INVESTIGACIÓ I RESULTATS

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

Fumar altera el funcionalisme de la cadena respiratòria mitocondrial i augmenta la peroxidació lipídica en limfòcits perifèrics humans

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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.

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

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).

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).

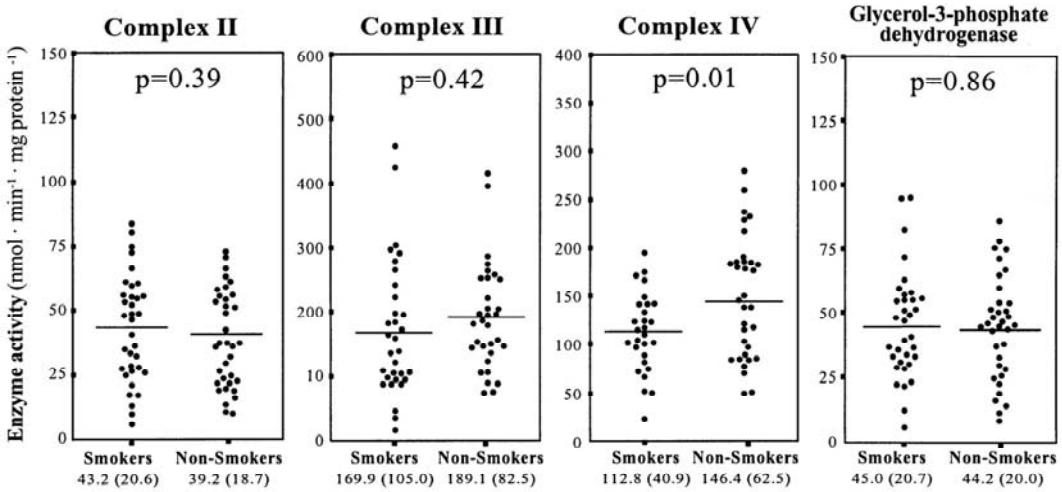

Fig. 1. Individual enzyme activities from peripheral lymphocytes measured spectrophotometrically. Dots represent the individual values and bars represent the means. Numbers under the *x*-axis denote means (±SD).

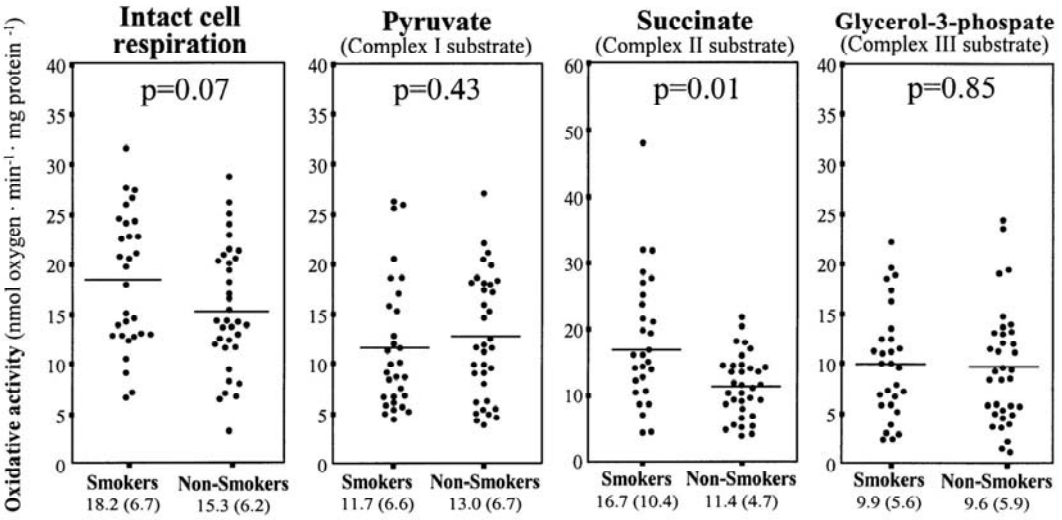

Fig. 2. Intact cellular oxidation and oxidative activities with the different substrates from peripheral lymphocytes measured polarographically. Dots represent the individual values and bars represent the means. Numbers under the *x*-axis denote means (±SD).

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 acute inhibition of succinate and ß-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

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.

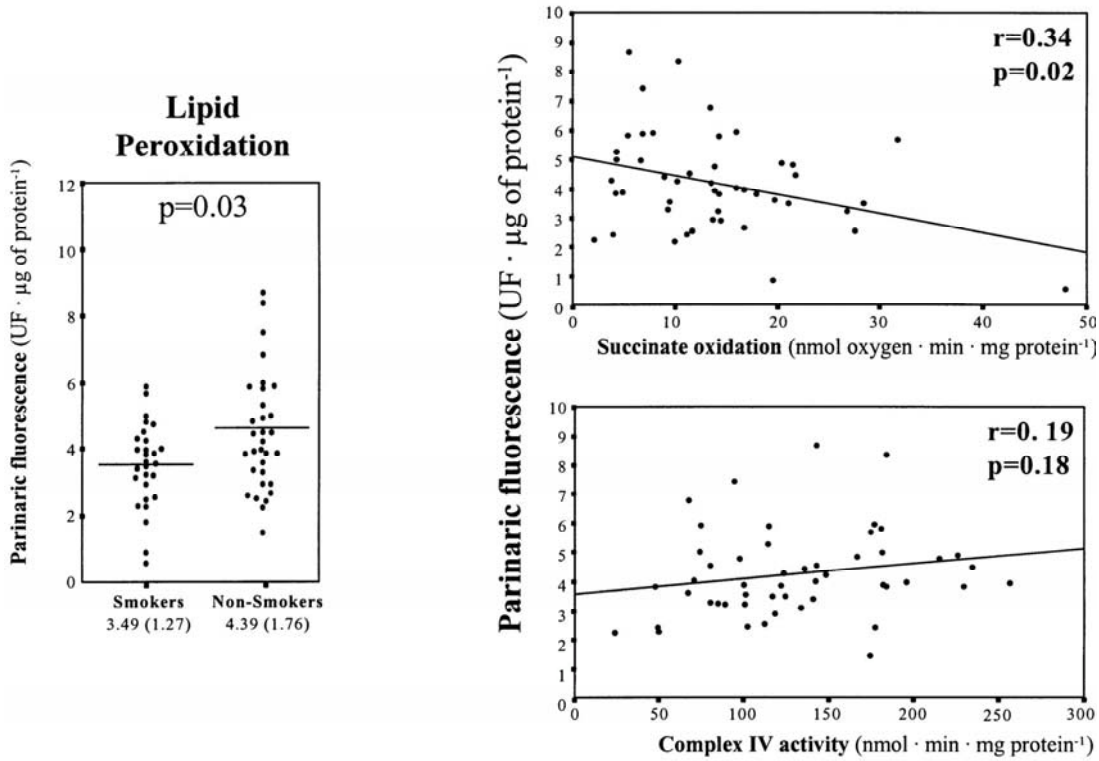

Fig. 3. Left, lipid peroxidation assessed by means of *cis*-parinaric acid. Dots represent the individual values and bars represent the means. Numbers under the *x*-axis denote means (±SD). Right, dot diagrams with the regression lines between lipid peroxidation and succinate oxidation (top) and complex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

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 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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SÍNTESI DELS RESULTATS

1.- Els fumadors presenten una inhibició de l'activitat enzimàtica del complex IV de la cadena respiratòria mitocondrial demostrable en limfòcits perifèrics, mentre que l'activitat dels complexos II i III no es veu afectada pel fet de fumar.

2.- L'oxidació del succinat (que entra a la cadena respiratòria a través del complex II) es troba incrementada en el grup de fumadors respecte als no fumadors. L'activitat oxidativa de les cèl·lules intactes també es troba augmentada en els fumadors, si bé en aquest cas no s'assoleix significació estadística respecte als no fumadors.

3.- La peroxidació dels lípids de les membranes limfocitàries, que és una conseqüència de la producció excessiva de radicals lliures que provoquen dany oxidatiu, es troba augmentada en les persones fumadores.

4.- Aquest dany oxidatiu correlaciona amb el grau d'activació de l'oxidació del succinat i, en menor mesura, amb la inhibició del complex IV.

5.- Per tant, els fumadors presenten una alteració difusa del funcionalisme de la cadena respiratòria mitocondrial, la qual podria ser l'origen d'una producció endògena incrementada de molècules d'elevat poder oxidant que produirien dany oxidatiu en les membranes d'aquestes persones. Aquesta producció endògena augmentada de radicals lliures s'afegiria a les substàncies oxidants exògenes que arriben a l'organisme dels

fumadors amb el fum del tabac i, en conjunt, podrien contribuir a potenciar l'acció d'altres carcinògens ben reconeguts.

6.- En aquest primer treball es llença la hipòtesi que entre les molècules contingudes al fum del tabac (més de 4000 substàncies), el monòxid de carboni és un bon candidat com a causant principal d'aquestes alteracions, donada la seva capacitat d'unir-se al complex IV i inhibir-lo, tant *in vitro* com *in vivo*. No obstant, altres components del fum del tabac, com ara l'àcid cianhídric (també tòxic del complex IV) també podrien col·laborar en la producció d'aquests efectes.

7.- En tot cas el tabaquisme és un altre factor a controlar en el moment de definir els intervals de normalitat en l'activitat del complex IV i en l'oxidació del succinat, així com a l'hora de construir grups control per als estudis de funcionalisme mitocondrial.

2

"Effect of smoking cessation on mitochondrial respiratory chain function"

Efecte del fet de deixar de fumar envers el funcionalisme de la cadena respiratòria mitocondrial

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ARTICLE

Effect of Smoking Cessation on Mitochondrial Respiratory Chain Function

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ABSTRACT

Objective. Chronic smoking has been associated with diverse mitochondrial respiratory chain (MRC) dysfunction in lymphocytes, although inhibition of complex IV activity is the most consistent and relevant finding. These mitochondrial abnormalities have been proposed to contribute to pathogenesis of diseases associated with tobacco consumption. We assessed MRC function in peripheral lymphocytes from heavy smokers after cessation in smoking habit. *Patients and Methods*. We studied MRC function from peripheral lymphocytes of 10 healthy chronic smoker individuals (age 43 ± 6 years; 50% women) before cessation of tobacco consumption (t_0), and 7 (t_1) and 28 (t_2) days after cessation. Smoking abstinence was ascertained by measuring carboxyhemoglobin levels and carbon monoxide (CO) concentration in exhaled breath. Ten healthy nonsmoker individuals matched by age and gender were used as controls. Lymphocytes were isolated by Ficoll's gradient, and protein content was determined by Bradford's technique. MRC function was studied through double means: 1) individual enzyme activities of complex II, III, and IV were analyzed by means of spectrophotometry; 2) oxygen consumption was measured polarographically using pyruvate, succinate, and glycerol-3-phosphate (complex I, II, and III substrates, respectively) after lymphocyte permeabilization. Enzyme and oxidative activities were corrected by citrate synthase activity. *Results*. Smokers showed a significant decrease in complex IV activity ($p = 0.05$) and also in respiration of intact lymphocytes ($p = 0.05$) compared to controls. Eight chronic smokers remained abstinent during the study. Smoking cessation was associated with a significant recovery of complex IV ($p = 0.01$) and complex III ($p = 0.05$) activities. Oxidative activities did not show any change during the study. *Conclusion*. Chronic smoking is associated with a decrease of complex IV and III activities of MRC, which return to normal values after cessation of tobacco smoking.

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INTRODUCTION

Smoking habit is directly connected with a vast number of human diseases involving most organs and tissues. Mechanisms by which smoking causes its deleterious effects are varied and not completely elucidated. Mitochondria have been proposed as one of the targets of tobacco smoke compounds (1). These organella are present in nearly all cellular lines and play an important role in two critical cell functions: they are the common pathway for a number of initiators of apoptosis (a physiological process by which cells are removed when their DNA is damaged by noxious agents), and their respiratory chain is the main source of cellular energy liberated as ATP molecules.

Cigarette smoke contains short and long-lived free radicals and also can stimulate endogenous cellular production of other highly reactive oxygen species (ROS), both inducing protein oxidation, lipid peroxidation (2), and mitochondrial DNA damage (3). This oxidative damage further results in mitochondrial depolarization and final cellular apoptosis (4,5). On the other hand, components of enzyme mitochondrial machinery, especially those integrating MRC, can be directly disturbed by tobacco smoke at different steps (6–8), which could result in a decrease in ATP synthesis (8). Although mitochondrial respiratory chain (MRC) abnormalities have been described in chronic smokers, the reversibility of these effects after smoking cessation has not been previously ascertained. Therefore, we assessed MRC function in peripheral lymphocytes from chronic smokers and assessed changes associated with smoking cessation.

PATIENTS AND METHODS

We included 10 chronic smokers who entered the "Tobacco Deshabituation Unit" of our hospital. Their cigarette consumption was between one and three packs a day, at least during the last five years before this study. All of them were otherwise healthy people with no previous history of metabolic or neuromuscular diseases. In all cases, a complete blood count and chemistry screen (glucose, creatinine, sodium, potasium, calcium, and creatinphosphokinase) were performed and normality of all tests was required to be included in the protocol.

To perform mitochondrial studies, 30 mL of venous blood were obtained in active smokers, and also 7 and 28 days after smoking cessation. Lymphocytes were isolated through a Ficoll's gradient and the protein content of the final lymphocyte suspension was measured using the Bradford's protein-dye binding principle (9).

Smoking status was ascertained at the three times of the study by measuring carbon monoxide content in exhaled air (COEA) by means of an electrochemical transducer (Dräger Pac III, Dräger Safety Inc, Pittsburgh, PA), and blood levels of carboxyhemoglobin (COHb) through CO-oximetry (Blood gas Analyzer CIBA-CORNING 800 System, Switzerland). COHb levels less than 2% and COEA levels less than 10 ppm were respectively required to consider the individual as abstinent. As control values, we used those obtained from 10 healthy nonsmoker individuals matched by age, gender, and physical activity with respect to the smokers group. All individuals gave their informed consent and the Ethic Committee of our hospital revised and accepted the present protocol.

MRC function from lymphocytes was assessed using a double approach. First, we determined the individual enzyme activity for complex II (succinate–ubiquinone reductase, EC 1.3.99.1), complex III (ubiquinol–cytocrome *c* reductase, EC 1.10.2.2), and complex IV (cytochrome *c* oxidase, EC 1.9.3.1) spectrophotometrically (UVIKON 920, Kontron®, Switzerland). Second, oxygen consumption was polarographically determined in intact lymphocytes (Hansatech Instruments Limited®, Norfolk, England). We studied spontaneous respiration, as well as respiratory rates using pyruvate, succinate, and glycerol-3-phosphate as substrates for complex I, II, and III, respectively. The concentration of substrates, complete methodology, and experimental conditions used in all these assays have been described previously (10–13).

In order to account for any eventual difference in the mitochondrial content of peripheral lymphocytes, results were expressed as absolute and relative activities as well. Relative activities were obtained in relation of citrate synthase (EC 4.1.3.7) activity, an enzyme of Krebs' cycle which is considered as a good marker of mitochondrial content. The measurement of citrate synthase activity was carried out spectrophotometrically under the conditions described elsewhere (14).

Results are expressed as percentages, mean ± standard deviation, and 95% confidence intervals using SPSS 10.0 statistical package. Comparisons between the three determinations performed in smokers and the control values of nonsmokers were performed by unpaired t-test. No corrections were made for multiple comparisons. The changes of enzyme activities and oxygen consumption of smoker individuals at the different times of the study (immediately before and 7 and 28 days after smoking cessation) were assessed by

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a repeated measures ANOVA using the general lineal model of SPSS. Differences were considered significant when p value was less than 0.05.

RESULTS

We initially included 10 smoker individuals, although only 8 subjects (43 ± 6 years; 50% women) remained abstinent until the end of the study and yielded all necessary data to be finally included in the study. COHb and COEA in active smokers were 5.1 ± 2.3% and 28.1 ± 7.6 ppm, respectively, which were significantly higher than those observed in controls (0.5 ± 0.3% and 3.6 ± 2.0 ppm, respectively; $p < 0.001$ for both). After smoking cessation, COHb and COEA values decreased to 0.6 ± 0.3% and 3.1 ± 2.0 ppm respectively, at day 7, and 0.8% ± 0.3 and 3.1 ± 2.2 ppm respectively, at day 28, which fitted into normal ranges.

Mitochondrial content of lymphocytes at the beginning of the study did not differ between both groups, as judged by citrate synthase activities (smokers: 127 ± 60 nmol/min/mg protein; controls: 136 ± 24 nmol/min/mg protein; p = NS). Cessation of the smoking habit was not associated with any significant change in citrate synthase activity in the smokers group (127 ± 40 nmol/min/mg protein on day 7 and 110 ± 23 nmol/min/mg protein on day 28).

Results were similar irrespective of whether they were expressed as absolute or relative activities. Relative enzyme activities are presented in Fig. 1. In active smokers, only complex IV activity was significantly lower than that of controls (33% inhibition; CI: 5% to 61%; $p < 0.05$). After smoking cessation, complex IV activity showed a progressive and significant recovery up to normal values after 28 days ($p = 0.002$). At that time, a 56% (CI: 10% to 102%) of complex IV recovery respect to initial values was observed (Fig. 2). Complex III activity showed a slight decrease (21% inhibition; CI: −9% to 51%; p = NS) in active smokers compared to controls, and cessation of smoking was associated with a statistically significant increase in its activity over time ($p = 0.04$).

Polarographic studies in lymphocytes from active smokers showed a significant decrease in spontaneous oxygen consumption compared to controls (26% inhibition; CI: 6% to 46%; $p < 0.05$); however, no differences were observed when respiratory state 3 rate was measured for each particular substrate (Fig. 3).

The behavior of oxidative activities after smoking cessation remained relatively stable with all substrates, and we did not observe any significant change during the study.

DISCUSSION

The present study confirms that complex IV enzyme activity is inhibited in smokers (7), and that this inhibition progressively disappears shortly after smoking habit cessation. Additionally, our data indicate that complex III activity is also slightly inhibited by chronic

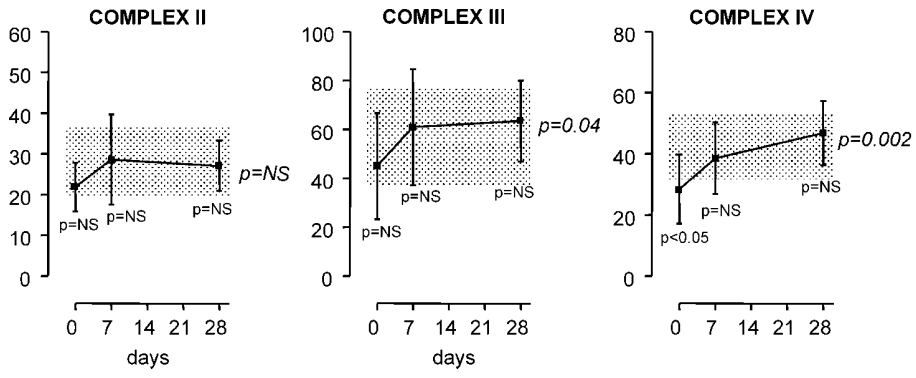

Figure 1. Relative enzyme activities (means with 95% CI) of smokers before (day 0) and after stopping (days 7 and 28) to smoke. There are no units for the Y axis because they represent the ratio between the absolute complex activity (× 100) and citrate synthase activity. Shadowed squares denote 95% CI for the mean of control values (nonsmoker individuals). P values under the bars refer to the comparisons between each measure in smoker individuals and the control values (unpaired t test), while p values at the right of each graphic refer to the comparisons of the three measures performed in the smoker individuals along the time (general lineal model for repeated measures). NS: not significant.

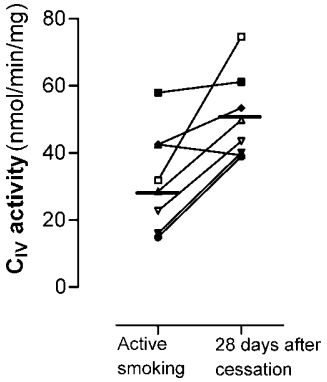

Figure 2. Initial and final complex IV absolute activities for each individual patient. Bars denote the means.

smoking and exhibits a progressive, statistically significant increase coinciding with smoking cessation. These changes in MRC complexes seem to be accompanied of a decrease in spontaneous oxidative cellular capacity, while oxidation of selected substrates remains unaltered. In whole, we consider that these findings add further evidence that MRC is an additional target of cigarette smoke.

Our data agree with most of the previously reported investigations in animal models. Some in vivo and in vitro studies on animal mitochondria have demonstrated that complex IV is inhibited by inhaling cigarette smoke, and that there is a close relationship with the length of smoke exposure (8,15–17). In these animal models, oxidative capacity of mitochondria resulted a decrease in substrates that entered complex I (17–19), II (17,18) or IV (19) level. Gairola et al. (20), based on the spectroscopic measurement of the cytochromes in their reduced state, proposed that multiple cytochrome constituents should be involved in the interaction of whole cigarette smoke and intact mitochondria.

However, Örlander et al. (21,22) and ourselves (7) in humans have consistently demonstrated that complex IV inhibition is the main alteration in chronic smokers. Similarly, Smith et al. (6) studied the activity of complexes of MRC in mitochondria obtained from platelets of chronic smokers and found 24% and 8% decrease in complex I and IV activities, respectively, although the inhibition was statistically significant only for the former. Therefore, different complexes of MRC seem to be inhibited in chronic smokers, and we believe that the differences observed could depend on the specific tissue involved. Similar findings among tissues have been described in some mitochondria-related neurodegenerative diseases (23,24).

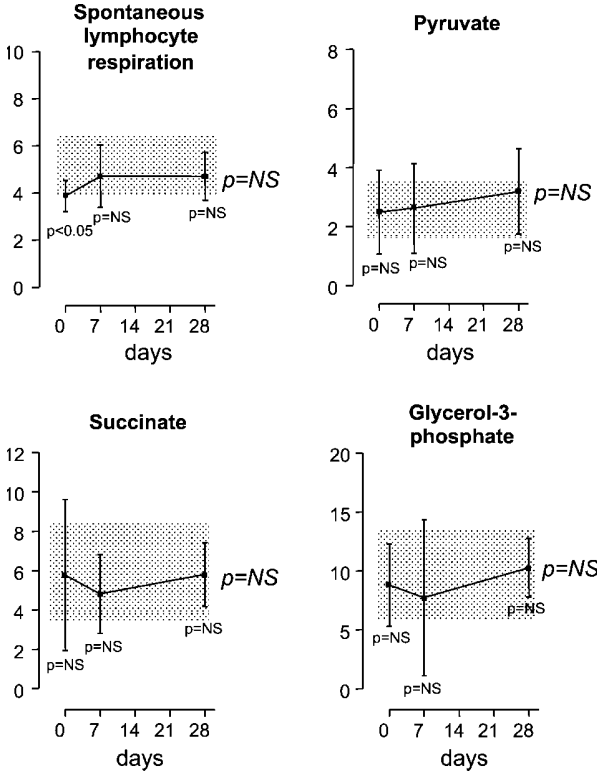

Figure 3. Relative oxidative activities (means with 95% CI) of smokers before (day 0) and after stopping (days 7 and 28) to smoke. There are no units for the Y axis because they represent the ratio between the absolute oxidative activity (× 100) and citrate synthase activity. Shadowed squares denote 95% CI for the mean of control values. P values under the bars refer to the comparisons between each measure in smoker individuals and the control values (unpaired t test), while p values at the right of each graphic refer to the comparisons of the three measures performed in the smoker individuals along the time (general lineal model for repeated measures). NS: not significant.

The most interesting finding in our study is the reversibility of complex IV inhibition after smoking cessation. However, the ultimate significance of this complex IV inhibition induced by smoking, although reversible, has not yet been determined. In fact, long-term inhibition of complex IV activity could lead to a MRC dysfunction, decreased ATP generation, enhanced ROS production, DNA damage (25), and eventually enhanced apoptosis (26) or abnormal cell proliferation (27). Therefore, reversibility of the MRC dysfunction after smoking cessation, even after many years of heavy smoking habit, does not necessarily imply that long-term damage can be avoided and all those persistent changes could play a role in some smoking-related diseases, as

can be seen in clinical practice when patients develop cancer or progressive lung diseases even many years after stopping the smoking habit. To support this hypothesis, we have also demonstrated an increase in oxidative damage of lymphocyte membranes of chronic smokers (7), which additionally could potentiate MRC dysfunction at different levels leading to greater cellular damage as part of a vicious circle.

The exact mechanism for complex IV inhibition, the most consistent finding, is a matter of debate. It has been suggested that CO or other of the thousands of molecules contained in cigarette smoke could contribute to such an inhibition. In experimental studies on animals CO has been largely recognized as a competitive inhibitor of complex IV (7,28,19). Although direct toxicity on complex IV has not been definitively confirmed in humans, this hypothesis seems very likely since we have repeatedly observed that the activity of complex IV is severely depressed in patients acutely poisoned by CO, and that this activity slowly recuperates after removing the patient from the CO exposure (30,31). In addition, in an in vitro model we have recently found that complex IV of human muscle mitochondria is inhibited by progressive concentrations of CO (32).

In conclusion, although the inhibition of mitochondrial complex IV activity in chronic smokers is reversible, long-term inhibition of MRC function could be involved in the development of multiorganic alterations even after smoking cessation.

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SÍNTESI DELS RESULTATS

1.- Els limfòcits de fumadors crònics sans presenten una inhibició de l'activitat enzimàtica del complex IV de la cadena respiratòria mitocondrial, respecte els controls no fumadors sans. Les dades d'aquest estudi confirmen les dades trobades a l'estudi previ.

2.- En abandonar el consum de tabac, l'activitat del complex IV es recupera de forma progressiva i estadísticament significativa fins a assolir valors estrictament normals a les 4 setmanes d'haver deixat de fumar.

3.- Tot i que l'activitat del complex III es troba lleugerament disminuïda als fumadors respecte els no fumadors, aquestes diferències no son estadísticament significatives. Una vegada més es confirmen les dades trobades prèviament, on l'activitat dels complexos II i III no es veien significativament alterades pel fet de fumar. Tanmateix, el fet de deixar de fumar s'associa amb un augment significatiu de la seva activitat.

4.- Malgrat l'alteració en l'activitat del complex IV en fumadors és reversible en abandonar el tabac, es desconeix quin és l'efecte d'una inhibició mantinguda durant el temps que ha estat fumador. De fet, l'alteració crònica de la cadena respiratòria mitocondrial podria induir dany cel·lular i tissular, el qual podria perdurar inclòs en desaparèixer la inhibició de la COX. A més, la pròpia alteració mitocondrial secundària al dany oxidatiu podria perpetuar la disfunció de la cadena respiratòria com a un cercle viciós.

5.- El mecanisme exacte de la inhibició del complex IV en fumadors (que és la troballa més consistent en tots els estudis de funcionalisme mitocondrial en relació al tabac), es desconeix. El monòxid de carboni podria ser una de les substàncies implicades en aquesta inhibició.

3

"Reversible inhibition of mitochondrial complex IV activity in peripheral blood mononuclear cells following acute smoking"

Inhibició reversible de l'activitat del complex IV mitocondrial de mononuclears de sang perifèrica deguda a fumar de forma aguda

Jose-Ramon ALONSO, Francesc CARDELLACH, Jordi CASADEMONT, Óscar MIRÓ.

European Respiratory Journal 2003; en premsa.

EUROPEAN RESPIRATORY SOCIETY
September 22, 2003

Ref: ERJ-00382-2003.R2

Dear Dr. Alonso:

We are pleased to inform you that your manuscript entitled "Reversible inhibition of mitochondrial complex IV activity following acute smoking" has been accepted for publication in the European Respiratory Journal. To avoid another revision, we have slightly modified the title of the manuscript so as to reflect the findings described in the text.

Old title: Reversible inhibition of mitochondrial complex IV activity following acute smoking.

New title: Reversible inhibition of mitochondrial complex IV activity in peripheral blood mononuclear cells following acute smoking.

The manuscript has been sent to our Publications Office in Sheffield, UK. Please address all queries about your manuscript to the Sheffield Office:

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146 West Street
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Yours sincerely,

Dr. Irfan Rahman
European Respiratory Journal

Prof P.J. Sterk and Prof. K.F. Rabe
Chief Editors

Eur Respir J 2003; 00: 000-000.

Reversible inhibition of mitochondrial complex iv activity in peripheral blood mononuclear cells following acute smoking

Jose-Ramon ALONSO, Francesc CARDELLACH, Jordi CASADEMONT, Óscar MIRÓ.

Mitochondrial Research Laboratory, Department of Internal Medicine, Hospital Clínic, IDIBAPS. University of Barcelona, Barcelona, Catalonia, Spain.

Smoking causes a decrease of mitochondrial complex IV activity in chronic smokers. However, it is not known if this toxic effect is due to the acute effect of cigarette smoke itself or it is a secondary phenomenon related to other smoking factors. We assessed mitochondrial respiratory chain function in peripheral blood mononuclear cells of 15 healthy non-smoker individuals before smoking (t_0), immediately after smoking 5 cigarettes in 45 minutes (t_1) and 24 hours later (t_2). Blood carboxyhemoglobin (COHb) and carbon monoxide concentrations in exhaled air (COEA) were determined to ascertain smoke inhalation status. After acute smoking, COHb raised from 0.5±0.3% to 3.3±1.5%, and COEA from 2.9±2.5ppm to 26.1±9.9ppm. Complex II and III enzyme activities did not change along the study. Complex IV activity showed a 23% inhibition at t_1 but it returned to initial (t_0) levels at t_2. A decay in oxygen consumption was observed after the correction for mitochondrial content. Lipid peroxidation of cell membranes remained unchanged. Short time smoking causes an acute and reversible mitochondrial complex IV inhibition in human mononuclear cells. These results suggest that smoke itself is one of the causes for the decrease of complex IV activity observed in chronic smokers. Possible clinical relevance is discussed.

Key words: smoking, carbon monoxide toxicity, mitochondrial diseases.
Grants: Supported by PGC PM99-0038 and Premi Fi de Residència 1999 of Hospital Clínic i Provincial de Barcelona.

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INTRODUCTION

Tobacco consumption causes damage in lungs and many other tissues and organs. Its devastating consequences are mediated by some of the more than 4000 compounds contained in tobacco smoke (ref.1). Some of these compounds constitute themselves a major exogenous source of reactive oxygen species (ROS), as well as exhibit a capacity for increasing endogenous ROS production via activation of inflammatory-immune processes (ref.2). The major consequence for cell biology of the increase in ROS content is to enhance oxidative damage of some biological molecules of crucial relevance for cellular functions such as DNA, proteins and lipids which leads to a deterioration or loss of their physicochemical properties and functions (ref.3). Increasing attention has been paid in the recognition of relevance that such an oxidative damage could play in tobacco-mediated diseases, since greater lipid peroxidation (ref.4), DNA oxidation and DNA repair activity (ref.5,6,7) have been demonstrated in smokers compared to non-smokers (ref.8).

Mitochondria is one of the most important endogenous sources of ROS, since side reactions of the mitochondrial respiratory chain (MRC) with molecular oxygen directly generate such toxic oxygen metabolites (ref.9). Some of the chemicals contained in tobacco smoke have been suggested to interfere with different compounds of the MRC electron transport (ref.10) which could result in an increased production of ROS. However, until to date there are only few studies on the relationship between tobacco consumption and mitochondrial activity in humans.

We have recently demonstrated a decrease in mitochondrial cytochrome *c* oxidase activity in peripheral blood mononuclear cells (PBMC) from chronic smokers associated with an increased membrane lipid peroxidation (ref.11). However, it is not known if such decrease in cytochrome *c* oxidase activity is due to the effects of tobacco smoke itself or, alternatively,

it is a collateral event associated to damage in other organs induced by the smoking habit. In order to progress in this question, in the present study we planned to investigate the acute effects of smoking in MRC from human PBMC. If acute smoking has effects on MRC function, it would implicate that such mitochondrial dysfunction could be of pathophysiological relevance in some smoking-related diseases.

INDIVIDUALS AND METHODS:

Individuals selection

We selected 20 non-smoker young healthy individuals with similar physical activity (ref.12,13) from an advertisement in the University Campus. All of them gave their informed written consent to be included in the study. Ethical Committee of our Hospital approved present protocol.

Proceedings

The 20 selected individuals were allocated in a diet without excess of antioxidants for 2 weeks (ref.14,15). Sincerity about non-smoker status was ascertained by means of two non-programmed determinations of carboxyhemoglobin (COHb) levels (Blood gas Analyzer CIBA-CORNING 800 System, Switzerland) and carbon monoxide in exhaled air (COEA) levels by means of an electrochemical transducer (Dräger Pac III, Dräger Safety Inc, Pittsburgh, PA, USA). All of them had COHb levels under 2% and COEA under 6 ppm. After this period, they smoked 5 filtered cigarettes (nicotine 0.8mg, tar 11mg) in 45 minutes. We excluded 5 individuals who did not increase by 3 times their basal levels of COHb at the end of the smoking.

In all 15 remaining subjects (25±4 years; 9 men) we determined COEA and we obtained 30mL of peripheral blood before smoking (t_0), one minute after the last cigarette (t_1) and 24 hours later (t_2). Three mL of each sample were used to measure COHb levels. PBMC (lymphocytes and monocytes) were isolated by means of Ficoll's gradient. The final protein concentration was quantified according to Bradford's method (ref.16).

Mitochondrial assays

We choosed PBMC for mitochondrial assays because it is a standard procedure for the investigation of respiratory chain disorders in humans (ref.17), it is a minimal invasive test for volonteers, and eventually we could try to establish a relationship with COHb levels either COEA levels if any.

Enzyme activities: Measurement of enzyme activity of individual complexes of the MRC was performed spectrophotometrically (UVIKON 920, Kontron®, Switzerland). We determined complex II, III, and IV activities following Rustin et al. methodology (ref.17), slightly modified for complex IV measurement (ref.18). Complex I and V cannot be currently measured with safety in PBMC (ref.17).

Oxidative activity: Oxygen utilization was measured polarographically in 250 µl of standard medium (pH 7.4) with a Clark electrode in a water-jacketed cell at 37ºC (Hansatech Instruments Limited®, Norfolk, England) according to Rustin et al. procedure (ref.17).

Correction by mitochondrial content: Relative activities were figured out dividing absolute enzyme and oxidative activities by citrate synthase activity (ref.19,20), which was assessed spectrophotometrically (ref.17).

Peroxidation of lipid membranes.

Loss of cis-parinaric acid fluorescence was used to measure lipid peroxidation (ref.21). We placed 100µg of PBMC protein into 3mL of phosphate buffered serum (PBS) containing cis-parinaric acid (5mM) (Molecular Probes®, Eugene, OR, USA). Loss of fluorescence was measured with a fluorescence spectrophotometer (Hitachi F-2000, Japan) in the dark at 37ºC for 30 minutes at regular intervals of 3 minutes.

Statistical analysis

Data were processed using a SPSS software. Mean ± standard deviation and percentages were used for quantitative and qualitative variables, respectively. Comparisons between determinations at t_0, t_1 and t_2 were carried out using a t-test for paired data after assessing the normality of the distribution (Kolmogorov-Smirnov's test) and the equality of variances (Levene's test). For comparison of *cis*-parinaric fluorescence curves at t_0, t_1 and t_2, we used an ANOVA model for repeated measures to search for intergroup differences along time. Values of $p<0.05$ were considered statistically significant.

RESULTS

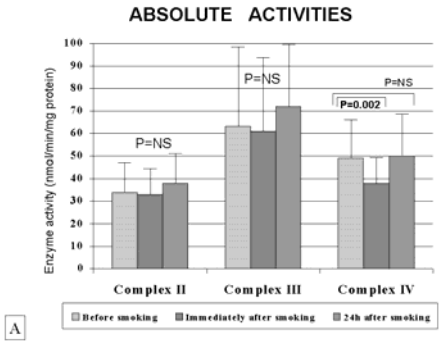
COHb levels of participants increased from 0.5±0.3% at basal conditions (t_0) to 3.3±1.5% immediately after smoking (t_1) ($p<0.001$). An identical pattern was recorded for COEA, which raised from 2.9±2.5 ppm (t_0) to 26.1±9.9 ppm (t_1) ($p<0.001$). Accordingly to these findings, cigarette smoke inhalation was considered as effective. Twenty-four hours after the last cigarette (t_2), both markers of tobacco smoke exposition returned to initial (t_0) values (0.6±0.3% for COHb and 3.9±2.3 ppm for COEA; p=NS respect to t_0 levels for both).

PBMC and mitochondria content did not differ along the times of study among the different

samples. PBMC content (mg of protein/ml) was 15.3 ± 3.6 at t_0, 13.98 ± 4.21 at t_1 and 14.2 ± 5.0 at t_2 (p=NS), and cytrate syntase activity (nmol/min/mg of PBMC protein) was 143.4 ± 35.8 at t_0, 145.3 ± 45.3 at t_1 and 134.9 ± 36.7 at t_2 (p=NS).

Effects of smoking on MRC are showed in figures 1 and 2. Smoking caused an immediate decrease of complex II, III and IV enzyme activities, but such decrease was statistically significative only for complex IV (cytochrome *c* oxidase), which went down from 49.4±17.1 nmol/min/mg protein at t_0 to 38.1±11.7 at t_1 (p=0.002), and returned to basal levels 24 hours later (50±19.3 nmol/min/mg protein at t_2) (Fig.1A).

After the correction of absolute enzyme rates by citrate synthase activity in order to obtain their relative activities, again only cytochrome *c* oxidase activity showed a same pattern of decrease associated with acute smoking, which went down from 34.7±8.4% at t_0 to 25.7±7.1% at t_1 (p<0.001) (Fig.1B).



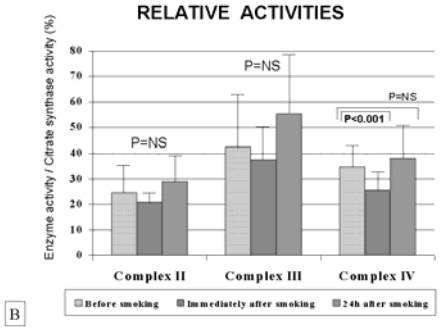
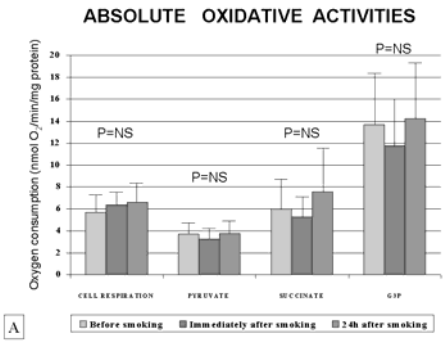


Figure 1. Absolute (A) and relative (B) enzyme activities

Study of oxygen consumption by PBMC showed no changes in spontaneous cell respiration or substrate oxidation along the time when absolute activities were considered (Fig. 2A). However, according to relative oxidative activities (Fig. 2B), an overall decrease of oxygen consumption after smoking (t_1) was observed with all substrates, when compared to t_0 values, being significant with pyruvate (p=0.01) and glycerol-3phosphate (p<0.01). In spite of this decrease of oxygen consumption, we observed a tendency to enhance spontaneous cell oxidation rate (Fig. 2B), that became significative at t_2.



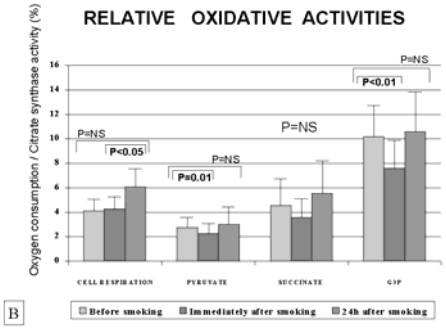


Figure 2. Absolute (A) and relative (B) oxidation rates

Finally, the study of lipid peroxidation of peripheral mononuclear cell membranes showed the same curves of loss of fluorescence of *cis*-parinaric acid before smoking, immediately after smoking, and 24 hours after smoking (p=NS) (Fig. 3).

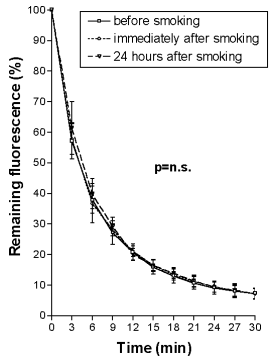


Figure 3. Curves of loss of fluorescence of *cis*-parinaric acid.

DISCUSSION

Present study relies on the effects of acute smoking on MRC components of PBMC of healthy, non-smoker people. Volonteers were healthy people with a similar physical activity (ref.12,13), and we reasonably excluded any illness by means of medical history, physical examination and routine blood analyses. Smoke inhalation was clearly effective in the studied individuals, as judged by the increase of COHb and COEA levels following smoking (ref.22,23,24). Then, we assumed that the individuals had absorbed enough CO to detect any potential change in the studied parameters. On these experimental premises, we observed a significant inhibition in absolute (23%) and relative (30%) cytochrome *c* oxidase activity. With regard to relative oxidative activities, oxygen consumption with pyruvate and glycerol-3-phosphate substrates, showed a significant inhibition according to citrate synthase activity. All these mitochondrial changes disappeared after 24 hours of smoking abstinence, when blood COHb and COEA levels had also returned to basal values. These findings can not be explained by any variability in mitochondrial yield of the samples at different times of the study, since citrate synthase activity was similar in all of them. Therefore, the observed effects should arise from direct and/or indirect actions of one or more of the components of tobacco smoke.

Gvozdjáková et al. (ref.25,26) have demonstrated a decrease in cytochrome *c* oxidase activity of heart muscle mitochondria from rabbits inhaling cigarette smoke; they also noted that this decrease was higher in proportion to the length of the smoke exposure. In humans, Örlander et al. (ref.27,28) found a decrease in cytochrome *c* oxidase activity from skeletal muscle mitochondria from chronic smokers, but they do not give any information about MRC function after smoking cessation. More recently, we have reported that tobacco is a confounding factor in studies concerning MRC function (ref.12) and we have also demonstrated a 23% of inhibition of cytochrome *c* oxidase activity in PBMC of heavy smokers with an average of COHb of 4.8% (ref.24). Therefore, our data in non-smokers add further evidence to all those studies and clearly indicate that smoking itself causes an immediate and transient alteration of cytochrome *c* oxidase activity.

Our overall results have shown a 23% of inhibition cytochrome *c* oxidase together with a significant decrease in oxygen consumption of MRC for pyruvate and glycerol-3-phosphate substrates and non-significant for succinate substrate, as well as significant enhanced spontaneous respiratory activity in intact PBMC after 24 hours of smoking cessation. This increase in cell respiration was an unforeseen finding because it accounted 24 hours after smoke exposure, when the rest of MRC parameters were already normal. Hipothetical steps at which such an up-regulation could account include changes in non-mitochondrial oxidation, ATPase activity and/or mitochondrial proton leak (ref.24). In any case, those results suggest that such a degree of cytochrome *c* oxidase inhibition in short time smoke inhalation is high enough to cause a disturbance of mitochondrial oxidative processes and a measurable but reversible decay in mitochondria energy production.

Although pathological effects after chronic exposure to some chemical agents are not always mediated through the same cellular and molecular pathways as the acute reaction, in our acute model of smoking we have found similar MRC inhibition than we did in chronic smokers (ref.24). However, in our opinion the ultimate toxic effect of smoking is more severe in chronic exposure since an enhanecd lipid peroxidation was found in heavy smokers (ref.24) but not after acute smoking, indicating a ultimately failure in adaptative processess in the former. So, the effect of cytochrome *c* oxidase inhibition on cell function *"in vivo"* in long term smoking habit, together with other many disturbing tobacco-related factors, such as hypoxia, vitamin and antioxidant deficiencies, cyanide and other toxic substances, could contribute to a overall decrease of oxidative capacity, resulting in high free-radical production, mitochondrial DNA damage and cell death or proliferation (ref.29,30,31). All these events would be specially harmful in target organs and tissues, like lungs and bronchial epithelium.

The specific relevance of this cytochrome *c* oxidase inhibition in whole cell metabolism remains unclear, since normally cells only use 10-20% of the V_{max} of the enzyme, and therefore to inhibit cellular respiratory rates one would need a greater inhibition than observed. Although this MRC dysfunction can not explain by itself the pathophysiology of smoking consequences, we believe that chronic and persistent inhibition should be considered as another factor to cellular injury, specially in cells already damaged by other physiopathological mechanisms of cigarette smoke. Additionally, effects of tobacco smoke (either acute of chronic) are not exerted equally on all tissues. For example, respiratory tract epithelium and lungs have a higher exposure to physical and chemical effects of tobacco compounds, while consequences in other organs

are rather related to their energy dependence status. Thus, in respiratory tract epithelium and lungs, a higher enzyme activity inhibition could lead to a higher mitochondrial dysfunction that could contribute to less removal of bronchial secretions (because bronchial epithelium ciliar missfunction), less response to infections (because macrophage missfunction), and carcinogenesis (ref.32,33). Moreover, in high energy dependent tissues, such as brain, heart and muscle, an abnormal cytochrome *c* oxidase activity could contribute to the damage associated with tobacco consumption via a chronic decrease on whole mitochondria oxidative capacity (ref.34,35,36,37,27,28). Finally, as seen in many hypoxic circumstances (ref.38), arteriopathy and chronic obstructive pulmonary disease could also contribute to mitochondrial injury.

Some authors have suggested that CO could be responsible for the decrease of cytochrome *c* oxidase activity (ref.27). This hypothesis is supported by our findings in patients suffering from acute, pure CO poisoning (COHb levels around 20%), in whom we demonstrated a severe and persistent inhibition of cytochrome *c* oxidase activity which ranged from 50 to 90%. This has been recently confirmed in *in vitro* conditions in our laboratory (ref.39). The binding of CO to cytochrome aa_3, a component of cytochrome *c* oxidase, is the responsible for its deletereous effect (ref.40,41,42) on this MRC enzyme (ref.11). Since acute smoking could be considered some kind of acute CO poisoning, although not pure (other components of cigarette smoke, such as cyanide (ref.43) could also contribute to cytochrome *c* oxidase inhibition) and of a lower intensity, we believe that most considerations generally accepted for true CO poisoning could be also argued for some of the disturbances that people with chronic smoking habbit will develope.

In conclusion, the decrease of mitochondrial complex IV activity that we have found in PBMC from acute smokers suggest that smoke itself is one of the causes of some enzyme inhibition observed in chronic smokers. However, the ultimate consequences of such long-term enzyme inhibition in tobacco-related diseases of lung and other tissues in chronic smokers remain to be established.

ACKNOWLEDGEMENTS

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SÍNTESI DELS RESULTATS

1.- El fet de fumar de forma aguda en persones no fumadores, provoca de forma immediata una disminució en l'activitat enzimàtica dels complexos II, III i IV en limfòcits circulants, que només resulta significativa pel complex IV.

2.- Aquesta disminució d'activitat enzimàtica del complex IV desapareix a les 24 hores d'haver estat exposat al fum del tabac, coincidint amb la normalització dels paràmetres que indiquen exposició al fum de tabac.

3.- A més, es troba una disminució del consum d'oxigen dels limfòcits per a alguns substrats, si analitzem els valors corregits pel contingut mitocondrial.

4.- Malgrat la disminució del consum d'oxigen, s'observa una tendència a augmentar l'activitat oxidativa cel·lular espontània que arriba a fer-se significativa a les 24h d'haver estat exposat al fum del tabac.

5.- La peroxidació lipídica de les membranes limfocitàries, que seria conseqüència de la producció excessiva de radicals lliures que provoquen el dany oxidatiu, és similar en tots els moments de l'estudi. No s'ha pogut doncs demostrar en el nostre grup de voluntaris no fumadors un augment del dany oxidatiu a diferència dels fumadors crònics.

6.- El CO del fum del tabac podria ser un dels responsables de la inhibició de l'activitat del complex IV, donada la seva afinitat pel citocrom aa_3 (component del complex IV) i les similituds observades en quant a funcionalisme mitocondrial en pacients intoxicats per monòxid de carboni. No obstant, altres components del fum del tabac com ara l'àcid cianhídric també hi podrien col·laborar en la producció d'aquests efectes.

7.- Podem concloure per tant, que el fum del tabac per se és probablement causant de les alteracions descrites en el funcionalisme de la cadena respiratòria mitocondrial dels fumadors. D'aquesta manera, podem descartar raonablement que les troballes en fumadors crònics fossin secundàries a la hipòxia crònica o a altres alteracions coexistents que poguessin presentar.

4

"Análisis *ex-vivo* de la función mitocondrial en pacientes intoxicados por monóxido de carbono atendidos en urgencias"

Anàlisi ex–vivo de la funció mitocondrial en pacients intoxicats per monòxid de carboni atesos a urgències

Óscar MIRÓ, **Jose-Ramon ALONSO**, Sònia LÓPEZ, Anna BEATO, Jordi CASADEMONT, Francesc CARDELLACH.

Medicina Clínica (Barcelona) 2003; en premsa.

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ANÁLISIS *EX VIVO* DE LA FUNCIÓN MITOCONDRIAL EN PACIENTES INTOXICADOS POR MONÓXIDO DE CARBONO ATENDIDOS EN URGENCIAS

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Fundamento: Diversos estudios experimentales en animales han demostrado que el monóxido de carbono (CO) tiene capacidad para unirse al complejo IV de la cadena respiratoria mitocondrial (CRM) y distorsionar el funcionalismo mitocondrial, Sin embargo, se desconoce si esta circunstancia se produce en la práctica clínica diaria de los pacientes que acuden a urgencias tras haber sufrido una intoxicación aguda por CO. Objetivo: Evaluar desde diferentes perspectivas si existe algún tipo de disfunción mitocondrial en los pacientes que han sufrido una intoxicación aguda por CO. Pacientes y métodos: Se incluyeron 10 pacientes que acudieron a urgencias tras sufrir una intoxicación aguda por CO (carboxihemoglobina inicial 20,4±6,0%). Siete de ellos recibieron tratamiento con oxigenoterapia hiperbárica. A todos ellos se les extrajo 20 mL de sangre durante la fase aguda de la intoxicación (t_0), y a los 3-5 días (t_1) y 10-14 días (t_2) del evento agudo. A partir de estas muestras se aislaron linfocitos para realizar los estudios mitocondriales, que consistieron en la determinación del contenido mitocondrial a través de la actividad de la citrato sintasa (nmol/min/mg proteina), la actividad enzimática (nmol/min/mg proteina) de los complejos III y IV de la CRM (ambos contienen citocromos), la actividad oxidativa (nmol oxígeno consumido/min/mg proteina)espontánea y estimulada mediante la administración de piruvato y succinato, y la cuantificación de la peroxidación lipídica utilizando ácido cis-parinárico. Estos parámetros se cuantificaron tanto por célula (valores absolutos) como por mitocondria (valores relativos). Los resultados se compararon con los valores control históricos de nuestro laboratorio procedentes de 130 individuos. Resultados: Durante la fase aguda de la intoxicación (t_0), no se observaron cambios en el contenido mitocondrial de los pacientes con respecto al grupo control, pero sí una inhibición significativa de la actividad enzimática de los complejos III y IV de la CRM asociada a un descenso de todas las actividades oxidativas, tanto si la estimación se hacía por célula como si se hacía por organela (mitocondria). Aunque todas estas actividades se recuperaron a lo largo del tiempo (t_1 y t_2), solo en el caso del complejo IV y de la actividad oxidativa estimulada por piruvato dicha recuperación resultó estadísticamente significativa. No obstante, ni siquiera en estos casos las actividades finales alcanzaron los valores control. Aunque se observó una tendencia a incrementarse la peroxidación lipídica, este aumento no alcanzó significación estadística. Conclusiones: En el presente estudio confirmamos *ex vivo* la existencia de una inhibición de la actividad de la CRM en pacientes intoxicados por CO que consultan a un servicio de urgencias. Dicha disfunción continúa siendo detectable transcurridos 14 días del evento agudo. Esta inhibición del funcionalismo mitocondrial podría desempeñar algún papel patogénico en los síntomas y signos tardíos que en ocasiones presentan estos enfermos.

TITLE: *EX VIVO* ANALYSIS OF MITOCHONDRIAL FUNCTION IN PATIENTS ATENDED IN AN EMERGENCY DEPARTMENT DUE TO CARBON MONOXIDE POSONING

Background: Many experimental studies in animals have demonstrated that carbon monoxide (CM) has the ability to bind to complex IV of the mitochondrial respiratory chain (MRC) inhibiting its function, It is unknown, however, if such circumstance is also present in patients that are admitted to an emergency department because an acute CM poisoning. Objetive: To evaluate from different points of view if mitochondrial function is abnormal in patients admitted because of an acute CM poisoning. Patients and methods: Ten patients with an acute CM poisoning admitted in an emergency department were included in the study. Initial carboxyhaemoglobin was 20,4±6,0%. Seven received hyperbaric-oxygen therapy. From all them, lymphocytes from 20 mL of blood were obtained at admission (t_0), and on days 3-5 (t_1), and 10-14 (t_2). Mitochondrial content was estimated through citrate synthase activity (nmol/min/mg protein). Enzymatic activity of complexes III and IV (both containing cytochromes) were measured in nmol/min/mg protein, and oxidative activity in intact cells as well as following addition of pyruvate an succinate, in nmol of consumed oxygen/min/mg protein. Lipid peroxidation was ascertained by means of cis-parinaric fluorescence. All values were given in absolute values, and corrected by mitochondrial content (relative values). Results were compared with figures obtained from 130 historical normal individuals. Results: During acute poisoning (t_0), there were no changes in mitochondrial content. On the contrary, there was a significant inhibition in enzymatic activity of complexes III and VI, and a decrease in all oxidative activities, both considering absolute or relative values. Although all the activities tended to recuperation with time (t_1 y t_2), statistical significance was only obtained for complex IV when stimulated with pyruvate. However, in any case the final activities reached normal control values. Although lipid peroxidation tended to increase, statistical significance was not obtained. Conclusions: In the present study we confirm that an inhibition of MRC can be demonstrated *ex vivo* in patients attended in an emergency department due to acute CM poisoning. The inhibition is still present 14 days after the acute event. Such mitochondrial dysfunction may play a pathogenic role in persisting or delayed sings and symptoms that occasionally these patients refer.

Key words: Carbon monoxide, intoxication, mitochondria, respiratory chain, emergency.

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Introducción

Se calcula que la intoxicación aguda por monóxido de carbono (CO) tiene una incidencia en los países desarrollados de 17,5 casos por cada 100.000 habitantes y es aún hoy en día un diagnóstico poco reconocido e infradiagnosticado en los servicios de urgencias[1]. Esto es así a pesar que la intoxicación por CO continúa siendo en muchos de estos países, incluyendo España, la primera causa de muerte por intoxicación[2-4]. En los casos fatales, la muerte sobreviene por la hipoxia tisular que produce la unión reversible y competitiva del CO al grupo heme de la hemoglobina, desplazando de él al oxígeno. Así, cuando lo habitual es que los valores de carboxihemoglobina (COHb) se sitúen por debajo del 3% en personas no fumadoras, cuando éstos alcanzan el 50% se considera que la muerte se produce de manera prácticamente indefectible[5]. En cambio, cuando el grado de intoxicación por CO no es de una magnitud suficiente para causar el fallecimiento del individuo, la unión del CO a la hemoglobina es revertida con el paso de las horas (el tiempo de vida media de dicha unión se sitúa entre 5 y 6 horas). La rapidez de este proceso de reversibilidad puede verse acelerado por la administración de oxígeno a una concentración del 100% y/o a una presión superior a la atmosférica, las cuales son las principales medidas terapéuticas a administrar en aquellos casos atendidos en urgencias[6].

Sin embargo, y a pesar que la mayoría de los pacientes que han sufrido una intoxicación aguda no mortal por CO pueden ser dados de alta de los servicios de urgencias a las pocas horas, una proporción de ellos puede desarrollar secuelas neurológicas, especialmente cognitivas que, o bien están presentes ya en ese momento, o bien aparecen o se intensifican durante los días o semanas posteriores a la intoxicación. Aunque con menor frecuencia, esto se observa incluso en pacientes que han sufrido una intoxicación moderada por CO (niveles de COHb entre el 20 y el 30%) y es por ello que recientemente se ha defendido la utilización de la oxigenoterapia hiperbárica en ellos en aras a prevenir el desarrollo de este síndrome neurológico tardío[7].

La explicación fisiopatológica de este síndrome aún no es bien conocida. Entre los mecanismos que se cree que pudieran jugar algún papel se encuentra la citotoxicidad causada por la unión del CO a otras moléculas del organismo que, aparte de la hemoglobina, contienen el grupo heme. Entre ellos, se encuentran la mioglobina y los citocromos y, entre estos últimos, el citocromo bc_1 y el citocromo aa_3, que forman parte de algunos de los complejos proteicos que integran la cadena respiratoria mitocondrial (CRM, figura 1). Esta CRM es la encargada del trasporte de electrones de elevado poder reductor, generados en diferentes vías catabólicas, con la finalidad última de aprovechar la energía de este transporte altamente endergónico para la síntesis de ATP. Es fácil intuir que la interrupción del normal funcionamiento de esta CRM por la acción del CO pudiera conllevar a un déficit energético celular y a un incremento de las reacciones oxidativas intracelulares y contribuir, en última instancia, a la disfunción tisular y a la aparición de síntomas y signos.

No obstante, debido a la inaccesibilidad del tejido diana (sistema nervioso central, SNC) para realizar estudios mitocondriales en los individuos intoxicados por CO, esta hipótesis sólo ha podido ser contrastada en modelos experimentales animales. De los resultados de estos estudios se desprende que la hipótesis del daño mitocondrial como mecanismo efector para la aparición de sígnos y síntomas tardíos tras la intoxicación por CO es una opción muy plausible[8-12]. Durante los últimos años nuestro grupo ha iniciado una serie de aproximaciones para intentar contrastar esta teoría en humanos. Para ello, se ha estudiado el funcionalismo mitocondrial de linfocitos de sangre periférica de pacientes intoxicados de forma aguda por CO y que han acudido al servicio de urgencias. Dado que los resultados preliminares han mostrado que los linfocitos de sangre periférica pueden constituir un buen modelo para el análisis de los efectos toxicológicos del CO[13,14], se diseñó el presente estudio con la finalidad de realizar una valoración integral de los efectos que produce el CO sobre la CRM en la práctica clínica no experimental. La hipótesis del mismo es que en pacientes intoxicados de forma aguda por CO es posible detectar una inhibición de la actividad de aquellos complejos de la CRM que contienen citocromos y que esta inhibición tiene repercusión en el funcionalismo global de dicha CRM e incluso llegar a causar un incremento del daño oxidativo.

Pacientes y Métodos

Pacientes:

Se incluyeron en el estudio 10 pacientes que acudieron al servicio de urgencias con sintomatología derivada de la exposición aguda al CO y cuyos valores de COHb fueran superiores al 10%. Se excluyeron aquellos que padecían otras enfermedades o que estuviesen recibiendo fármacos. Las características clínicas de los pacientes se recogen en la tabla 1. En todos los casos la exposición a CO podía

considerarse "pura", ya que la fuente de exposición había sido sistemas de calefacción o calentadores con combustión incompleta por una ventilación insuficiente. En siete de éstos existían criterios suficientes para indicar oxigenoterápia hiperbárica para completar el tratamiento y así se hizo. A todos estos pacientes se les extrajo, previamente a recibir tratamiento, 20 mililitros de sangre venosa para la realización de los estudios mitocondriales que se detallan más adelante. El protocolo fue aprobado por el Comité Ético de Investigación de nuestro centro, y se obtuvo el consentimiento informado de los pacientes incluidos.

	Intoxicados por CO (n=10)	Controles (n=130)
Edad (años)	39±19	37±12
Sexo (mujer)	40%	35%
Fumador habitual	40%	44%
Valores de carboxihemoglobina		
-t_0	20,4±6,0	2,2±2,5*
-t_1	1,7±0,9	N.P.
-t_2	1,6±1,2	N.P.
Síntomas agudos		
-Disnea	40%	N.P.
-Náusea	60%	N.P.
-Cefalea	100%	N.P.
-Torpor mental	80%	N.P.
-Debilidad	70%	N.P.
-Inestabilidad	30%	N.P.
-Ataxia	20%	N.P.
Síntomas crónicos		
-Disnea	0%	N.P.
-Náusea	30%	N.P.
-Cefalea	30%	N.P.
-Torpor mental	10%	N.P.
-Debilidad	10%	N.P.
-Inestabilidad	0%	N.P.
-Ataxia	0%	N.P.
Tratamiento hiperbárico	70%	N.P.

N.P.: no procede.

*p<0,001

Tabla 1: Principales características clínicas de los pacientes incluidos en el estudio. CO: monóxido de carbono; t_0: intoxicación aguda; t_1: 3-5 días tras la intoxicación aguda; t_2: 10-14 días tras la intoxicación aguda.

Diseño:

El diseño del presente estudio contemplaba dos fases diferenciadas. En la primera, transversal, los resultados obtenidos en los pacientes en fase aguda de la intoxicación por CO (t_0) se compararon con un grupo control formado por 130 individuos sin patología conocida y que habían realizado una donación voluntaria de sangre, los cuales constituyen los valores de referencia históricos de nuestro laboratorio. La segunda fase del diseño, longitudinal, consistió en la repetición de los estudios mitocondriales a los pacientes intoxicados por CO al cabo de 3 a 5 días (t_1) y de 10 a 14 días (t_2) de haber sido tratados y dados de alta.

Aislamiento de linfocitos:

El aislamiento de linfocitos se realizó mediante centrifugación en un gradiente de densidad de Ficoll (Histopaque®-1077, Sigma Diagnostics). Los linfocitos así obtenidos fueron resuspendidos en 100-150 µL de solución salina tamponada con fosfato, y la concentración de células se estimó mediante la determinación del contenido proteico siguiendo el principio de unión colorante-proteína propuesto por Bradford[15].

Cálculo del contenido mitocondrial:

Se realizó mediante la determinación espectrofotométrica (UVIKON 922, Kontron®, Suiza) a 412 nm de la actividad de la citrato sintasa (EC: 4.1.3.7), que es una enzima del ciclo de Krebs que se encuentra en la matriz mitochondrial y cuya concentración permanece altamente constante en el interior de la mitocondria. Por dicho motivo, diversos autores consideran que la actividad de esta enzima es un buen marcador del contenido mitocondrial de una célula o tejido[16,17].

Determinación de las actividades enzimáticas:

Se determinaron mediante espectrofotometria la actividad de los complejos III (EC 1.10.2.2; el cual contiene citocromo bc_1) y el complejo IV (EC 1.9.3.1; el cual contiene citocromo aa_3) de la CRM. Los experimentos se realizaron a 37ºC siguiendo la metodología propuesta por el grupo de Rustin[17] ligeramente modificada para el complejo IV[18]. Los resultados obtenidos en cuanto a actividad absoluta (nmol/min/mg proteína) reflejan la actividad enzimática por célula. También se calcularon las actividades relativas de estos complejos dividiendo por la actividad de la citrato sintasa, las cuales son un reflejo de las actividades enzimáticas por organela (mitocondria).

Determinación de las actividades oxidativas:

La actividad oxidativa se determinó mediante polarografía (nmol oxígeno consumido / min / mg proteína) con un electrodo de Clark a 37ºC (Hansatech Instruments Limited®, Norfolk, Reino Unido). En condiciones experimentales previamente definidas[16,19] se midió el consumo de oxígeno (respiración espontánea) por parte de los linfocitos intactos, y posteriormente el consumo de oxígeno obtenido tras permeabilizar la membrana celular con digitonina al 1% y añadir glutamato-malato (substrato que cede electrones al complejo I) y succinato (substrato que cede electrones al complejo II). Mediante la utilización de glutamato-malato se valora el correcto transporte de electrones del complejo I a la coenzima Q, al complejo III, al citocromo c, al complejo IV y al agua; mientras que mediante el succinato se valora el corrector transporte de electrones del complejo II a la coenzima Q, al complejo III, al citocromo c, al complejo IV y al agua (ver figura 1). Al igual que para las actividades enzimáticas, los valores absolutos de actividad oxidativa fueron divididos también por la citrato sintasa para obtener los valores

relativos, ya que mientras los primeros expresan la actividad oxidativa por célula, los segundos la expresan por organela.

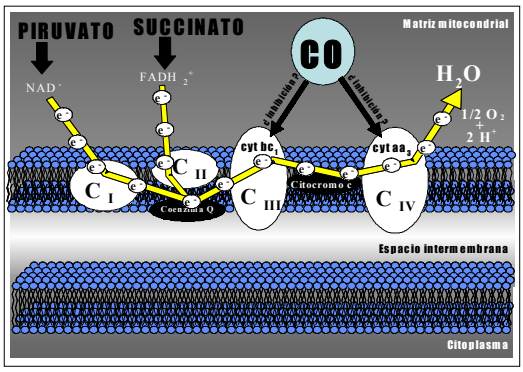

Figura 1: Esquema de la cadena respiratoria mitocondrial, la cual se encuentra integrada por cuatro complejos proteicos multienzimáticos (C_I: complejo I; C_{II}: complejo II; C_{III}: complejo III; C_{IV}: complejo IV) y dos transportadores móviles de electrones (coenzima Q y citocromo c). Piruvato y succinato son dos substratos que se utilizan en el estudio de la actividad oxidativa mitocondrial y que actuan como dadores de electrones en los puntos que muestra el esquema. El resultado final que se registra en los estudios polarográficos es el consumo de oxígeno asociado a la administración de los mencionados substratos. Las abreviaturas cyt bc_1 y cyt aa_3 corresponden a citocromo bc_1 y citocromo aa_3, respectivamente, y son los lugares donde potencialmente podría unirse el monóxido de carbono (CO).

Daño oxidativo:

Se valoró mediante la determinación del grado de peroxidación lipídica de las membranas linfocitarias. Para ello se utilizó el ácido cis-parinárico, el cual se une a los ácidos grasos haciéndose fluorescente. Esta fluorescencia se pierde cuando existe peroxidación de los ácidos grasos, de manera que una mayor pérdida de fluorescencia del mismo es sinónimo de una mayor peroxidación lipídica. Para la realización de los experimentos, se colocaron 100 µg de proteína linfocitaria en una cubeta con 3 mL de PBS nitrogenizado que contenía ácido cis-parinárico 5 µM (Molecular Probes®, Eugene, OR, USA), se incubó a 37ºC en oscuridad y posteriormente se determinó cada 3 minutos la fluorescencia de la muestra durante un total de 15 minutos con longitudes de onda de 318 nm de excitation y 410 nm de emisión[20,21].

Análisis estadístico:

Los datos cualitativos se expresan como porcentajes y los cuantitativos como media ± error estándar de la media. Para la comparación de las variables cualitativas se utilizó el modelo lineal general para muestras independientes (estudio transversal) o muestras repetidas (estudio longitudinal y estudio de la curvas de cis-parinárico). En todos los casos, se consideró que existían diferencias estadísticamente significativas cuando el valor de p fue inferior a 0.05.

Resultados

Durante la fase de intoxicación aguda por CO (t_0), la estimación del contenido mitocondrial a través de la determinación de la actividad de la citrato sintasa no mostró cambios respecto al contenido mitocondrial del grupo control (figura 2, superior). Sin embargo, la cuantificación de las actividades enzimáticas de los complejos III y IV de la CRM (los dos que contienen citocromos) mostró descensos significativos en ambos casos, siendo del 50% el grado de inhibición para el primero y del 65% para el segundo (figura 2, centro). Finalmente, respecto a la capacidad oxidativa mitocondrial medida en forma de consumo de oxígeno en condiciones óptimas, se pudo observar un descenso significativo de la misma con porcentajes de inhibición que oscilaban entre el 58% y el 64% según las condiciones del experimento (esto es, si se valoraba el consumo de oxígeno de manera espontánea o estimulado con la administración de piruvato o de succinato). Cuando estos resultados de actividades enzimáticas y oxidativas se expresaron no por célula sino por organela (dividiendo los valores absolutos por la actividad de la citrato sintasa) se observó igualmente un descenso en todos los parámetros mitocondriales evaluados con respecto al grupo control (figura 3). No obstante, los porcentajes de inhibición resultaron inferiores (oscilaron entre el 30% y el 65%) y, para el caso particular de la actividad oxidativa estimulada con succinato, dicha inhibición no alcanzó la significación estadística.

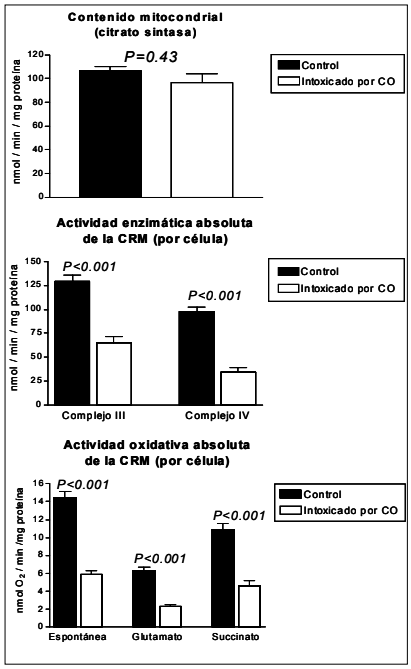

Figura 2: Resultados (media y error estándar de la media) obtenidos en los controles y en los pacientes en el momento agudo (t_0) de intoxicación por monóxido de carbono (CO) por lo que respecta a contenido mitocondrial (arriba), actividad enzimática (centro) y actividad oxidativa (abajo). Los resultados se expresan como actividades absolutas (por célula). CRM: cadena respiratoria mitocondrial.

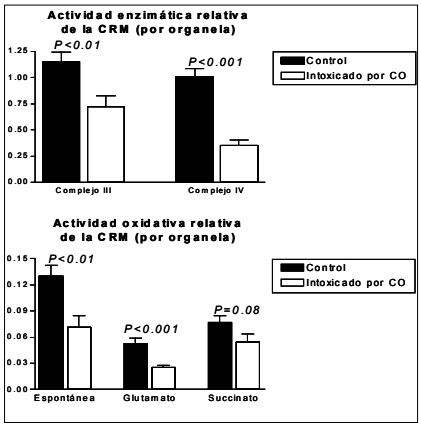

Figura 3: Resultados (media y error estándar de la media) obtenidos en los controles y en los pacientes en el momento agudo (t_0) de intoxicación por monóxido de carbono (CO) por lo que respecta a actividad enzimática (arriba) y actividad oxidativa (abajo). Los resultados se expresan como actividades relativas (por organela), las cuales se obtienen a partir de dividir la actividad absoluta por la actividad de la citrato sintasa (marcador de contenido mitocondrial). CRM: cadena respiratoria mitocondrial.

Las figuras 4 y 5 presentan la evolución de las actividades enzimáticas y oxidativas (respectivamente) de los pacientes intoxicados por CO durante los días posteriores a la intoxicación (t_1 y t_2), y cuando los niveles de COHb se habían normalizado. Aunque en todos los casos se observó una recuperación de la actividad, ésta recuperación sólo resultó significativa para el complejo IV y para la actividad oxidativa estimulada con piruvato, lo cual sucedía tanto si se valoraba a nivel celular como a nivel organular. A pesar de ello, prácticamente en ningún caso las actividades finales llegaron a alcanzar los valores del grupo control.

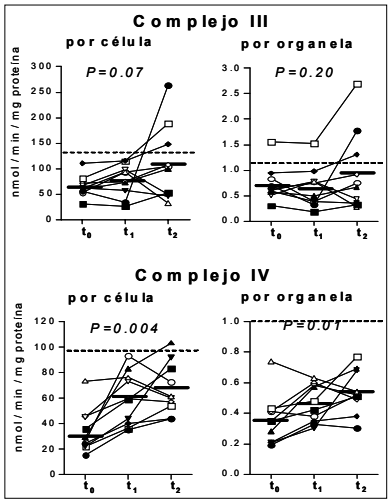

Figura 4: Evolución a lo largo del tiempo (t_0: intoxicación aguda; t_1: 3-5 días tras la intoxicación aguda; t_2: 10-14 días tras la intoxicación aguda) de las actividades enzimáticas de los pacientes intoxicados de forma aguda por CO, expresadas tanto por célula (valores absolutos) como por organela (valores relativos). Las líneas contínuas muestran las medias de los pacientes en los diferentes momentos del estudio, y la línea discontinua la media del grupo control.

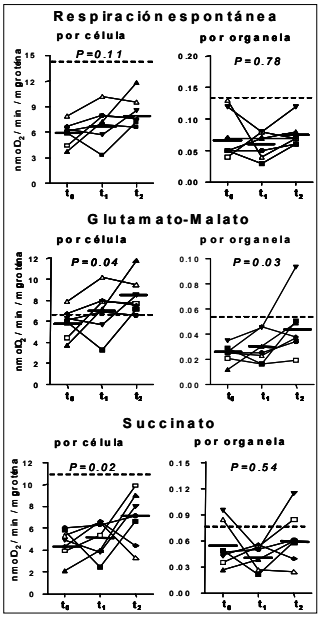

Figura 5: Evolución a lo largo del tiempo (t_0: intoxicación aguda; t_1: 3-5 días tras la intoxicación aguda; t_2: 10-14 días tras la intoxicación aguda) de las actividades oxidativas de los pacientes intoxicados de forma aguda por CO, expresadas tanto por célula (valores absolutos) como por organela (valores relativos). Las líneas contínuas muestran las medias de los pacientes en los diferentes momentos del estudio, y la línea discontinua la media del grupo control.

Respecto a la valoración del daño oxidativo, las curvas de fluorescencia del ácido cis-parinárico (figura 6) mostraron un incremento de la peroxidación de los lípidos de membrana (mayor pérdida de fluorescencia) tanto durante la fase aguda de la intoxicación por CO (t_0) como durante los días posteriores (t_1 y t_2), si bien ninguno de estos incrementos de peroxidación alcanzó una significación estadística.

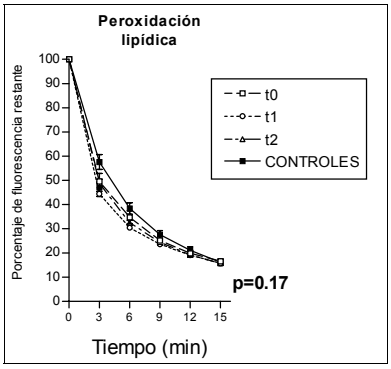

Figura 6: Curvas de fluorescencia (media y error estándar de la media) del ácido cis-parinárico obtenidas en el grupo control y en el grupo de pacientes intoxicados por monóxido de carbono (CO) en los diferentes momentos del estudio (t_0: intoxicación aguda; t_1: 3-5 días tras la intoxicación aguda; t_2: 10-14 días tras la intoxicación aguda). A mayor pérdida de fluorescencia, mayor peroxidación lipídica de las membranas.

Discusión

Los principales hallazgos del presente estudio de funcionalismo mitocondrial *ex vivo* en pacientes intoxicados de forma aguda por CO han sido la demostración que el CO causa una inhibición profunda y sostenida de los complejos III y IV de la CRM, y que dicha inhibición repercute en la capacidad oxidativa mitocondrial. Estas inhibiciones presentan una lenta recuperación durante los días posteriores a la intoxicación aguda, de manera que transcurridos 14 días del evento aún se detectan actividades disminuidas con respecto al grupo control. En conjunto, esta disfunción mitocondrial podría ayudar a explicar la persistencia de síntomas y signos tardíos tras una intoxicación aguda por CO a pesar que los niveles de COHb hayan sido completamente normalizados.

Si bien la alteración mitocondrial del complejo IV (citocromo c oxidasa) de la CRM ya había sido sospechada por el propio Haldane a comienzos de este siglo[22], no fue hasta 1939 cuando se comprobó por primera vez *in vitro* la unión del CO al citocromo a_3 de dicho complejo IV[23]. Sin embargo, tuvo que esperarse hasta la década de los 80 para que una serie de experimentos llevados a cabo por el grupo de Piantadosi y Brown constataran *in vivo* en modelos animales esta unión del CO al complejo IV[8-12]. Además, estos autores encontraron que el CO es capaz de inhibier el metabolismo cerebral aún después de haberse normalizado los niveles de COHb, e incluso aunque se administrase de forma concomitante oxigenoterapia hiperbárica[10]. En 1998 nuestro grupo presentó los primeros estudios *ex vivo* en humanos realizados en 3 pacientes que habían presentado una intoxicación por CO y que sugerían que esta inhibición del complejo IV de la CRM tenía relevancia clínica[13]. Los datos que aquí se presentan, obtenidos a partir de estudios en linfocitos de sangre periférica, confirman definitivamente esta hipótesis. Por otra parte, aunque es bien conocido que el complejo III de la CRM contiene citocromo bc_1, no hemos encontrado referencias en la bibliografía en las que se objetive que dicho complejo también resulte inhibido de forma significativa por el CO, por lo que éste es un aspecto en el que deberá profundizarse en un futuro.

Una cuestión interesante de nuestros resultados es por qué, a pesar de la utilización de terapia hiperbárica en la mayoría de casos (7 de 10), este tratamiento no consigue normalizar de forma absoluta las alteraciones mitocondriales detectadas. Una explicación podría ser que, a pesar de que la afinidad del CO y del oxígeno *in vitro* es menor para los citocromos que para la hemoglobina, existe una diferente capacidad *in vivo* del oxígeno para alcanzar estas moléculas. Además, el tratamiento hiperbárico persigue como objetivo normalizar las cifras de COHb (que además se utiliza como marcador del tratamiento) y es posible que, cuando este objetivo se alcanza, la unión del CO a moléculas tisulares (y entre ellas los citocromos) aún no haya sido completamente revertida. Los datos experimentales anteriormente comentados de la ineficacia del tratamiento hiperbárico para revertir el descenso del metabolismo cerebral causado por el CO[10] apoyarían esta hipótesis. Dado que el presente estudio tiene un número de casos limitado, no es posible establecer comparaciones entre los grupos tratados y no tratados con terapia hiperbárica y serán necesarios estudios posteriores que valoren si tratamientos más prolongados con terapia hiperbárica podrían ser capaces de modificar nuestros hallazgos. De hecho, recientemente se ha demostrado que las sesiones repetidas con oxigenoterapia hiperbárica reducen el riesgo de padecer sequelas cognitivas durante las semanas siguientes a una intoxicación aguda por CO[7].

En cualquier caso, lo que parece evidente a partir de nuestros experimentos es que la capacidad oxidativa mitocondrial resulta marcadamente afectada por la inhibición enzimática de los complejos III y IV. Cuando existe una inhibición de algún complejo de la CRM, el flujo de electrones a través de ella se ve enlentecido y decrece el consumo de oxígeno. Cuando el complejo afectado es el III o el IV (como sucede en nuestro caso) este descenso del consumo de oxígeno se hace evidente tanto si se mide la respiración celular espontánea como la estimulada mediante substratos que ceden electrones al complejo I (piruvato) o al complejo II (succinato) (ver figura 1). El resultado esperable ante un descenso de la actividad oxidativa es que parte del potencial reductor contenido en estos electrones se derive hacia la oxidación incompleta del oxígeno y la consiguiente formación de radicales libres que lesionarán moléculas biológicas. De hecho, ya en condiciones normales hasta un 5% del oxígeno consumido por la mitocondria se convierte en radicales oxidativos[24]. Sin embargo, este último extremo (el del incremento del daño oxidativo), no ha podido ser demostrado en el presente estudio, al menos por lo que se refiere a la peroxidación lipídica, ya que si bien se encontró una tendencia a estar incrementada, las diferencias con el grupo control no resultaron estadísticamente significativas. Es posible que el corto tiempo de exposición de los pacientes intoxicados por CO no sea suficiente para que se produzca el daño oxidativo y, en este sentido,

hemos podido comprobar como en personas que presentan de forma crónica valores elevados de COHb (fumadores crónicos) este incremento de la peroxidación lipídica sí que se produce[20].

Una hipótesis alternativa para explicar nuestros resultados es que la existencia de estas inhibiciones fuese debida, no a la unión del CO con los citocromos, sino una consecuencia directa de la hipoxia tisular sufrida durante el evento agudo secundaria a la unión del CO con la hemoglobina. Sin embargo, existen estudios experimentales que demuestran que la actividad del complejo IV de la CRM no se encuentra inhibida tras 72 horas de hipoxia tisular (conseguida a través de mecanismos distintos a la intoxicación por CO)[25]. Dado que todas las alteraciones encontradas en el presente estudio persistían en mayor o menor grado 14 días después de la intoxicación, creemos más plausible que sea la unión del CO a los grupos prostéticos de los citocromos contenidos en los complejos III y IV de la CRM la responsable última de la disfunción de la CRM mitocondrial detectada en el presente estudio[26].

Quisiéramos resaltar el hecho que los resultados que aquí se presentan han sido obtenidos a partir del estudio de linfocitos extraídos de sangre periférica. La mayoría de estos linfocitos circulan en sangre en su forma inactivada, por lo que su actividad metabólica es baja y las mitocondrias no están sometidas a una demanda de actividad. Además, su contenido en mitocondrias es reducido pues se estima en 15-20 organelas por célula[27]. Por todo ello, es posible que nuestros resultados subestimen lo que en realidad acontece en los tejidos diana, como es el SNC con un elevado nivel metabólico. En este sentido, dado que las neuronas son altamente dependientes del metabolismo celular aerobio y que son células postmitóticas en las que el daño mitocondrial resulta más difícil de eliminar, parece lógico pensar que la inhibición enzimática y oxidativa en estas células sea mayor y/o más mantenida que la que hemos observado en los linfocitos y que incluso en este tejido dicha inhibición pueda conducir a un incremento del daño oxidativo, hecho este último que no hemos podido constatar en los linfocitos.

Aunque el número de pacientes estudiados pueda parecer bajo, la homogeneidad de los mismos en cuanto a la fuente de exposición al CO, la extensa valoración mitocondrial que se ha realizado y la consistencia interna de los resultados obtenidos creemos que otorgan fiabilidad a nuestros resultados. Además, el hecho que con este número limitado ya se obtengan diferencias significativas habla en favor de la existencia de todas estas disfunciones que el presente estudio describe. Ante estos resultados, cabría plantearse la conveniencia de, además de la oxigenoterapia hiperbárica, administrar de forma concomitante cofactores y vitaminas que se han mostrado eficientes en algunos casos de enfermedades mitocondriales primarias[28] con la finalidad de minimizar, tanto cuantitativa como cualitativamente, el desarrollo de un síndrome neurológico tardío[14]. Sin duda, este será un campo de investigación a tener en cuenta durante los próximos años, y futuros trabajos utilizando modelos in vitro profundizarán a buen seguro en los mecanismos patogénicos de la intoxicación por CO. En cualquier caso, y como conclusión, creemos que nuestros datos demuestran claramente que la CRM es una diana relevante en pacientes intoxicados de forma aguda con CO y que, a través de este mecanismo, podrían explicarse algunos síntomas tardíos observados en estos pacientes.

Agradecimientos:

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SÍNTESI DELS RESULTATS

1.- Les activitats enzimàtiques dels complexos III i IV (ambdós contenen citocroms) resulten molt disminuïdes en els pacients intoxicats per monòxid de carboni, mesurades en limfòcits de sang perifèrica.

2.- Aquesta disminució d'activitat enzimàtica s'acompanya d'un descens de l'activitat oxidativa mitocondrial.

3.- Durant la seva evolució després de la teràpia indicada en cada cas, les activitats enzimàtiques i l'activitat oxidativa van anar millorant, però la recuperació només resulta significativa pel complex IV. Malgrat aquesta millora progressiva, en cap cas les activitats finals van arribar a les assolides pel grup control.

4.- En quant al dany oxidatiu, es pot observar un increment de la peroxidació dels lípids de membrana a la fase aguda i els dies següents, però aquesta troballa no arriba a tenir significació estadística.

5.- Podem concloure que el monòxid de carboni produeix una inhibició profunda i sostinguda dels complexos III i IV de la cadena respiratòria mitocondrial, i que aquesta inhibició repercuteix en la capacitat oxidativa mitocondrial. La disfunció mitocondrial millora progressivament però lentament després de l'intoxicació, independentment que els nivells de COHb hagin estat normalitzats prèviament.

6.- A través del mecanisme d'afectació del funcionalisme mitocondrial, es podrien explicar alguns símptomes tardans de la intoxicació per monòxid de carboni.

5

"Oxidative damage on lymphocyte membranes is increased in patients suffering from acute carbon monoxide poisoning"

El dany oxidatiu sobre membranes limfocitàries es troba augmentat en pacients intoxicats per monòxid de carboni

Óscar MIRÓ, **Jose-Ramon ALONSO**, Jordi CASADEMONT, Diana JARRETA, Alvaro URBANO-MARQUEZ, Francesc CARDELLACH.

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Oxidative damage on lymphocyte membranes is increased in patients suffering from acute carbon monoxide poisoning

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Abstract

Increased oxidative damage seems to be a relevant mechanism in the pathophysiology of patients with an acute carbon monoxide (CO) poisoning. We have investigated the degree of membrane oxidative damage through the assessment of lipid peroxidation in circulating lymphocytes from five patients acutely intoxicated by CO. Since mitochondria are a major source of reactive oxygen species and mitochondrial cytochrome *c* oxidase (COX) has been reported to be inhibited after acute CO poisoning, we have also assessed the lymphocyte COX activity and its relationship with the degree of lipid peroxidation. Data were compared with those from 32 non-smoker healthy controls comparable in terms of age, gender and physical activity. In intoxicated patients, we have found a significant increase of lipid peroxidation compared to control individuals ($P < 0.05$), as well as a marked COX inhibition ($P < 0.001$). Both parameters showed a positive, nearly significant correlation ($r = 0.81$, $P = 0.09$). We conclude that oxidative damage of lymphocyte membranes is increased after acute CO poisoning, and suggest that such increase could be partially mediated by mitochondrial COX inhibition caused by CO.

Keywords: Carbon monoxide; Lipid peroxidation; Oxidative stress; Oxidative damage; Mitochondria

1. Introduction

Toxic effects of carbon monoxide (CO) poisoning are mostly attributed to its high affinity for binding to hemoglobin, which causes a severe limitation in erythrocyte oxygen transport leading to a reduced tissue oxygen availability (Coburn, 1979). However, other mechanisms not related to tissue hypoxia have also been described as involved in the harmful effects of CO poisoning (Ernst and Zibrak, 1998), like the reoxygenation injury (Zhang and Piantadosi, 1992), the conversion of xanthine dehydrogenase to xanthine oxidase (Thom, 1992) and the nitric oxide-derived oxidants (Thom et al., 1997), all of them causing oxidative stress.

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In this sense, mitochondrial dysfunction could constitute an additional mechanism for explaining the physiopatology of CO intoxication. Indeed, cytochromes contained in complex III (succinate-coenzyme Q reductase) and complex IV (cytochrome *c* oxidase, COX) of mitochondrial respiratory chain (MRC) are other potential targets for CO, since they contain, like hemoglobin, heme groups. We have recently reported an inhibition of mitochondrial COX of lymphocytes from three patients with acute CO poisoning, and we found that the extent of this inhibition correlated with the severity of symptoms. Moreover, COX activity returned to its normal level much more slowly than did carboxyhemoglobin levels after appropriate treatment, and we suggested that MRC inhibition could be of crucial relevance in the persistence of some symptoms (Miró et al., 1998b). According to this hypothesis, an increase in oxidative damage should be expected to be present in patients suffering from an acute CO poisoning. Therefore, present work investigates the presence of oxidative damage on membranes from lymphocytes of patients after acute CO poisoning.

2. Patients and methods

2.1. Patient and control individual selection

We have studied five patients with acute CO poisoning, three of them from a poorly ventilated heating device, and the other two from fire smoke inhalation. All of them were otherwise healthy people until the time of CO poisoning. As controls, we used 32 non-smoker healthy people who were comparable with intoxicated patients in terms of age, gender and physical activity. All of them accepted to be enrolled in the study and signed informed consent.

2.2. Isolation of lymphocytes

In all cases, 20 ml of peripheral venous blood were obtained prior to the start of any therapy in order to determine the carboxyhemoglobin (COHb) level with a CO-oxymeter, and to isolate lymphocytes by means of a Ficoll gradient as explained elsewhere (Miró et al., 1998b). Lymphocytes were resuspended in 100 μl of phosphate buffered serum (PBS) and protein concentration was quantified following the principle of protein–dye binding proposed by Bradford (1976).

2.3. Assay of cytochrome c oxidase (EC 1.9.3.1) activity

It was performed spetrophotometrically following the methodology proposed by Rustin et al. (1994) slightly modified (Miró et al., 1998a). Experiments were carried out at 550 nm in a cuvette containing 1 ml of isoosmotic medium (phosphate buffer 10 mM plus sucrose 0.3 M and bovine serum albumine (BSA) 1 mg/ml, pH 6.5), 10 μg of lymphocyte protein and reduced cytochrome *c* 10 μM as electrons donor. The external membrane of mitochondria were permeabilized with laurylmaltoside 2.5 mM. Under these conditions, the oxidation rate of cytochrome *c* was then measured. This rate is the result of two processes: the specific enzymatic oxidation of cytochrome *c* catalyzed by COX (which supposes more than 95% of total oxidation rate), and the non-specific non-enzymatic oxidation of cytochrome *c* due to factors other than COX activity itself (which supposes less than 5% of total rate). In order to distinguish between both processes, we used the specific COX inhibitor KCN (200 μM) to stop the enzymatic reaction catalyzed by COX, being residual (non-enzymatic) activity remaining after the addition of KCN subtracted to total rate.

2.4. Quantification of lipid peroxidation

Oxidative damage of lymphocyte membranes was measured through the assessment of lipid peroxidation using *cis*-parinaric acid. *Cis*-parinaic acid is a fatty acid that contains four conjugated double bonds, which render it naturally fluorescent and which are attacked in lipid peroxidation reactions. Accordingly, *cis*-parinaric acid fluorescence is consumed in lipid peroxidation reactions. Because it readily incorporates into membranes, its losing of fluorescence is used to indirectly

Table 1
Characteristics of individuals included in the study

	Patients ($n = 5$)	Controls ($n = 32$)	P value
Age (years)	42 ± 18	37 ± 13	0.72
Gender female (%)	2 (40%)	18 (56%)	0.63
Impaired physical activity (%)	1 (20%)	4 (13%)	0.54
Carboxyhemoglobin levels (%)	25.5 ± 12.4	1.1 ± 0.3	<0.001

monitor the degree of lipid peroxidation. For this purpose, 30 μg of lymphocyte protein were placed into 1 ml of PBS containing *cis*-parinaric acid (5 μM) (Molecular Probes, Eugene, OR), and incubated for 30 min in the dark at 37°C. Afterwards, fluorescence at 318-nm excitation and 410-nm emission was measured as described (Hedley and Chow, 1992; Barrientos et al., 1997; Miró et al., 1999), and *cis*-parinaric acid fluorescence was used to determine the chemical process of lipid peroxidation. The greater lipid peroxidation exists, the less fluorescence is detected.

2.5. Statistical analysis

Qualitative data were expressed as percentages and quantitative data as mean $\pm$ 1 S.D. For comparison, we have used the Fisher's exact test and unpaired t-test, respectively. The relationship between quantitative variables was assessed by means of simple linear regression analysis. In all cases, values of $P < 0.05$ were considered as statistically significant.

3. Results

Table 1 summarizes the epidemiological data of patients and controls. Four out of five acutely CO intoxicated patients required hyperbaric oxygen therapy.

As shown in Fig. 1, patients acutely intoxicated by CO exhibited a significant increase in lipid peroxidation compared to control group (2.77 ± 0.81 vs. 4.56 ± 1.78 fluorescence units per μg of lymphocyte protein, respectively; $P < 0.05$), and also an inhibition of COX activity (33.0 ± 8.3 vs. 149.5 ± 61.0 nmol of oxidized cytochrome c per

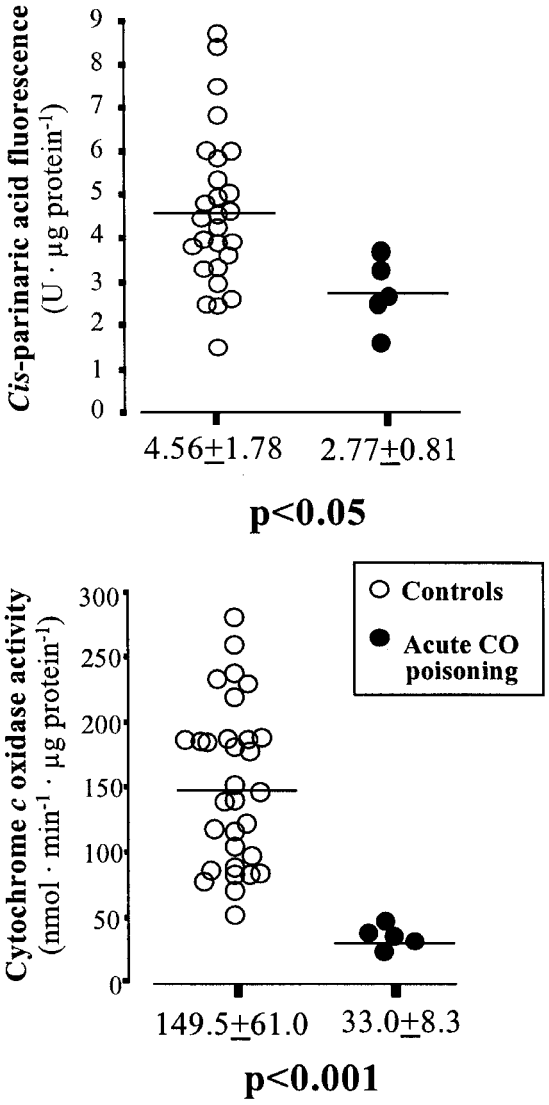

Fig. 1. *Cis*-parinaric acid fluorescence of lymphocyte membranes (upper) and mitochondrial respiratory chain complex IV activity (lower) from patients suffering from acute CO poisoning and controls. In the former measurement, the greater lipid peroxidation exists, the less fluorescence is detected. Numbers under the X-axis denote mean $\pm$ 1 S.D.

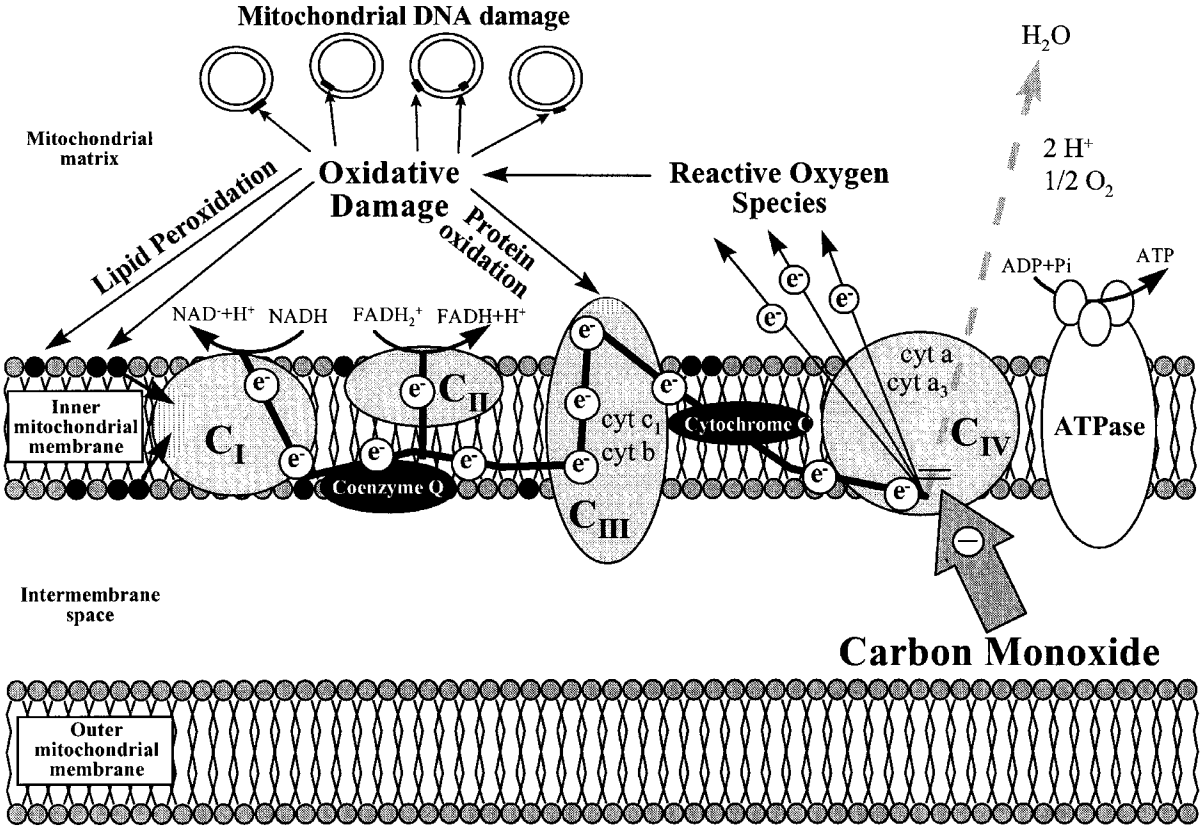

Fig. 2. Proposed scheme for the effects of carbon monoxide on mitochondrial respiratory chain. C_I, complex I; C_{II}, complex II; C_{III}, complex III; C_{IV}, complex IV; cyt, cytochrome; e^-, electron.

min and μg of lymphocyte protein, respectively; $P < 0.001$) (Fig. 1). Moreover, a positive, nearly significant correlation was found between the extent of oxidative damage (lipid peroxidation) and the level of MRC impairment (COX inhibition) ($r = 0.81$, $P = 0.09$).

4. Discussion

Our results demonstrate that an increased lipid peroxidation of lymphocyte membranes is present in patients acutely intoxicated by CO. These findings are in agreement with previous experimental studies where significant increases in reactive oxygen species (ROS) oxidizing essential nucleic acids, proteins and lipids have been found, and it is thought that they are of crucial relevance in the pathophysiology of CO intoxication (Thom, 1990; Ernst and Zibrak, 1998). However, to our knowledge, there are no previous studies confirming such hypothesis in humans.

One hypothetical origin of such increased oxidative damage is mitochondrial dysfunction. These organella are a major source for ROS due to the great number of oxidoreduction reactions that take place inside them during the electron transit through the MRC. COX inhibition during acute CO poisoning, leading to a blockage of this electron flux and to an enhanced ROS production and oxidative stress, could explain the increase in lipid peroxidation observed in our study (Morrow et al., 1995). Lipid peroxidation can propagate itself as a chain reaction, and a single initiating free radical results in the peroxidation of a large number of unsaturated fatty acids. Additionally, lipid peroxides are themselves unstable and break down to produce some toxic aldehydes that can further damage membrane proteins (Hedley and Chow, 1992) (Fig. 2). Therefore, mitochondrial dysfunction, oxidative stress and lipid peroxidation must be interpreted as components of a vicious circle in which the effect continues feeding the

cause. Although any conclusion reached from the analysis of only five CO intoxicated patients is tentative, our data suggest that the enhanced oxidative damage could be caused, at least in part, by the inhibition of COX.

CO poisoning produces both immediate and delayed neuronal injury in selective regions of the brain which is not readily explained on the basis of tissue hypoxia (Penney et al., 1989). In fact, 14% of severely intoxicated patients experience acute neuronal injuries, and a delayed impairment occurs in an additional 12% (Thom and Kleim, 1989). Piantadosi et al. (1997) demonstrated in rats that cerebral oxidative stress is not a direct effect of decreased tissue oxygen concentration during in vivo CO-mediated hypoxia, and that at least a part of ROS generated in brain originate primarily from mitochondria (Zhang and Piantadosi, 1992). The decreased mitochondrial COX activity and the enhanced oxidative damage of lipid membranes partially related to this enzyme inhibition in humans acutely intoxicated by CO support that MRC inhibition could play a central role in the increase of oxidative stress in those patients, and suggest that similar mechanisms could reasonably take place in other tissues, such as brain. Further evidence into this field will provide a better understanding of the pathophysiology of human CO poisoning which in turn could result in a potential improvement in its management.

Acknowledgements

We specially thank all our colleagues who collaborated generously with the present study. This work was supported by the grants DGICYT PM95-0105 and CICYT SAF 95-191. Josep R. Alonso was supported by the Hospital Clínic (Premi Fi de Residència, 1998), and Diana Jarreta by La Marató de TV-3 (2102-97).

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SÍNTESI DELS RESULTATS

1.- L'activitat enzimàtica del complex IV de la cadena respiratòria mitocondrial es troba marcadament disminuïda en els pacients intoxicats de forma aguda per monòxid de carboni.

2.- Existeix un augment del dany oxidatiu, mesurat amb el grau de peroxidació lipídica de les membranes de limfòcits circulants, en els pacients intoxicats de forma aguda per monòxid de carboni.

3.- Sembla haver una correlació positiva entre el dany oxidatiu i la inhibició del complex IV, tot i que no arriba a ser estadísticament significatiu.

4.- Suggerim que l'augment del dany oxidatiu en pacients intoxicats de forma aguda per monòxid de carboni podria ser parcialment explicat per la inhibició del complex IV deguda al propi monòxid de carboni.

6

"Carbon monoxide specifically inhibits cytochrome *c* oxidase of human mitochondrial respiratory chain"

El monòxid de carboni inhibeix específicament la citocrom c *oxidasa de la cadena respiratòria mitocondrial humana*

Jose-Ramon ALONSO, Francesc CARDELLACH, Sònia LÓPEZ, Jordi CASADEMONT, Óscar MIRÓ.

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Carbon Monoxide Specifically Inhibits Cytochrome C Oxidase of Human Mitochondrial Respiratory Chain

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Abstract: Carbon monoxide (CO) toxicity is the result of a combination of tissue hypoxia and direct CO-mediated damage at a cellular level, since not all the signs and symptoms presented can be explained only by the formation of carboxyhaemoglobin. Mitochondria, specially the electron transport chain, seem to be the target for CO at a subcellular level. However, the direct effect of CO in individual complexes of the human mitochondrial respiratory chain has not been completely elucidated. We here studied the *in vitro* effect of CO on individual complexes of the mitochondrial respiratory chain of human mitochondria. We obtained muscle tissue from 10 healthy people who underwent orthopaedic surgery for hip replacement. Isolated mitochondria were incubated for 5 min. under CO concentrations of 50, 100 and 500 ppm. Afterwards, enzymatic activities of individual complexes of the mitochondrial respiratory chain were assessed *in vitro* and compared with those obtained in basal (synthetic air without CO) conditions. Cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain) activity showed a decrease from 836±439 nmol/min./mg of mitochondrial protein after air incubation to 670±401, 483±182, and 379±131 nmol/min./mg after 50, 100 and 500 ppm of CO incubation, respectively (20%, 42% and 55% decrease in cytochrome *c* oxidase activity). This gradual decrease in cytochrome *c* oxidase was found to be statistically significant ($P<0.001$). Other complex activities showed no any significant variation. Carbon monoxide is toxic for mitochondria in man, altering the mitochondrial respiratory chain at the cytochrome *c* oxidase level. This inhibition in cytochrome *c* oxidase may play a role in the development of the symptoms observed in acute CO poisoning, and in some diseases related to smoking.

Carbon monoxide (CO) is a colourless, odourless and non-irritant toxic gas produced by the incomplete combustion of hydrocarbons. Although a very modest amount of CO is normaly produced in man by the catabolism of haemoglobin, larger amounts absorbed from exogenous sources can lead to poisoning and eventually death (Myers 1990; Horner 2000). Clinical signs and symptoms of acute CO poisoning are not specific, but include tachycardia, tachypnoea, headache, nausea, vomiting, dizziness, weakness, difficulty in concentration or confusion, visual changes, syncope, seizures, abdominal pain and muscle cramping (Ernst & Zibrak 1998).

The pathophysiology of CO toxicity appears to be the result of a combination of tissue hypoxia and direct CO-mediated damage at a cellular level. Haemoglobin has a high affinity for CO, leading to formation of carboxyhaemoglobin (Rodkey *et al.* 1974). Hypoxia is due to the formation of carboxyhaemoglobin that reduces the transport of oxygen and impairs the release of oxygen from oxyhaemoglobin in tissues (Roughton & Darling 1944). Since there is no precise relationship between carboxyhaemoglobin values and the symptoms presented (Coburn 1979; Hardy & Thom 1994), other pathophysiological mechanisms for CO toxicity have been suggested, such as reoxygenation injury, lipid peroxidation and oxidative stress (Thom 1990; Zhang & Piantadosi 1992), as well as binding to cellular proteins including myoglobin and cytochromes (Coburn 1979; Zhang & Piantadosi 1992). In this sense, we have reported a marked and sustained inhibition of cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain) activity in human lymphocytes after acute CO poisoning (Miró *et al.* 1998a).

Tobacco smoke is an important source of CO. Accordingly, the more a person smokes, the higher the carboxyhaemoglobin levels (Puente-Maestu *et al.* 1998), with known adverse effects on oxygen transportation by haemoglobin (McDonough & Moffatt 1999). However, the direct effects of smoke CO at a cellular level and its implication in diseases to related smoking remain to be completely elucidated. Similar to other investigators we have found an inhibition in mitochondrial respiratory chain function, mainly on mitochondrial cytochrome *c* oxidase, due to tobacco smoke (Örlander *et al.* 1979; Gvozdjáková *et al.* 1984; Larsson & Örlander 1984; Gvozdjak *et al.* 1987; Pryor *et al.* 1992; Miró *et al.* 1999). These findings suggest that the CO of tobacco smoke itself might play a role in this mitochondrial dysfunction.

Although CO is known to be toxic to mitochondria (Coburn 1979), only a few studies have evaluated its pathogenic mechanism at a subcellular level in animal models (Piantadosi *et al.* 1985; Brown & Piantadosi 1990; Zhang & Pianta-

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dosi 1992), and confirmation in man has not been completely achieved. In addition, only a few studies based on CO poisoning have ruled out the effect of the coexisting hypoxia (Haab 1990), and studies with smokers have not excluded the participation of other substances in the observed mitochondrial effect.

Our aim was to investigate the specific effect of CO itself on the mitochondrial respiratory chain of human mitochondria in the absence of any confounding factor.

Material and Methods

Patients. We obtained muscle samples from the quadriceps muscle (vastus lateralis) from patients submitted to orthopaedic surgery (hip prothesis placement) under regional anaesthesia (Miró *et al.* 1999). We excluded patients with a previous diagnosis of neuromuscular disease and/or familial history of neuromuscular disorder. All the individuals were similar in age and physical activity and all were non-smokers in order to avoid factors that have been considered to be confounding variables in studies on mitochondrial function (Cardellach *et al.* 1989; Smith *et al.* 1993; Boffoli *et al.* 1994; Barrientos *et al*. 1996). Non-smokers were considered to be individuals that had never smoked or had abstained from smoking during the previous year. We included only persons who walked on the street every day, following the Steinbrocker classification (Steinbrocker *et al.* 1949). The subjects were informed about the study protocol and all provided written informed consent to participate. The study was approved by the Ethics Committee of our hospital. A total of 10 persons were included in the study.

Obtaining mitochondria. The muscle samples were obtained at the begining of the orthopaedic procedure and placed into media with 20 mM Tris (pH 7.2), 250 mM sucrose, 40 mM KCl, 2 mM EGTA and 1 mg/ml of bovine serum albumin (BSA) at 4°, and processed in less than 5 min.

Muscle mitochondria were isolated using a standard methodology employed in our laboratory (Cardellach *et al.* 1991 & 1992), slightly modified according to Rustin *et al.* (1994) for minute amounts of human skeletal muscle, as previously reported (Miró *et al*. 1998a, b & c). The pellet of crude mitochondria was resuspended in media with 20 mM Tris (pH 7.2), 250 mM sucrose, 40 mM KCl and 2 mM EGTA. All the steps were carried out at 4°. Protein concentration was determined by the Bradford method (Bradford 1976). The approximate time between skeletal muscle biopsy and mitochondrial isolation (ready to start biochemical assays) was about 45 min. Spectrophotometric studies were carried out immediately afterwards.

Biochemical studies. According to different conversion charts of CO in exhaled air (COEA) to carboxyhaemoglobin and data from smokers, allowed professional exposures to CO, and patients with CO poisoning, synthetic air (N_2 79% O_2 21% CO 0 ppm) was used as the gas control, and concentrations of 50 ppm, 100 ppm and 500 ppm CO (Linde Abelló®, Barcelona, Spain) for the determinations.

Samples were incubated for 5 min. at 37° under these different gas mixtures of CO-enriched air. Measurement of the specific activity of the following complexes of the respiratory chain was performed by spectrophotometry (UVIKON 922, Kontron, Zurich, Switzerland): NADH-ubiquinone reductase (complex I), succinate-ubiquinone reductase (complex II), ubiquinol-cytochrome *c* reductase (complex III), and cytochrome *c* oxidase (complex IV). A

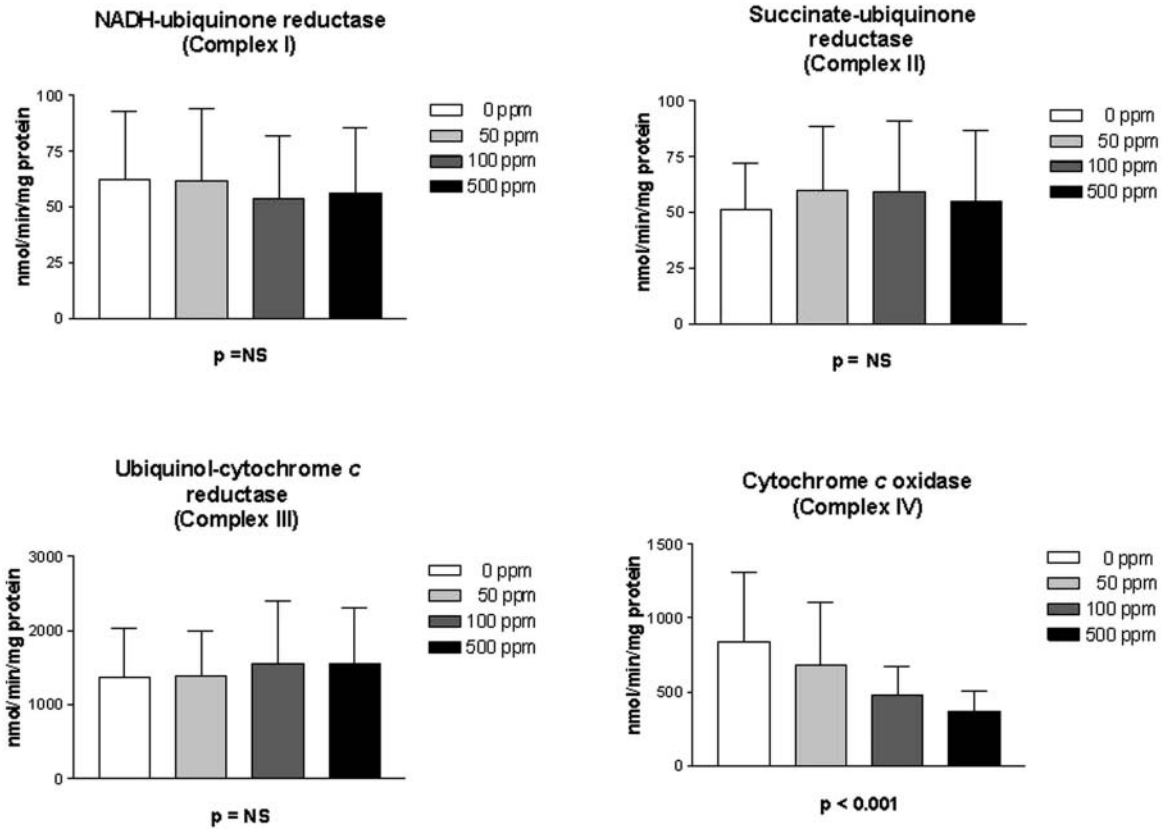

Fig. 1. Absolute enzyme activities.

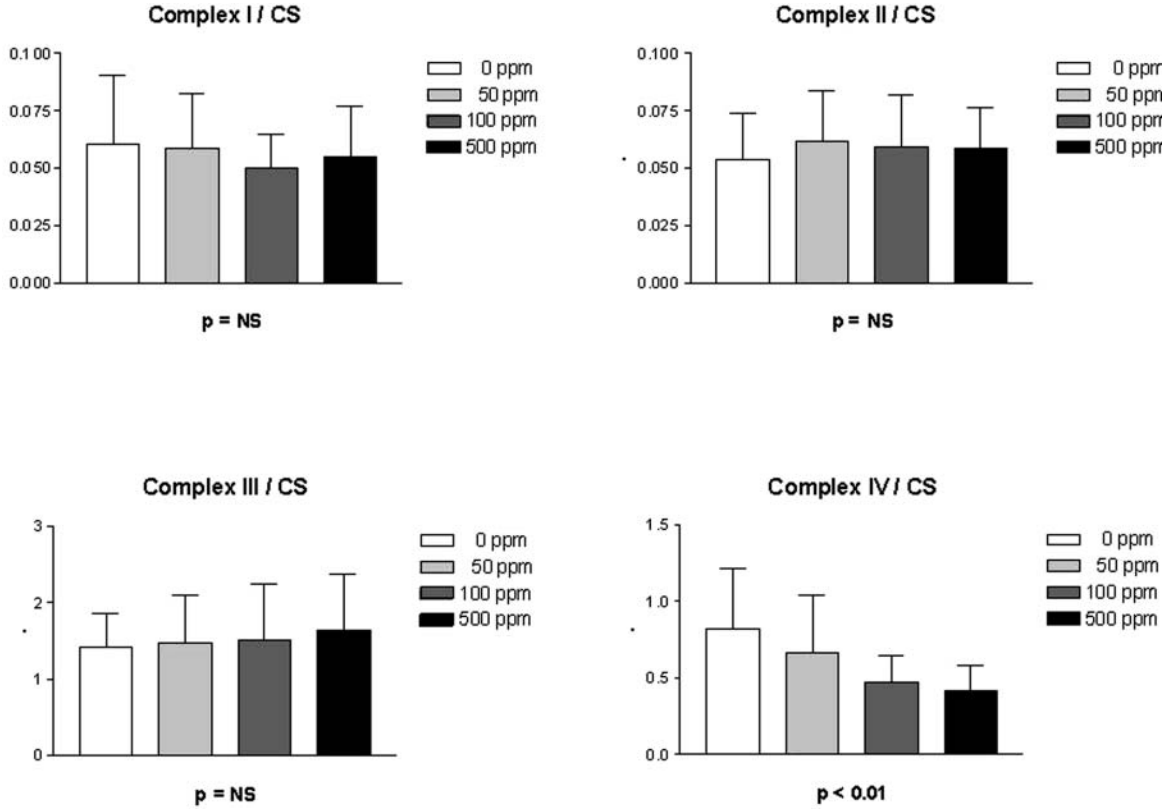

Fig. 2. Relative enzyme activities.

total of 10–40 µg of mitochondrial protein was required to determine the activity of each complex, except for complex IV, for which 2–4 µg was used (Miró *et al*. 1998a, b & c). Assays were performed at 37° in a total volume of 1 ml according to Chretien *et al.* (1994) and Rustin *et al.* (1994). Absolute enzyme activities were expressed as nmols of reduced or oxidized substate/min./mg of mitochondrial protein. Relative enzyme activities were presented as ratios related to cytrate synthase activity (Rustin *et al.* 1994).

Statistical analyses. Data were processed using a SPSS software. Results from quantitative variables were expressed as mean± standard deviation. We used one-way ANOVA for repeated measures to determine differences among them, and checked for a linear trend using a polynomial contrast of first degree. Values of $P<0.05$ were considered as statistically significant.

Results

Biopsies were obtained from 6 men and 4 women. Similar to previous studies (Miró *et al.* 1999) we did not find gender differences in enzyme activities (results not shown). Fig. 1 shows the activity of the mitochondrial respiratory chain enzymes after exposure to the different CO concentrations. Increased concentrations of CO showed a progressive and significant decrease in cytochrome *c* oxidase activity ($P<0.001$) with no changes being observed in other complex enzyme activities. Basal activity was 836±439 nmol/min./mg of mitochondrial protein, which decreased to 670±401 nmol/min./mg of mitochondrial protein after CO incubation at 50 ppm, to 483±182 nmol/min./mg of mitochondrial protein at 100 ppm, and lastly to 379±131 nmol/min./mg of mitochondrial protein at 500 ppm, representing a decrease of 20%, 42% and 55% respectively in cytochrome *c* oxidase activity.

After correcting absolute enzyme rates by citrate synthase activity in order to obtain their relative activities, again only cytochrome *c* oxidase (complex IV) activity showed the same pattern of inhibition associated with increasing exposures of CO concentrations ($P<0.05$) (fig. 2).

Discussion

The present study reports the effects of exposures to increased CO concentrations on mitochondrial respiratory chain components of human mitochondria. We incubated aliquots of different concentrations of CO following standard procedures for gas exposure. According to Henry's law, incubation media were saturated with each gas mixture. Thus, it was assumed that the samples had absorbed sufficient CO to detect any acute potential effect arising from CO exposure in the studied parameters. On the other hand, muscle samples were obtained from persons during similar physical activity, and illnesses that could interfere with mitochondria activity were excluded by evaluation of medical history, physical examination and routine blood analyses.

We observed a significant, progressive inhibition in cytochrome *c* oxidase activity parallel to increased concen-

trations of CO in each medium while other enzyme activities did not show any change in relation to the presence of CO. Cytochrome *c* oxidase inhibition by CO could explain previously reported findings on mitochondrial function in animals inhaling tobacco smoke (Gvozdjáková *et al.* 1984), smokers (Örlander *et al.* 1979; Larsson & Örlander 1984; Miró *et al.* 1999) and patients with acute CO poisoning (Miró *et al.* 1998a, b & c). Since oxygen was always present and no other toxic substance was added in the media, this inhibition may be explained by a reduction of prosthetic group cytochrome a_3 of cytochrome *c* oxidase by CO (Ellis *et al.* 1986).

The progressive inhibition of cytochrome *c* oxidase activity by increased exposures of CO concentrations may produce an overall decrease in mitochondrial respiratory chain function. Such a disturbance of mitochondrial respiratory chain function could lead to a decay in mitochondria energy production and, eventually, to cellular injury or dysfunction, especially in "high metabolic rate" tissues exposed to CO such as the central nervous system, heart muscle or skeletal muscle. Indeed, muscular weakness and impaired heart function have been reported and demonstrated in CO poisoning (Tritapepe *et al.* 1998; Raub *et al.* 2000; Yanir *et al.* 2002) and in heavy smokers (Örlander *et al.* 1979; Gvozdjakova *et al.* 1984). On the other hand, the disturbance in mitochondrial respiratory chain function may also lead to a major production of reactive oxygen species (Cadenas & Davis 2000). Our results support previously reported data that suggest a higher oxidative stress on cells after CO exposure which may explain some of the clinical features of CO poisoning not only due to increased carboxyhaemoglobin levels, i.e. delayed neuropsychiatric syndrome (Thom 1993). In fact, repeated sessions of hyperbaric oxygenotherapy in patients with nearly normal carboxyhaemoglobin seem to reduce the incidence of the syndrome, indicating that subcellular effects of CO should be taken into account in the pathophysiology of this syndrome (Weaver *et al.* 2002; Cardellach *et al.* 2003).

There are some limitations in establishing an exact correlation between CO levels used in *in vitro* studies and the clinical symptoms of acute CO poisoning *in vivo*. Carbon monoxide absortion depends on minute ventilation, relative CO/O_2 concentrations in the environment and the length of exposure (Forbes *et al.* 1945). On the other hand, after being absorbed, CO distribution depends on tissue perfusion, as any substance in the blood-stream. These two factors make it impossible to correlate *in vitro* CO concentrations with similar concentrations in blood. Moreover, tissue sensitivity differs according to its metabolic rate (Ray 1997), and there is always a certain degree of hypoxia because of carboxyhaemoglobin which may magnify the effects of CO. Finally, CO and hypoxia can lead to cell death, and thus, irreversible tissue damage.

In conclusion, we demonstrate that CO by itself is toxic to human mitochondria and alters the mitochondrial respiratory chain at the cytochrome *c* oxidase level. This inhibition may play a role in some CO-related alterations explained not only by hypoxia, such as acute CO poisoning, and diseases to related smoking. Our data on mitochondrial respiratory chain dysfunction by CO agree with previous findings by our group and those of others, but no quantitative correlations may be made because of the design of the study. In spite of these limitations, we believe that our findings contribute to the understanding of the pathogenic mechanisms for the clinical features involved in these situations. However, the severity of the clinical manifestations depends not only on the degree of poisoning but also on the presence of other factors such as tissue hypoxia and previous tissue function, i.e. underlying illness.

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SÍNTESI DELS RESULTATS

1.- L'activitat enzimàtica del complex IV de la cadena respiratòria mitocondrial presenta una inhibició progressiva i estadísticament significativa paral·lelament a l'increment de la concentració de monòxid de carboni a la qual son exposats els mitocondris aïllats.

2.- Cap altre complex enzimàtic mostra variacions.

3.- En conclusió, el monòxid de carboni per si mateix es un tòxic pel mitocondri en humans, afectant el funcionalisme de la cadena respiratòria mitocondrial a nivell del complex IV.