). These observations make mitochondria theoretically important players in cigarette smoke-induced cellular damage. Therefore, we hypothesized that cigarette smoke will disturb the mitochondrial respiratory chain function, leading to a decreased mitochondrial membrane potential ($\Delta\psi_m$) and a decreased ATP production. We furthermore hypothesized that this cigarette smoke-induced ATP depletion is crucial in the routing of apoptotic-triggered cells and may switch them toward dying from necrosis.

Therefore, we set out to identify the acute effect of cigarette smoke extract (CSE) on mitochondrial respiratory chain function in isolated mitochondria and mode of cell death in human bronchial epithelial cells. The consequences of the cigarette smoke-induced mitochondrial dysfunction might be of great importance in understanding the presence of an altered cell death process in COPD.

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MATERIALS AND METHODS

Chemicals. 2,4-Dinitrophenol (DNP) was obtained from Merck (Haarlem, The Netherlands), and sodium succinate, ATP, ADP, potassium cyanide, staurosporin (STS), and oligomycin were from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands).

Preparation of CSE. Kentucky 2R4F research-reference filtered cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY) where smoked via a peristaltic pump. Just before the experiments, the filters were cut from the cigarettes. Each cigarette was smoked in 5 min with a 17-mm butt remaining. Four cigarettes were bubbled through 50 ml of cell growth medium or mitochondrial respiration buffer, and this solution was regarded as 100% strength CSE.

Cell cultures. Human primary bronchial epithelial cells were collected and cultured by the methods described by Borger et al. (8). For the experiments described, these cells were used at 80–90% confluency in *passage 2*. Beas-2b lung epithelial cells were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 with 25 mM HEPES, L-glutamine (BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated FCS (BioWhittaker), and 20 µg/ml gentamycin (Centafarm Services, Etten-Leur, The Netherlands). Before the experiments, both the human primary bronchial epithelial cells and the Beas-2b cells were cultured for 16 h in serum-free RPMI 1640 medium.

Isolation of mitochondria. Mitochondria were isolated from fresh pig's liver (obtained from the local slaughterhouse) with the use of a commercial mitochondria isolation kit (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands). Briefly, fresh liver tissue (obtained within 1 h of death) was washed twice with two volumes of extraction buffer (10 mM HEPES, 200 mM mannitol, 70 mM sucrose, and 1 mM EGTA, pH 7.5). The liver was cut into small portions and homogenized with 10 vol extraction buffer containing 2 mg/ml delipidated BSA (Sigma-Aldrich Chemie), using a pestle and glass tube. The homogenate was centrifuged at 600 g for 5 min. The supernatant was collected and centrifuged at 11,000 g for 10 min. After this, the supernatant was removed and the pellet was resuspended in 10 vol of extraction buffer and centrifuged at 600 g for 5 min. Finally, the supernatant was centrifuged at 11,000 g for 10 min, after which the supernatant was removed and the isolated mitochondria were resuspended in respiratory buffer (120 mM KCl, 5 mM K_2PO_4, 3 mM HEPES, 1 mM EGTA, brought to pH 7.2 with 5 mM KH_2PO_4). Mitochondrial protein was estimated by the Bradford method (Bio-Rad Laboratories, Veenendaal, The Netherlands) in accordance with the manufacturer's instructions. To stabilize the mitochondria, respiration buffer was supplemented with 0.2% delipidated BSA (wt/vol).

Detection of $\Delta\psi_m$. Primary bronchial epithelial cells were stained with 5 µg/ml JC-1 probe (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instruction. Loaded cells were stimulated for 1 h with different concentrations of CSE at 37°C. Isolated mitochondria were stained with 0.2 µg/ml JC-1 probe for 10 min at 37°C. State III respiration was reached after addition of 5 mM sodium succinate and 1 mM ADP. Mitochondria were stimulated for 15 min at 37°C with different concentrations of CSE. Epithelial cells and isolated mitochondria were monitored with an excitation wavelength of 485 nm through a 590-nm band-pass filter in a FL600 fluorescent plate reader (Bio-Tek Instruments, Abcoude, The Netherlands).

Measurement of ATP. Intracellular ATP levels of epithelial cells were quantified after treatment with Triton X-100. For mitochondrial measurement of ATP, the isolated mitochondria (final protein concentration of 100 µg/ml) were resuspended in respiration buffer. The experiments were performed in state III respiration. Different CSE concentrations were tested, whereas DNP (final concentration of 20 µM) served as negative control. Mitochondria were incubated for 15 min at 37°C. At the end of the incubation period, ATP synthesis was stopped by freezing the samples in −196°C nitrogen. The ATP levels were measured by the Enliten ATP assay from Promega (Leiden, The Netherlands) and a Berthold microplate luminometer (Berthold Detection Systems, Pforzheim, Germany).

Mitochondrial respirometry. Mitochondrial respiration was measured according to the method of 't Hart et al. (39a). Briefly, mitochondria (final concentration of 2 mg/ml) were resuspended in a 95% oxygen-saturated respiration buffer supplemented with 0.2% (wt/vol) delipidated BSA (Sigma-Aldrich Chemie) in a 1-ml respiration chamber. Stimuli were preincubated for 1 min. State III respiration was started after addition of succinate (final concentration of 5 mM) and ADP (final concentration of 0.5 mM).

Complex I and II activity. The activity of NADH dehydrogenase (complex I) was assayed by the method of Minakami et al. (28). The enzyme activity was calculated by subtracting the residual activity remaining after the addition of the specific inhibitor (2 µM rotenone). Succinate dehydrogenase (complex II) was determined by enzymatic methods according to the method Slater and Bonner (37).

Mitochondrial swelling. We performed mitochondrial swelling, according to the method of Barzu et al. (7), as an index for mitochondrial viability. Briefly, mitochondria were resuspended in respiration buffer (without EGTA) containing 4 mg mitochondrial protein/ml. The mitochondria were "energized" with succinate (final concentration of 5 mM). The suspension was pipetted into the wells (100 µl/well) of a 96-well polystyrene microtiterplate (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Different CSE concentrations were added, and $CaCl_2$ (1 mM) was used as a positive control. Immediately after addition of the stimuli, the plate was measured in an EL808 spectrophotometer using a 550-nm filter (Bio-Tek Instruments). Measurements were repeated every 30 s for a period of 30 min.

Caspase-3 and -7 activities. Beas-2b cells were preincubated for 1 h with or without 2 µM STS. Thereafter, all cells were washed and incubated for 4 h with different concentrations of CSE. Caspase-3 and -7 activities were determined with the "caspase-glo 3/7" luminescent assay kit from Promega and a Berthold microplate luminometer. Caspase-3 and -7 activities of the Beas-2b cells were determined according to the manufacturer's instruction.

Flow cytometric analysis of cell death. Surface exposure of phosphatidylserine and plasma membrane disruption was stained by annexin-V-FITC and propidium iodide according to the manufacturer's instruction (IQ Products, Groningen, the Netherlands). Cells were analyzed by flow cytometry (Calibur, Becton Dickinson Medical Systems, Heidelberg, Germany).

Statistical analysis. Data were analyzed with SPSS/PC$^+$ software (SPSS Benelux, Gorinchem, The Netherlands). Repeated-measures ANOVA was used was used for assessment of decline in dose-response CSE experiments. Comparisons between different experimental groups were performed with a one-sample *t*-test (see Figs. 1 and 2) and the nonparametrical Mann-Whitney U test (see Figs. 5, *A* and *B*, and 6*C*). $P < 0.05$ was considered significant. Results are presented as means ± SE unless otherwise mentioned.

RESULTS

CSE disturbs mitochondrial function in human primary bronchial epithelial cells. To investigate the acute effect of CSE on the mitochondrial respiratory chain function, we examined whether exposure of human primary bronchial epithelial cells to CSE would affect $\Delta\psi_m$. Exposure of increasing concentrations of CSE for 1 h caused a significant dose-dependent decrease of $\Delta\psi_m$ ($P < 0.0001$; Fig. 1*A*). Furthermore, intracellular ATP levels were measured to evaluate the consequences of the decreased mitochondrial depolarization. CSE caused a significant dose-dependent decrease of intracellular ATP ($P = 0.0021$; Fig. 1*B*). The protonophore DNP, used as a positive control, showed, as expected, a depolarization of
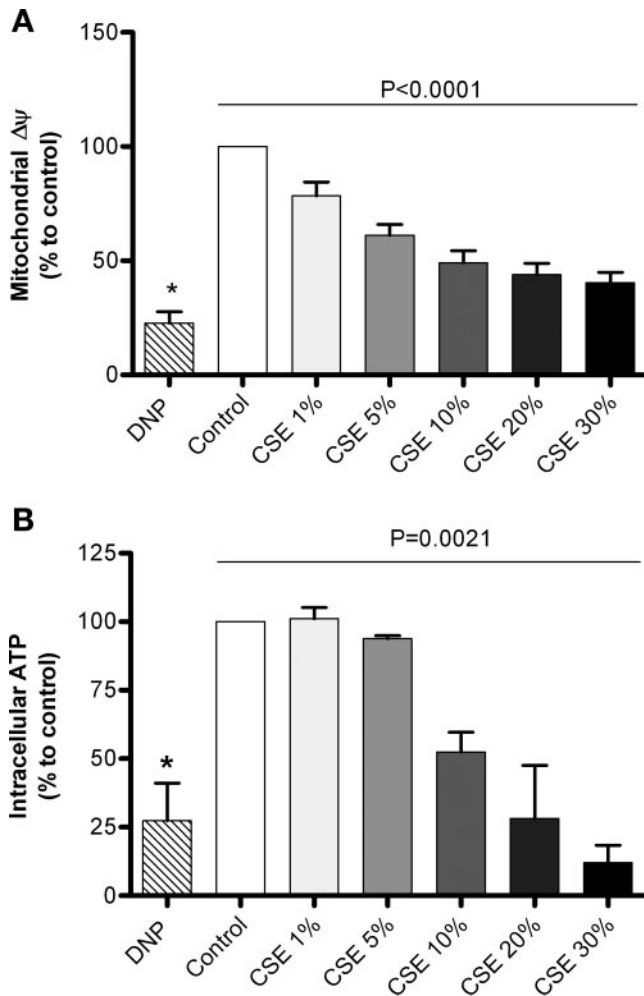
Fig. 1. Cigarette smoke extract (CSE) induces a loss of mitochondrial function in human primary bronchial epithelial cells. Effects of increasing concentrations of CSE on mitochondrial inner membrane potential ($\Delta\psi_m$) (*A*) and intracellular ATP levels (*B*) in primary human bronchial epithelial cells exposed for 1 h ($n = 3$) are shown. *$P < 0.05$ for 2,4-dinitrophenol (DNP; 20 μM) vs. control. *P* values given above the horizontal lines represent the significance for the total decline of all CSE concentrations used (repeated-measures ANOVA).

the mitochondrial inner membrane and decreased intracellular levels of ATP in these epithelial cells (Fig. 1).

The mitochondrial respiratory chain is the target of CSE-mediated ATP depletion. In intact epithelial cells, we demonstrated that CSE disrupts mitochondrial function. To study whether CSE also directly affects mitochondria, we isolated fresh pig liver mitochondria. Exposure of isolated mitochondria to CSE caused no mitochondrial swelling under the experimental conditions, thus indicating mitochondrial viability (Fig. 2). However, we were able to demonstrate that CSE significantly depolarizes the mitochondrial inner membrane in a state III respiration (in the presence of ADP, change of −61.4% ± 2.4%; $P < 0.0001$; Fig. 3*A*). A similar effect was observed for state IV respiration (absence of ADP, data not shown). Because the ETC facilitates the transfer of hydrogen ions across the mitochondrial inner membrane, a decreasing $\Delta\psi_m$ in a state III respiration by CSE should negatively affect the proton gradient. To demonstrate this, we exposed isolated

mitochondria to different concentrations of CSE for 15 min and showed a significant dose-dependent decrease of the synthesis of ATP (change of −80.6% ± 4.3%; $P < 0.0001$; Fig. 3*B*).

CSE inhibits complex I and II of the ETC. We hypothesized that CSE is able to produce an ETC failure through inhibition of the respiratory chain, especially at the sites of input of energy (complexes I and II). We therefore tested the activity of both complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) in response to CSE. Low concentrations of CSE (0.05–10%) resulted in a dose-dependent inhibition of complex I activity (change of −79.7% ± 4.6%; $P < 0.0001$; Fig. 4*A*), whereas 1–30% of CSE caused a dose-dependent inhibition of complex II activity (change of −50.4% ± 3.4%; $P < 0.0001$; Fig. 4*B*).

CSE affects mitochondrial respiration. Blocking complexes I and II by CSE contributes to a decreased flow of electrons along the mitochondrial ETC, resulting in a decreased $\Delta\psi_m$ and decreased ATP production. This sequence of "ETC events" that occurs in response to CSE may result in a decreased mitochondrial respiration. To prove that CSE actually acts as a blocking agent, we incubated isolated mitochondria in an oxygraph sample chamber with a 95% oxygen-saturated respiration buffer (Fig. 5*A*). After addition of succinate as metabolic substrate, mitochondria started to respire in a state IV respiration (154.2 ± 15.4 ng $O_2 \cdot min^{-1} \cdot mg^{-1}$). Addition of ADP directly induces increased oxygen uptake (state III respiration; 494.6 ± 56.5 ng $O_2 \cdot min^{-1} \cdot mg^{-1}$) because ADP is rapidly converted into ATP. The respiratory control index (indicating the coupling between respiration and phosphorylation) was 3.2 ± 0.3 for these experiments (Fig. 5*A*). Thereafter, we investigated the effects on the mitochondrial respiration of additional exposure of the isolated mitochondria to CSE (Fig. 5, *B* and *C*). In Fig. 5*B*, an example of such a single experiment is shown. For this experiment, isolated mitochondria were preincubated with 30% CSE in respiration buffer. By using this approach, we demonstrated that CSE induced a blockade of the ETC dose dependently in state III respiration (change of 318.2 ± 29.9 ng $O_2 \cdot min^{-1} \cdot mg^{-1}$; $P < 0.05$). Even the addition of the protonophore DNP was not able to induce an increased oxygen uptake, which indicates a strong blockade of electrons along the ETC (Fig. 5*B*).
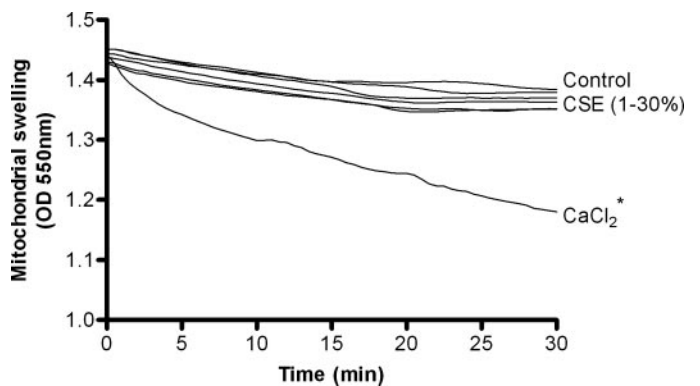

Fig. 2. CSE induces no mitochondrial swelling. Mitochondrial swelling assay was used to test the viability of mitochondria ($n = 4$). Mitochondria were incubated for 15 min with increasing concentrations of CSE. CaCl$_2$ was used as a positive control. OD, optical density. *$P < 0.05$.
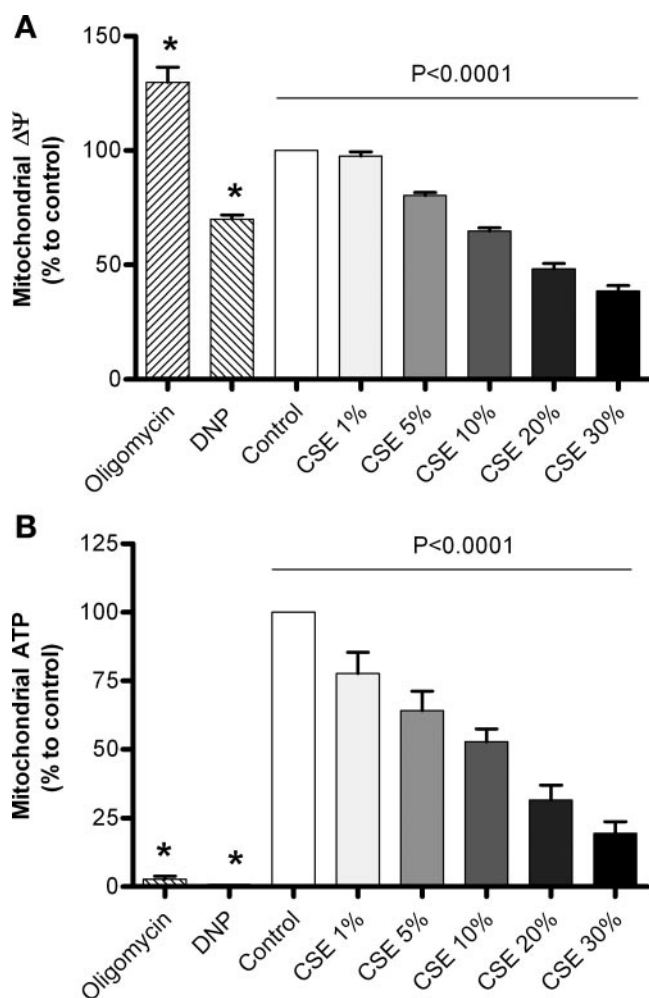
Fig. 3. CSE induces a decreased $\Delta\psi_m$ and depletes ATP in isolated mitochondria. Effects of increasing concentrations of CSE on $\Delta\psi_m$ (*A*) and ATP levels (*B*) in isolated mitochondria exposed for 15 min ($n = 6$) are shown. *$P < 0.05$ for 20 μM DNP or 5 μM oligomycin vs. control. All experiments were performed in a state III mitochondrial respiration. *P* values given above the horizontal lines represent the significance for the total decline of all CSE concentrations used (repeated-measures ANOVA).

CSE-induced ATP depletion is crucial in the routing of apoptotic-triggered cells to die by necrosis. We examined the mode of cell death in Beas-2b cells induced by the protein kinase inhibitor STS under conditions of intracellular ATP depletion caused by CSE. STS triggers apoptosis in lung epithelial cells without disturbing the function of the respiratory chain (3, 26). Therefore, Beas-2b cells were preincubated for 1 h with 2 μM STS, followed by incubation for 4 h with different concentrations of CSE. In this model, CSE concentrations $\geq$10% caused an intracellular ATP depletion of >50% within 1 h in primary epithelial cells (Fig. 1) and within 2 h in Beas-2b cells (Fig. 6, *A* and *B*). Caspase activation and apoptosis induction by STS were significantly prevented by CSE in a dose-dependent manner (Fig. 6, *C* and *D*). CSE concentrations $\geq$10% completely abolished the STS-induced caspase-3 and 7 activities (STS alone: 477.4 ± 69.88% of control; STS + CSE 10%: 118.3 ± 35.73%). Furthermore, the early apoptotic cells exposed to CSE switched their mode of cell death into necrosis by annexin-V and propidium iodide flow cytometry.

Addition of CSE to STS-treated cells resulted in decreased numbers of apoptotic cells (60.25 ± 15.2%; $P < 0.05$) and an increase of the cells going into necrosis for the highest concentrations of CSE used (29.83 ± 6.6%; $P < 0.05$). These results suggest that intracellular ATP levels decide the mode of cell death after giving the epithelial cells an apoptotic cell death signal.

DISCUSSION

In the present study, we investigated the effects of CSE on the function of the mitochondrial respiratory chain in human bronchial epithelial cells and isolated mitochondria. We were able to demonstrate that CSE acts as a blocking agent of complexes I and II of the ETC. The inhibition of the two entry points of the ETC caused a decrease in $\Delta\psi_m$ and proton motive force. As a consequence of that, the consumption of oxygen and production of ATP were diminished. Mitochondria play a major role in the generation of ATP, which is required for the cell to die by apoptosis (17). Our data demonstrate that early
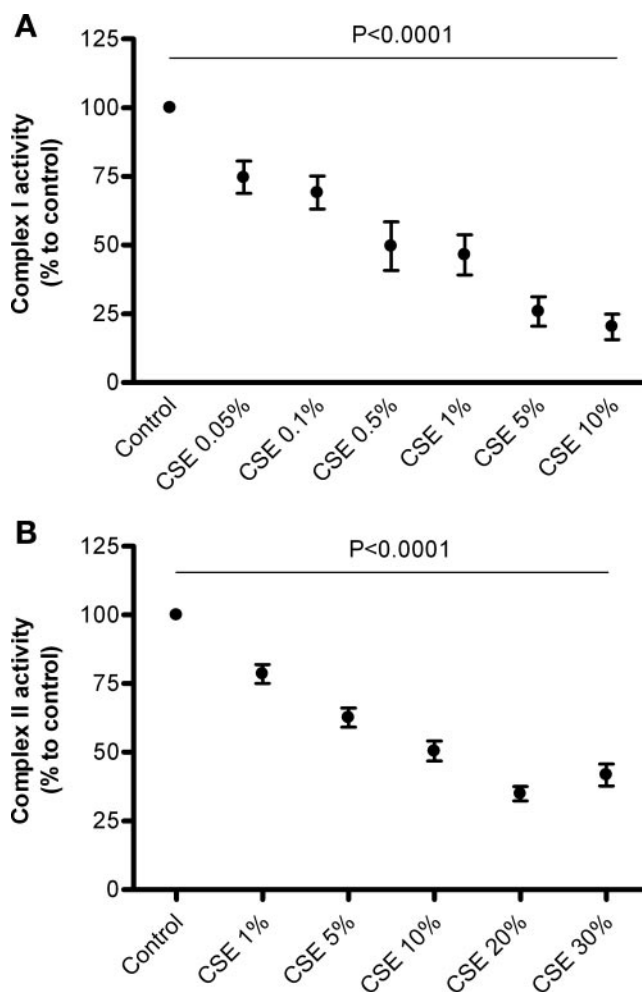

Fig. 4. Complexes I and II of the electron transfer chain are blocked by CSE. Effects of increasing concentrations of CSE on activities of NADH dehydrogenase (complex I; *A*) and succinate dehydrogenase (complex II; *B*) in isolated mitochondria ($n = 9$) are shown. *P* values given above the horizontal lines represent the significance for the total decline of all CSE concentrations used (repeated-measures ANOVA).
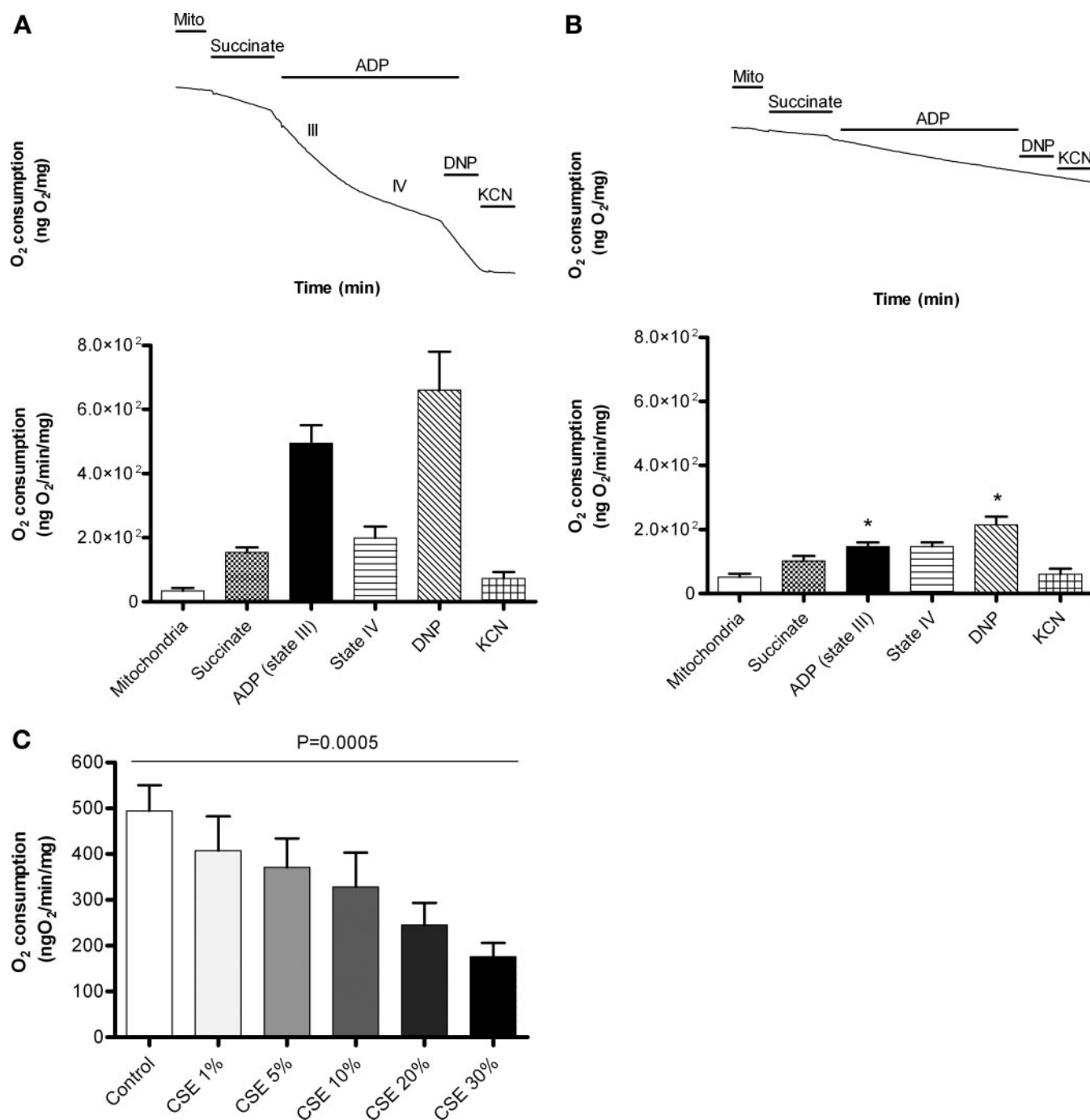
Fig. 5. Mitochondrial respiration is decreased by exposure to CSE. Mitochondrial respiration rate was measured with the oxygraph chamber method ($n = 4$). *A*: control experiment. *B*: CSE-pretreated mitochondria (1 min with 30% CSE before the respiration experiment was started). *C*: dose response of CSE-induced inhibition of the mitochondrial state III respiration rate ($n = 4$). Oxygen consumption plots are representative for 1 experiment of 4. Mito, mitochondria without addition of substrates; III, state III mitochondrial respiration; IV, state IV mitochondrial respiration; ADP, 5 mM ADP; DNP, 20 μM DNP; KCN, 20 μM potassium cyanide. *$P < 0.05$, comparison between the CSE and control experiment. P value given above the horizontal line in *C* represents the significance for the total decline of all CSE concentrations used (repeated-measures ANOVA).

apoptotic epithelial cells that become ATP deficient switch their mode of cell death from apoptosis into necrosis.

Until now, only a few studies have investigated the effects of cigarette smoke on mitochondrial function; however, these studies have not been able to elucidate its mechanism of inhibition. So far, no studies have been performed in epithelial cells. However, the inhibition of complexes I and II by ciga-

rette smoke has been observed in two previous studies using different models (2, 39). Anbarasi et al. (2) demonstrated a decrease in complex I and II activity in brain cells from rats inhaling cigarette smoke and suggested that the alteration in the phospholipids environment of the membrane could be responsible for the inhibition of the electron flow. Similarly, Smith et al. (39) demonstrated a decrease in complex I activity in
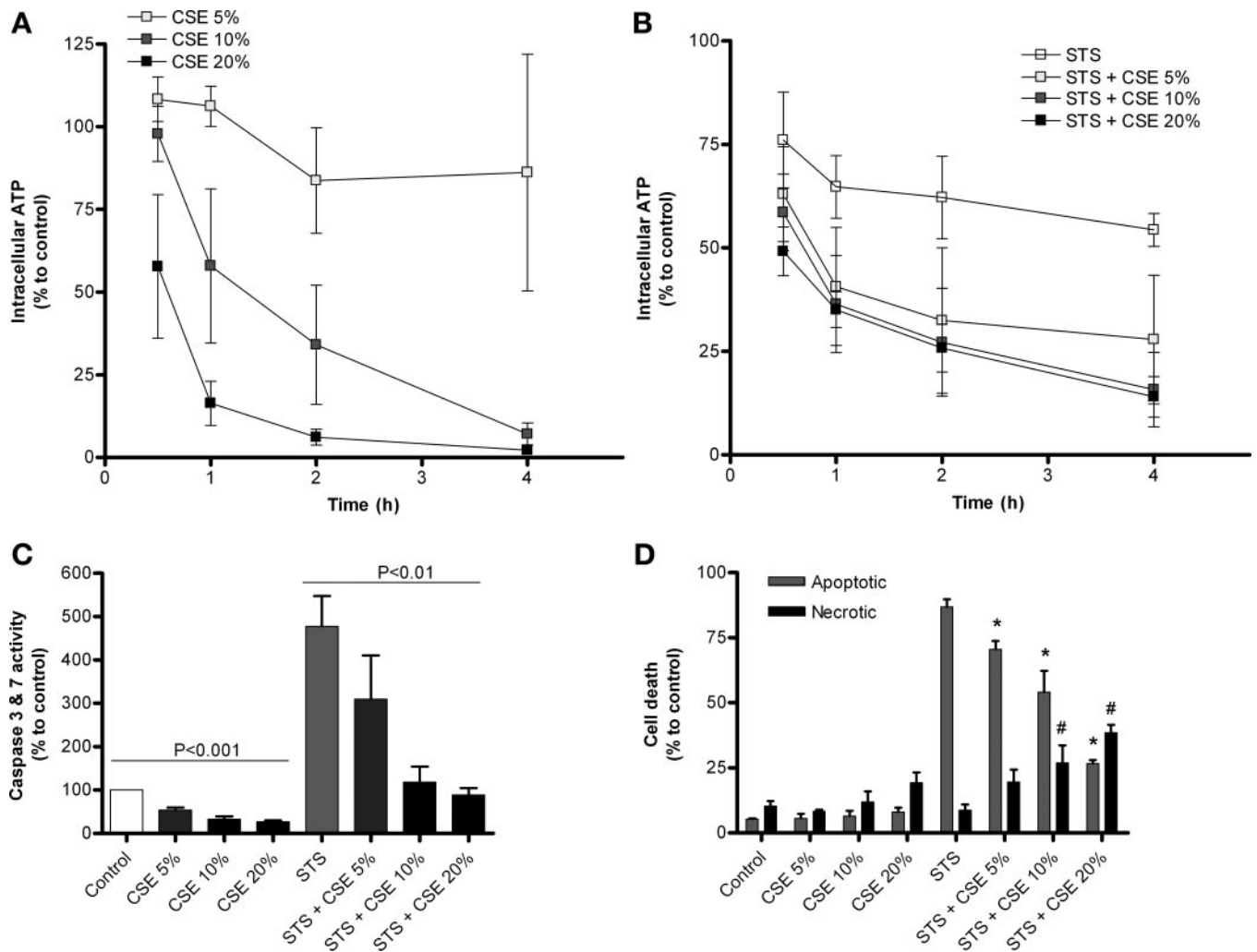
Fig. 6. CSE-induced depletion of ATP switches apoptosis to necrosis. *A* and *B*: effects of the experimental conditions [increasing length of the exposure to and concentrations of CSE (*A*) and staurosporin (STS; *B*)] on intracellular ATP levels in Beas-2b bronchial epithelial cells. Changes in mode of cell death occur after CSE treatment of Beas-2b cells preincubated for 1 h with 2 μM STS. *C* and *D*: cells were washed and incubated for 4 h with different concentrations of CSE. Caspase-3 and -7 activation (*C*) and apoptosis induction (*D*; assessed with annexin-V and propidium iodide FITC on flow cytometry) by STS was prevented by CSE in a dose-dependent manner ($n = 4$). *$P < 0.05$ for comparison between the STS + CSE and STS apoptosis control experiment. #$P < 0.05$ for comparison between the STS + CSE and STS necrosis control experiment.

platelets of a human smoking group compared with that of a nonsmoking control group. Besides the action of cigarette smoke on the activity of complexes I and II, it has also been shown that CSE exposure for 3 h induces a dose-dependent mitochondrial membrane depolarization in human isolated monocytes (4). Together, these limited results suggest that cigarette smoke can affect mitochondrial function in different cells and disease models.

Our results now clearly show that CSE inhibits mitochondrial function in primary human bronchial epithelial cells. Additionally, by using direct incubation of isolated mitochondria with CSE, we were able to show for the first time that CSE acts directly as a blocking agent of complexes I and II on the ETC without interference of other cellular metabolic activities. To confirm actual blockade, we tested the respiratory activity of isolated mitochondria and showed that the CSE-induced decrease in mitochondrial respiration could not be overruled with the protonophore (uncoupler) DNP.

The components that are actually responsible for the inhibition of complex I and II respiratory activity are not known. It is known that several compounds of cigarette smoke, like carbon monoxide and cyanides, can inhibit cytochrome-*c* oxidase at the level of complex IV activity (1, 12). However, CSE stored for a few weeks and therefore devoid of carbon monoxide still showed the same blocking effects (data not shown). Cigarette smoke contains over 4,000 components, and many of those might hypothetically affect the ETC function at many different sites, e.g., phenolic compounds, heavy metals (cadmium, arsenic, lead), gaseous molecules (nitric oxide, $ONOO^-$, carbon monoxide), nicotine, aromates (ubiquinones, acrolein), etc (32).

The final consequence of the CSE-induced ETC blockade is a loss of the capacity of the mitochondria to generate ATP. Because mitochondria play a crucial role in delivering energy for the apoptotic process, alteration of ATP levels may play a crucial role in the routing of cells to die by apoptosis or

necrosis (26, 31). The large amount of free radicals present in inhaled cigarette smoke will initially be able to induce an apoptotic signal in the exposed cells (9). In the presence of an intact energy system, the cells will be able to generate apoptotic death. In case of apoptotic death, the loss of cells will result in a repair response with limited inflammatory consequences (15). Our results show a decrease in caspase-3 and caspase-7 activity in STS-treated Beas-2b bronchial epithelial cells. These results conflict with the observation of Jiao et al. (23), who showed a clear induction of caspase-3 activity in A549 lung epithelial cells, but is in agreement with the report of Wickenden et al. (42), who showed that CSE inhibits caspase-3 activity after apoptosome formation but before actual caspase-3 cleavage. This difference might be explained by our recently published results, which showed that bronchial epithelial cells indeed can behave differently with respect to the initiation of apoptosis or necrosis (38). Apoptosis tends to occur when lower amounts of CSE are given or CSE is given for a shorter period, whereas cells go into necrosis when the CSE is applied for a longer time or at higher concentrations (38). We now show that, when early triggered apoptotic cells become deficient in ATP, they will switch from apoptosis to necrosis.

It is now increasingly recognized that cell death, and in particular apoptosis, may play an important role in the pathophysiology of COPD (24, 36). When apoptotic cells die, they are cleared by monocyte phagocytic activity without the release of proinflammatory substances, whereas necrotic cells release their cellular contents, resulting in an inflammatory response in the environment of these dying cells (44). One link between necrosis acting as a proinflammatory stimulus is through the passive release of the high-mobility group box-1 (HMGB1) protein by necrotic cells but not by apoptotic cells (35). HMGB1 is a nuclear factor and is a secreted protein. The secreted form is a potent inducer of inflammatory mediators like TNF-α (44). The role of necrosis-induced HMGB1 secretion in COPD is not established yet, but recently it has been shown that HMGB1 release may play a key role in the pathogenesis of acute lung injury (40). It can thus be hypothesized that the release of HMGB1 by cigarette smoke-induced necrotic cell death is of great importance in the pathophysiology of COPD and that modulation of this pathway might reveal new treatment modalities (33).

In conclusion, our study demonstrates that CSE induces a blockade of the mitochondrial ETC. This results in a loss of $\Delta\psi_m$, causing a decrease in ATP production. By this depletion of cellular energy, early apoptotic cells exposed to CSE showed a switch to a necrotic cell death. This cigarette smoke-induced mitochondrial dysfunction may become a new pathophysiological concept in the development of COPD and other cigarette smoke-induced diseases and may open new pathways for treatment modalities. Future studies can elucidate the role of cigarette smoke-induced mitochondrial dysfunction and mode of cell death in COPD.

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