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Smoking disturbs mitochondrial respiratory chain function and enhances lipid peroxidation on human circulating lymphocytes

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Mitochondria constitute a source of reactive oxygen species. We tested whether mitochondrial function from human circulating lymphocytes is affected by smoking habit and if this could be associated with an increase in oxidative damage of biological membranes. We prospectively studied 35 smokers and 35 non-smoking healthy individuals matched by age and sex, with a similar physical activity. Individual enzyme activity of complexes II, III and IV of the mitochondrial respiratory chain (MRC) and of glycerol-3-phosphate dehydrogenase activity were measured spectrophotometrically. Intact cell respiration and oxidative rates after addition of pyruvate, succinate and glycerol-3-phosphate were assessed polarographically. Lipid peroxidation of biological membranes was assessed measuring the loss of *cis*-parinaric acid fluorescence. Results are expressed as means (±SD). Smokers showed a significant decrease in complex IV activity compared with non-smokers (112.8 ± 40.9 versus 146.4 ± 62.5 nmol/min/mg protein, respectively; 23% of inhibition; $P = 0.01$), while the rest of the complexes of MRC were unaffected. Conversely, oxidative rate with succinate, but not with the other substrates, was enhanced in smokers compared with non-smokers (16.7 ± 10.4 versus 11.4 ± 4.7 nmol oxygen/min/mg protein, respectively; 46% of activation; $P = 0.01$). Lipid peroxidation of lymphocyte membranes was increased in smokers with respect to non-smokers (3.49 ± 1.27 versus 4.39 ± 1.76 units of fluorescence/mg protein, respectively; 21% of increase; $P = 0.03$) and this increase correlated positively with succinate oxidation activation ($R = 0.34$, $P = 0.02$) and, to a lesser extent, with complex IV inhibition, although it did not reach statistical significance ($R = 0.19$, $P = 0.18$). In smokers, the MRC function of lymphocytes is disturbed and correlates with the degree of oxidative damage of membranes. This mitochondrial dysfunction could contribute to increased endogenous production of reactive oxygen species and could play a role in tobacco carcinogenicity.

Introduction

Tobacco smoke is an environmental and personal pollutant that contains >4000 compounds, most of them with mutagenic and carcinogenic activity, on which are dependent its addictive and pathogenic properties (1). The classes of components identified in both the gas and the tar phases of cigarette smoke include alkenes, nitrosamines, aromatic and heterocyclic carbons and amines, which are well-known sources of reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion and hydroxyl radicals (2,3). In addition to the exogenous source of ROS that implies cigarette smoke by itself, endogenous production of ROS is also increased in smokers (4–6). The main effect of increased amounts of ROS on cell biology is an enhanced oxidative damage, which is expressed as either greater lipid peroxidation of biological membranes (7), and increased oxidative DNA damage (6,8–11) with higher DNA repair activity (9) in smokers compared with non-smokers. This DNA damage is considered of crucial relevance in cancer development (9,12–14).

Mitochondria are widely accepted as a major endogenous source of ROS, due to the great number of oxidoreduction reactions that take place inside these organelles during electron transport throughout the mitochondrial respiratory chain (MRC). Some chemicals contained in tobacco smoke or tar could theoretically interfere with the components of the MRC. For example, carbon monoxide (CO) has a great affinity to bind proteins containing a heme group, such as cytochromes (15,16), and acute CO poisoning causes a marked and sustained inhibition of enzymatic activity of cytochrome *c* oxidase (complex IV) activity (17). If tobacco smoke is able to produce an MRC impairment through an inhibition of some of its components, it could result in an increase of endogenous ROS production, which might eventually contribute to the deleterious cellular oxidative processes and favour mutagenic events finally leading to cancer development. However, despite this potential capability of interaction between tobacco and mitochondria, investigations regarding the effects of smoke habit on human MRC are scarce and only partially addressed to answer this question (18–20). To further investigate this hypothesis, we prospectively studied the effect of smoke habit on MRC function of circulating lymphocytes from smokers as well as on the integrity of its membranes.

Materials and methods

Patient selection

We prospectively included 35 smokers and 35 non-smoking healthy people from staff hospital, matched by age and sex, all under 60 years of age, participating in little or no intense physical activity during their spare time, and with a normal complete blood analysis. Smoking habit was measured as current number of cigarettes smoked per day and number of years of smoking. All patients were informed of the study protocol, which was approved by the Ethical Committee of our Hospital.

In all cases we obtained 20 ml of peripheral venous blood between 10 am and 12 pm. Smokers had smoked at least three cigarettes before sampling. Carboxyhemoglobin (COHb) blood levels were immediately quantified in all cases using a CO-Oximeter.

Lymphocyte isolation

Blood lymphocytes were isolated by means of successive centrifugations in a Ficoll's gradient, and the final protein concentration was quantified according to Bradford's method (21).

Abbreviations: CO, carbon monoxide; COHb, carboxyhemoglobin; MRC, mitochondrial respiratory chain; ROS, reactive oxygen species.

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Mitochondrial biochemical studies

Enzyme activities. Measurement of enzyme activity (expressed as nmol/min/mg of lymphocyte protein) of individual complexes of the MRC was performed spectrophotometrically (UVIKON 920, Kontron, Switzerland) at 37°C in a cuvette containing 1 ml of medium. We determined complex II (succinate-ubiquinone reductase), complex III (ubiquinol-cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase) activities following the method of Rustin *et al.* (22), slightly modified for complex IV measurement (23). Additionally, we also assessed the glycerol-3-phosphate dehydrogenase activity (an enzyme which feeds the MRC with one electron entering at complex III level) and citrate synthase activity (which is used as marker of mitochondrial content) as described elsewhere (22). In all cases, the enzyme activity was calculated by subtracting the residual activity remaining after the addition of the specific inhibitor (10 mM malonate for complex II activity; 1 µM antimycin A for complex III activity; 0.24 mM KCN for complex IV activity; and 20 mM 3-phosphoglycerate for glycerol-3-phosphate dehydrogenase activity).

Oxidative activity. Oxygen utilization was measured polarographically in 0.25 ml of standard medium (pH 7.4) containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4 and 0.25 mg bovine serum albumin with a Clark electrode in a water-jacketed cell at 37°C (Hansatech Instruments, Norfolk, UK). Intact cell oxidative activity was determined according to Rustin *et al.* (22) and oxidative rates (all expressed as nmol oxygen/min/mg lymphocyte protein) were assessed after the permeabilization of cellular membrane with digitonin and the addition of the following substrates: pyruvate (5 mM; complex I substrate) plus malate (1 mM), succinate (20 mM; complex II substrate) in the presence of rotenone (4 µM) and ATP (0.4 mM), and glycerol-3-phosphate (10 mM; complex III substrate) in the presence of rotenone (4 µM), ATP (0.4 mM) and malonate (20 mM). In order to distinguish between enzymatic and non-enzymatic oxidation, specific inhibitors for each substrate were used in both smokers and non-smokers (4 µM rotenone for pyruvate oxidation; 20 mM malonate for succinate oxidation; and 40 mM 3-phosphoglycerate for glycerol-3-phosphate oxidation). Then, specific mitochondrial oxidative activities were calculated after subtracting the non-mitochondrial rate from the state 3 rate.

Calculation of MRC oxidative enzyme activities ratios. In order to assess the balance among the different constituents of MRC, we calculated the ratios among all the oxidative and enzyme activities measured in present study.

Mitochondrial membrane lipid peroxidation studies

The lymphocyte membrane's integrity was measured through the assessment of lipid peroxidation. Thirty micrograms of lymphocyte protein were placed into 1 ml of phosphate-buffered saline (PBS) containing 5 µM *cis*-parinaric acid (Molecular Probes, Eugene, OR) and then incubated for 30 min in the dark at 37°C. Afterwards, fluorescence at 318 nm excitation and 410 nm emission was measured as described (24,25), and the remaining *cis*-parinaric acid fluorescence was used to determine the chemical process of lipid peroxidation. The more lipid peroxidation that occurs, the less fluorescence is detected.

Statistical analysis

Data were processed using SPSS software. Means (±SD) and percentages were used for quantitative and qualitative variables, respectively. Comparisons between groups were carried out using a *t*-test for independent data, after assessing the normality of the distribution and the equality of variances. Simple linear regression was used to find correlation between quantitative variables. *P*-values <0.05 were considered statistically significant.

Results

Mean ages (±SD) of smokers and non-smokers were 35 ± 12 and 34 ± 11 years, respectively. In both groups, 19 out of 35 (54%) individuals were women. Values for COHb blood levels were 4.8 ± 2.5% for smokers and 1.2 ± 0.3% for non-smokers ($P < 0.001$). In smokers, current tobacco consumption was 20 ± 9 cigarettes per day, and number of years of smoking was 13 ± 8.

Mitochondrial content, assessed by citrate synthase activity, did not differ between both groups (80.2 ± 27.6 nmol/min/mg protein for smokers and 88.9 ± 29.9 nmol/min/mg protein for non-smokers; *P*-value not significant).

The measurements of individual enzymatic activities of the MRC showed a decreased complex IV activity in smokers compared with non-smokers (112.8 ± 40.9 versus 146.4 ± 62.5 nmol/min/mg protein, respectively; 23% of inhibition; $P = 0.01$), while the rest of the complexes did not differ between both groups (Figure 1).

Smokers showed an increased mitochondrial oxidative rate with succinate compared with non-smokers (16.7 ± 10.4 versus 11.4 ± 4.7 nmol oxygen/min/mg protein, respectively; 46% of activation; $P = 0.01$); they also showed an increased intact cell respiration (18.2 ± 6.7 versus 15.3 ± 6.2 nmol oxygen/min/mg protein, respectively; 19% of activation), although it did not reach statistical significance ($P = 0.07$). Conversely, oxidation of pyruvate and glycerol-3-phosphate did not differ between groups (Figure 2). Percentage of non-mitochondrial oxygen uptake with respect to whole oxygen consumption during state 3 (which includes mitochondrial and non-mitochondrial oxygen uptake) was 16 ± 8% for glutamate, 10 ± 7% for succinate and 21 ± 13% for glycerol-3-phosphate, without differences between both groups.

MRC oxidative enzyme activities ratios are presented in Table I. Although the majority of altered ratios of smoker individuals included complex IV or succinate oxidation activities, there was also some additional altered ratios affecting other oxidative or enzyme activities.

Finally, lipid peroxidation of lymphocyte membranes was significantly increased in smokers compared with non-smokers (3.49 ± 1.27 versus 4.39 ± 1.76 units of fluorescence/µg protein, respectively; 21% of increment; $P < 0.05$) (Figure 3). Such an increase in lipid peroxidation positively correlated with the increase of succinate oxidation activity ($R = 0.34$, $P = 0.02$) and, in a weaker manner, with the inhibition of complex IV activity ($R = 0.19$, $P = 0.18$).

Discussion

The present study in human lymphocyte mitochondria demonstrates that smoking causes an inhibition of complex IV activity and an increase in the oxidative activity with succinate, a substrate entering at the complex II level of the MRC. These findings cannot be attributed to differences in mitochondrial population, since mitochondrial content did not differ between both groups. Therefore, these data indicate that smoking clearly disturbs the cellular MRC in smokers.

The inhibition of complex IV has been also observed in previous animal and human experiences. Gvozdjáková *et al.* (26) demonstrated a decrease in complex IV activity of heart muscle mitochondria from rabbits inhaling cigarette smoke, and noted that this decrease was higher with greater length of smoke exposure. Similarly, Örlander and co-workers (18,19) found a decreased complex IV activity in skeletal muscle mitochondria of smokers, and suggested that tobacco smoke components, especially CO, could be responsible for this decrease. Actually, this hypothesis has been confirmed in patients suffering from an acute CO poisoning, who develop a severe and persistent inhibition of complex IV activity (17). Consequently, complex IV seems to be a target for CO and (maybe) other components of tobacco smoke. Other authors (20) have found that smoke habit should also act against complex I of MRC, but since this complex cannot be reliably measured in lymphocytes (22), we could not confirm such an inhibition in our study.

Conversely, from the current available data, the ultimate effect of smoking on mitochondrial oxidative activity remains still controversial. In an *in vitro* study, Gairola and Aleem (27) showed that very small amounts of tobacco smoke cause an

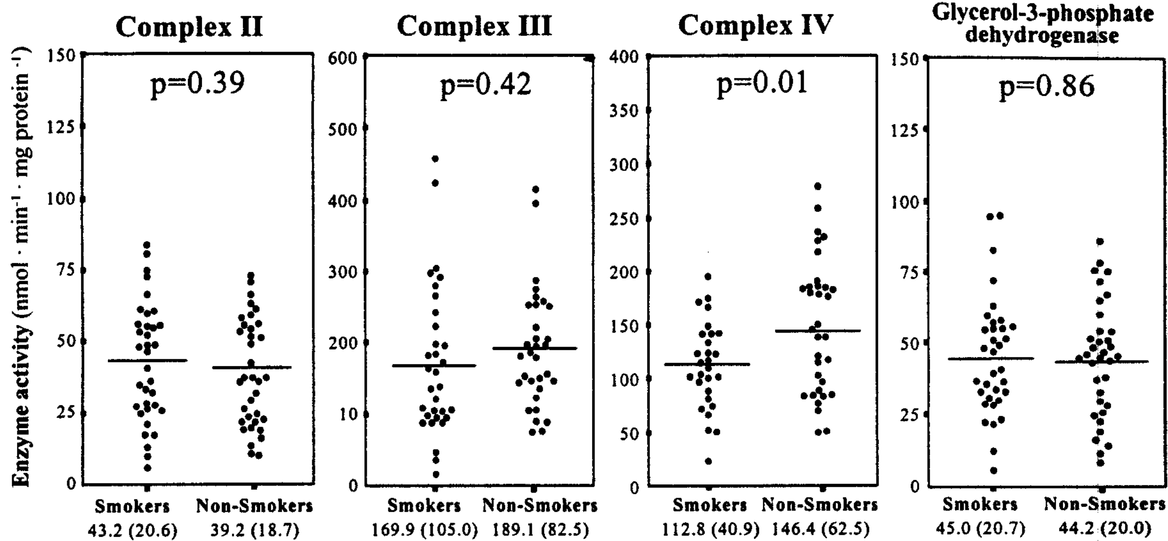

Fig. 1. Individual enzyme activities from peripheral lymphocytes measured spectrophotometrically. Dots represent the individual values and bars represent the means. Numbers under the *x*-axis denote means (±SD).

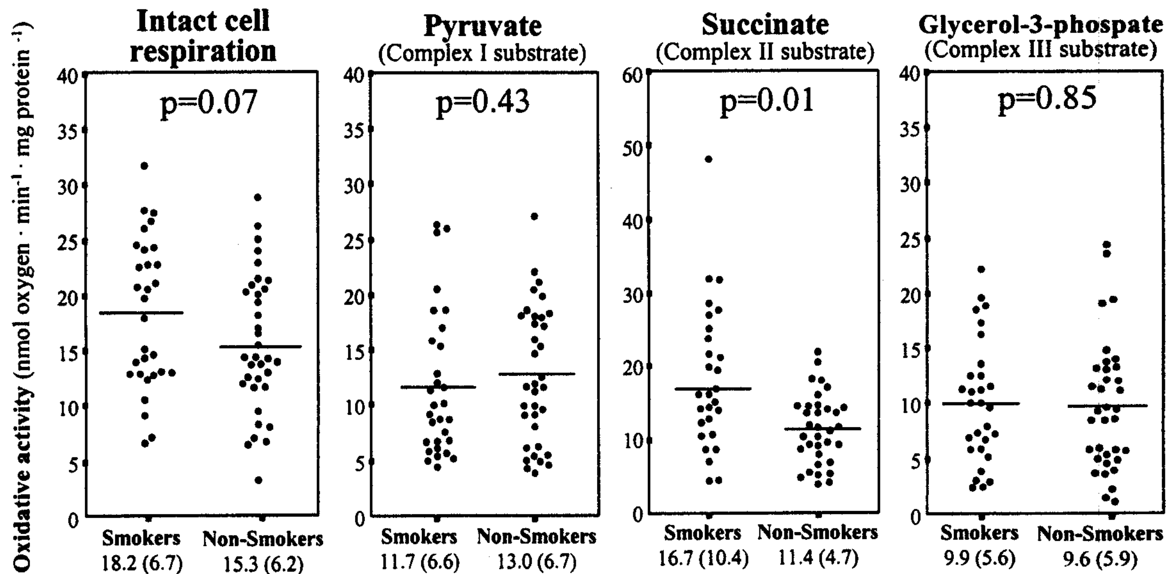

Fig. 2. Intact cellular oxidation and oxidative activities with the different substrates from peripheral lymphocytes measured polarographically. Dots represent the individual values and bars represent the means. Numbers under the *x*-axis denote means (±SD).

acute inhibition of succinate and β-hydroxybutyrate oxidation by rat liver mitochondria, and Gvozdjáková and co-workers (28–30) reported similar results in *in vitro* and *in vivo* animal studies. More recently, Pryor *et al.* (1) have found that the extracts of cigarette tar inhibit the electron flow across the MRC of rat liver mitochondria and beef submitochondrial particles, when substrates for either complex I, II or IV were used. These experimental results contrast with data coming from human *in vivo* assessment of mitochondrial oxidative activity of smokers. Polymorphonuclear leukocytes of smokers have been shown to have an increased glucose oxidation (31), and these people also have a higher 24 h energy expenditure (32) and 24 h oxygen consumption (8) (which indirectly estimate mitochondrial oxidative metabolism) compared with non-smokers. Then, our studies in the measurement of mitochondrial oxidative rates from human lymphocytes constitute the first direct evidence of such an increase. This effect may not be limited to circulating lymphocytes, since in a previous report assessing the effect of age on MRC of human skeletal muscle (33) we found that smoke habit increased oxidative rates, although the presence of other uncontrolled factors in that study (such as age, sex, physical activity or different kind of anesthesia employed for obtaining muscle) did not allow us to reach a definitive conclusion. Additionally, the alteration of several MRC oxidative enzyme activities ratios, which constitute a more sensitive method than isolated determinations for detecting MRC disturbances (34,35), found in smokers in the current study demonstrates that the respiratory chain is, on the whole, unbalanced by smoking habit. In this sense, the extensive lymphocyte washing carried out during the isolation process would have probably allowed detection of only permanent, but not transient, effects of smoking, so that real mitochondrial damage in smokers could be even more severe.

The concurrence of a decreased complex IV activity and an increased mitochondrial oxidative capacity in smokers could be considered as a consequence of compensatory up-regulatory

Table I. Ratio values of mitochondrial respiratory chain (MRC) oxidative activities and MRC enzyme activities in circulating lymphocytes

	C-II	C-III	C-IV	G3Pdh	CELLox	Pox	Sox
C-III	nsk: 0.32 ± 0.14 sk: 0.46 ± 0.39						
C-IV	nsk: 0.36 ± 0.16 sk: *0.52 ± 0.19*[c]	nsk: 1.7 ± 1.3 sk: 1.6 ± 0.6					
G3Pdh	nsk: 1.1 ± 0.8 sk: 1.2 ± 0.9	nsk: 4.3 ± 1.7 sk: *3.2 ± 1.1*[a]	nsk: 3.4 ± 1.7 sk: *2.6 ± 1.0*[a]				
CELLox	nsk: 2.9 ± 1.5 sk: 2.9 ± 1.3	nsk: 12.0 ± 5.1 sk: 10.3 ± 5.0	nsk: 9.2 ± 4.4 sk: *6.6 ± 2.3*[b]	nsk: 3.1 ± 1.2 sk: 2.6 ± 1.6			
Pox	nsk: 3.5 ± 1.4 sk: 3.8 ± 1.7	nsk: 12.5 ± 4.7 sk: *18.6 ± 11.5*[a]	nsk: 10.4 ± 3.2 sk: 11.5 ± 6.2	nsk: 3.8 ± 2.7 sk: 4.1 ± 2.9	nsk: 1.4 ± 0.6 sk: *1.8 ± 0.8*[a]		
Sox	nsk: 3.8 ± 1.7 sk: 3.5 ± 1.0	nsk: 15.2 ± 5.4 sk: *11.9 ± 6.9*[a]	nsk: 13.3 ± 6.0 sk: *8.1 ± 4.1*[b]	nsk: 3.3 ± 1.6 sk: 3.2 ± 1.6	nsk: 1.6 ± 0.9 sk: 1.4 ± 0.8	nsk: 1.4 ± 0.9 sk: *0.9 ± 0.5*[b]	
G3Pox	nsk: 5.5 ± 3.3 sk: 5.9 ± 3.1	nsk: 20.5 ± 9.0 sk: 18.3 ± 9.4	nsk: 15.6 ± 6.8 sk: 14.8 ± 7.7	nsk: 5.6 ± 3.4 sk: 5.5 ± 3.4	nsk: 2.0 ± 1.2 sk: 2.3 ± 1.2	nsk: 1.7 ± 1.2 sk: 1.5 ± 0.8	nsk: 1.2 ± 0.3 sk: *1.5 ± 0.5*[b]

C-II, complex II; C-III, complex III; C-IV, complex IV; G3Pdh, glycerol-3-phosphate dehydrogenase; CELLox, intact cell respiration; Pox, pyruvate oxidation; Sox, succinate oxidation; G3Pox, glycerol-3-phosphate oxidation; nsk, controls; sk, smokers.
[a] $P < 0.001$.
[b] $P < 0.05$.
[c] $P < 0.01$.

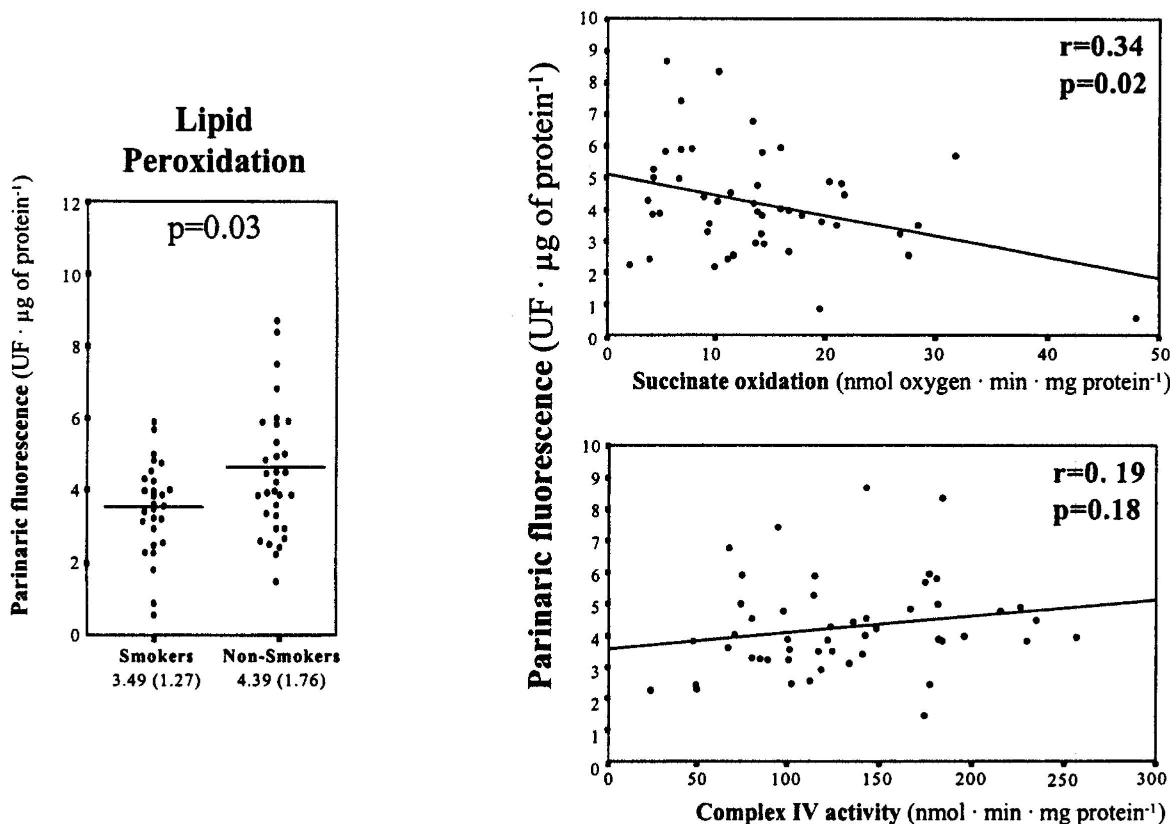

Fig. 3. Left, lipid peroxidation assessed by means of *cis*-parinaric acid. Dots represent the individual values and bars represent the means. Numbers under the *x*-axis denote means (±SD). Right, dot diagrams with the regression lines between lipid peroxidation and succinate oxidation (top) and complex IV activity (bottom).

mechanisms. We hypothesize that, in the presence of a chronic complex IV inhibition, mitochondrial homeostasis leads to an efficient up-regulation that allows the organelles to maintain their function. Actually, this hypothesis is in accordance with the kinetic basis of threshold effects observed in mitochondrial diseases (36,37) and also with animal studies (38), where the experimental complex IV inhibition leads to a decrease in mitochondrial respiration which exhibits a threshold behavior similar to that observed in mitochondrial diseases, remaining respiratory flux maximal until a low level of complex IV activity is reached.

The exact step involved in this compensation is difficult to pinpoint from our data, since the present study was not designed to address this specific issue. Therefore, the interpretation of differences in the oxidative activity by intact and permeabilized cells must be cautious because they could potentially include

the effect of non-mitochondrial oxidation, ATPase activities and mitochondrial proton leak. The subtraction of the remaining rate after the addition of the specific complex II inhibitor malonate (which would depend on the rate of ATP hydrolysis ocurring under these conditions) from that before this addition allows us to exclude any non-mitochondrial contribution to the differences in succinate oxidation between smoker and non-smoker groups. The potential roles of ATPase activities were not assessed, but we assume they should be the same for both groups, so the resulting effects finally balanced. The possibility of a decreased proton leak in smokers as the cause of the increased succinate oxidation activity deserves special comment. Proton leak reflects the passive flux of protons across the inner membrane leading to a mitochondrial oxygen consumption which is not coupled to ATP synthesis (39) and it is considered a means of reducing the harmful ROS production and secondary oxidative damage (40); therefore, a hypothetical decay in proton leak in smokers could explain the enhanced lipid peroxidation of membranes found in our study. In addition to all these chemical changes, disturbances in the mitochondrial membrane structure itself in smokers can not be definitively ruled out, since conformational changes making more active sites of complex II available on the expanded surface of inner membrane have been argued as causing succinate oxidative stimulation (41). Finally, it is possible that not only intracellular mechanisms, but also systemic factors, could account for the increased oxidative activity; for example, cathecolamines and glucagon are known to be sufficient for increasing respiratory rates (42), and it is widely accepted that these circulating hormones are increased in smokers (43–46).

Besides the alterations of MRC, we observed that lipid peroxidation of membranes was increased in smokers compared with non-smokers, and it correlated with those MRC alterations. The activation of mitochondrial oxidative processes in the presence of a decreased complex IV activity could result in an enhanced production of ROS, which ultimately might contribute to the lipid peroxidation of membranes that we observed in smokers. This finding agrees with recent investigations demonstrating an increased oxidative stress (5,6,47) and lipid peroxidation of several biological molecules (7,48–51) in smoker individuals. Lipid peroxidation can propagate as a chain reaction, and a single initiating free radical can result in the peroxidation of a large number of unsaturated fatty acids. Not only can this affect the physical stability of membranes, but the lipid peroxides are themselves unstable and break down to yield a range of toxic aldehydes that are capable of damaging membrane proteins (24). In fact, mitochondrial dysfunction, oxidative stress and lipid peroxidation are integrating a vicious circle in which the effect continues feeding the cause. The presence of severe MRC disturbances in the smokers could have crucial relevance in oxidative stress processes and, ultimately, in tobacco-related carcinogenesis in humans.

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