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ANTIOXIDANT VITAMIN SUPPLEMENTATION OF SMOKE-EXPOSED RATS PARTIALLY PROTECTS AGAINST MYOCARDIAL ISCHAEMIC/ REPERFUSION INJURY

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Our previous studies showed that exposure of rats to limited periods of cigarette smoke resulted in more severe myocardial damage when their hearts were subjected to myocardial ischaemia/reperfusion. The aim of this study was to determine whether supplementation of rats with antioxidant vitamins α-tocopherol and β-carotene was able to protect their hearts against the increase in ischaemia/reperfusion injury caused by smoke-exposure. The parameters measured were mitochondrial oxidative function, cellular levels of α-tocopherol and low molecular weight iron (LMWI). Supplementation with antioxidant vitamins resulted in significantly less mitochondrial functional oxidative damage compared to that observed in the controls. Supplementation did not affect the cellular LMWI content, suggesting that the generation rate of hydroxyl radicals was similar in both groups. The protective effect of α-tocopherol and β-carotene supplementation on the mitochondrial function against ischaemia/reperfusion could be due to their free radical scavenging action. Supplementation with antioxidant vitamins, therefore, had a beneficial effect on the excessive myocardial ischaemia/reperfusion injury of smoke exposed rats.

KEY WORDS: Mitochondria, α-tocopherol, low molecular weight iron, ischaemia, reperfusion.

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INTRODUCTION

Cardiac ischaemia is a relatively common clinical event, which is lethal if perfusion is not restored. Reperfusion is not without danger and can contribute to the damage induced by ischaemia. Smoking is an independent factor for the development of coronary heart disease.[1] Cigarette smoke contains vast amounts of free radicals ($\sim 10^{14}$ molecules/inhalation), which can directly and indirectly initiate and perpetuate the process of lipid peroxidation.[2] Giving free radical scavengers to smokers might, therefore, be beneficial. One such scavenger is α-tocopherol especially known for its proven effectiveness as a biological antioxidant, protecting the cell against oxidative stress.[3] α-Tocopherol also plays a role in membrane stability and fluidity [3]. The susceptibility of erythrocytes from smokers to *in vitro* peroxidation was markedly decreased when the smoker received α-tocopherol supplementation. If this susceptibility of the erythrocyte from smokers to peroxidative stress reflects that of the other cells, then smokers may benefit from increased α-tocopherol intake but this would have to be established by extensive trials.[4]

Humble *et al.*[5] demonstrated an increased risk for cardiovascular death in the wives

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of smoking men. Studies done by Pryor *et al.*, [6] Dobson *et al.*[7] and Glantz *et al.*[8] supported these findings and their conclusion was that passive smoking is an independent risk factor for coronary heart disease. We have previously shown that chronic limited periods of smoke-exposure of rats worsened the ischaemia/reperfusion injury to the mitochondrial oxidative function of rat hearts.[9] This was probably due to the depletion of cellular antioxidants as shown by the very low levels of α-tocopherol and the marked elevated low molecular weight iron (LMWI).

The aim of this study was to determine whether supplementation of rats during smoke-exposure periods with specifically antioxidant vitamins (α-tocopherol and β-carotene) would protect against, or at least minimize, the effect of smoke on the myocardial ischaemia/reperfusion injury.

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MATERIAL AND METHODS

Animals

Female rats (300–400 g) were used as experimental animals and subjected to small concentrations of cigarette smoke. The antioxidant vitamin supplemented group received α-tocopherol and β-carotene in their drinking water (0.84 g α-tocopherol and 0.3 g β-carotene in a litre of water) during the whole period of smoke-exposure (2 months).

Smoke-exposure

The model as described previously was used.[9] Rats were put in a 35 litre perspex cage with an inlet and an outlet. The outlet was connected to a vacuum pump and the inlet to a glass tube with two holes. A cigarette (nicotine 17 mg; tar 1.7 mg) was put in the hole farthest from the cage. Every 55 s the hole nearest to the cage was closed for 5 s and smoke was drawn into the cage for 5 s. Thereafter the finger was withdrawn and room air sucked into the cage for 55 s. The process was repeated until the cigarette burnt out (± 10 min). This procedure was repeated twice a day for 2 months. Carboxyhemoglobin concentration in rats subjected to 2 months of smoking and those that did not smoke were not statistically different.

Ischaemia/Reperfusion

Rat hearts were mounted on the "working rat heart model" as described earlier and subjected to (i) 10 min ischaemia (normothermic ischaemic cardiac arrest) and (ii) 10 min ischaemia (normothermic ischaemic cardiac arrest) followed by 5 min reperfusion.[10]

Isolation of Mitochondria

After termination of the experiments, the hearts were plunged into cold isolation medium consisting of 0.18 mM KCl, 0.01 M EDTA; the pH adjusted to 7.4 by addition of Tris base at 4°C. The mixture was homogenized using a Polytron PT 10 homogenizer, and the mitochondria were isolated as described previously.[11]

Mitochondrial Oxidative Phosphorylation Function

The oxidative phosphorylation rate of the isolated mitochondria was measured

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polarographically using a Gilson oxygraph. The following incubation medium was used: 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 8.5 mM K_2HPO_4, and 5 mM glutamate (Tris salt, pH 7.4) at 25°C, to which 3 $\pm$ 0.15 mg mitochondrial protein was added. To measure the QO_2 (state 3), 500 nmol ADP was added to initiate the reaction. The following parameters were measured: (a) ADP/O: nmol ADP consumed/nmol oxygen consumed; (b) QO_2, state 3: nmol oxygen consumed in the presence of ADP/mg mitochondrial protein/min; and (c) oxidative phosphorylation rate, OPR: nmol ADP consumed/mg mitochondrial protein/min (i.e., QO_2 (state 3) $\times$ ADP/O.[12]

Low Molecular Weight Iron (LMWI)

A modified method of Krause *et al.*[13] was used. Hearts were homogenised in 0.25 M sucrose, 5 mM Tris (pH 7.4) buffer and centrifuged for 10 min (755 g). A quarter volume TCA (20%) was added to the supernatant and allowed to stand for 10 min) whereafter it was centrifuged for 10 min at 1300 g. Supernatant (500 μl) was added to a tube containing 300 μl water, 50 μl 0.1% *o*-phenanthroline, 50 μl 0.1% ascorbic acid and 50 μl saturated ammonium acetate. After incubation for 10 min at 37°C to allow for maximum colour development, the absorbance was read at 510 nm against a blank containing all reagents except *o*-phenanthroline. A standard curve was used for calculation of the iron concentration, and the results expressed as nmol iron/mg protein.

Cellular α-Tocopherol, β-Carotene, Retinol and Retinol Palmitate

A modified method of Turnham *et al.*[14] was used. The heart ($\pm$ 1 g) was homogenised in 4 ml saline and samples were divided into two and α-tocopherol, retinol, retinol palmitate and β-carotene added to one of them for identification of the peaks. To a 2 ml sample, 1.5 ml SDS (10 mM) was added. After mixing, the samples were incubated at 70°C for 30 min under nitrogen, whereafter 3 ml ethanol was added, as well as 1.5 ml heptane. The same was vortexed for 5 min and centrifuged at 755 g for 1-2 min. The heptane layer was transferred to another tube. Another 1.5 ml heptane was used for a washing process to obtain a maximum extraction of vitamins, the two heptane layers were pooled and the heptane evaporated at 40°C under nitrogen. To each sample 1 ml 5% chloroform in methanol were added and the sample filtered through a 0.2 μm filter. The samples were stored at -20°C.

A HPLC system compromised of a Waters 501 pump, a Wisp 710 B autosampler, a Beckman System Gold Diode Array as detector and system Gold software were used. 3% Chloroform in methanol (filtered through 2 μm pore size filter) was used as mobile phase. We separated 100 μl extracts in mobile phase isocratically on a 250 $\times$ 4.6 mm reversed phase C_{18} cartridge column with 5 μm particles.

Protein Content

Protein content was measured by the method of Lowry *et al.*[15]

Statistical Analysis

All values were expressed as mean $\pm$ SEM and, where applicable, unpaired Student's *t*-test was used and the level of significance was set at $p < 0.05$.

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TABLE I
Effect of antioxidant vitamin supplementation on mitochondrial oxidative phosphorylation function. (QO_2 (state 3) (nmol O_2 consumed/mg protein/min)) of smoke-exposed rats subjected to myocardial ischaemia/reperfusion

	n	Unsupplemented controls	Vitamin supplemented
Unperfused control	6	126.08 ± 1.86	129.08 ± 4.09
Perfused control	4	124.93 ± 2.95	128.48 ± 5.86
10 min Ischaemia	4	84.37 ± 2.96[a]	115.14 ± 8.33[b]
10 min Ischaemia + 5 min reperfusion	4	80.19 ± 3.53[a]	104.80 ± 2.45[a,b]

Statistically significant ($p < 0.05$): [a]versus unperfused; [b]versus unsupplemented controls.

RESULTS

Mitochondrial Oxidative Phosphorylation Function

The mitochondrial function (QO_2 (state 3) and OPR) (Tables I and II) of rats that were supplemented with α-tocopherol and β-carotene were the same as those that were not supplemented. Control perfusion did not alter the QO_2 (state 3) in passive smoking, but a significant decrease was observed during ischaemia as well as ischaemia followed by reperfusion. Hearts of vitamin supplemented rats showed a lesser degree of impairment (±28% less in the case of ischaemia only and 24% less in the case of ischaemia followed by reperfusion (Table I)). ADP/O ratios stayed unaltered throughout smoke-exposure as well as ischaemia/reperfusion. OPR reflected the tendency of QO_2 (state 3) (Table II).

Low Molecular Weight Iron (LMWI)

Although the LMWI content (Table III) in the control hearts of unsupplemented rats (unperfused and perfused) were statistically lower in the hearts, the values were not statistically different during ischaemia and ischaemia followed by reperfusion. In both the supplemented and unsupplemented groups a statistical significant increase occurred in comprison to the unperfused control.

Cellular Vitamin Content

Retinol, retinol palmitate and β-carotene could not be detected in the hearts. The

TABLE II
Effect of supplementation on mitochondrial oxidative phosphorylation function (nmol ADP consumed/mg protein/min) of smoke-exposed rats subjected to myocardial ischaemia/reperfusion)

	n	Unsupplemented Control	Vitamin supplemented
Unperfused control	4	388.33 ± 11.09	387.24 ± 12.36
Perfused control	4	374.79 ± 10.36	382.87 ± 11.61
10 min ischaemia	4	247.20 ± 11.70[a]	339.66 ± 12.03[a,b]
10 min ischaemia + 5 min reperfusion	4	241.37 ± 10.11[a]	310.21 ± 9.87[a,b]

Statistical significance ($p < 0.05$): [a]versus unperfused; [b]versus unsupplemented controls.

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TABLE III
Effect of vitamin supplementation on cellular low molecular weight iron content (nmol/mg protein) of smoke-exposed rats subjected to myocardial ischaemia/reperfusion

	n	Unsupplemented controls	Vitamin supplemented
Unperfused control	6	0.485 ± 0.031	0.647 ± 0.028[b]
Perfused control	4	0.481 ± 0.030	0.724 ± 0.025[b]
10 min Ischaemia	4	0.950 ± 0.034[a]	1.109 ± 0.094[a]
10 min Ischaemia + 5 min reperfusion	4	1.067 ± 0.032[a]	1.159 ± 0.046[a]

Statistical significance ($p < 0.05$): [a]versus unperfused; [b]versus unsupplemented controls.

cellular α-tocopherol content (Table IV) of hearts of rats supplemented with the antioxidant vitamins was on average almost double that of the hearts from the unsupplemented rats. The hearts of the unsupplemented rats showed a decrease in α-tocopherol content during ischaemia and showed a further decrease when the ischaemia was followed by reperfusion. The antioxidant supplemented group's α-tocopherol content also dropped statistically significantly during ischaemia as well as ischaemia followed by reperfusion (±30%) in comparison to unperfused values of supplemented rats. The cellular α-tocopherol content of the supplemented group also showed a decrease but were at all times nearly double the values observed in the unsupplemented hearts.

DISCUSSION

Supplementation of rats with α-tocopherol and β-carotene during the smoke-exposure period resulted in a significant protection of mitochondrial oxidative function expressed as QO_2 (state 3) and OPR. LMWI was not lower in supplemented rat hearts than unsupplemented hearts. As LMWI is a catalyst in the Haber-Weiss and Fenton reactions and most likely responsible for the generation of hydroxyl radicals,[16] it might be reasonable to suppose that the same amount of hydroxyl radicals were formed in hearts of the supplemented group and the unsupplemented group during ischaemia and ischaemia followed by reperfusion.

Antioxidant vitamins like α-tocopherol and β-carotene scavenge free radicals[16] and are assumed in the process. The decrease observed in α-tocopherol in hearts of rats that were supplemented with α-tocopherol and β-carotene as well as those not

TABLE IV
Cellular α-tocopherol content (nmol/mg protein) of smoke-exposed rats subjected to myocardial ischaemia/reperfusion

	n	Unsupplemented controls	Vitamin supplemented
Unperfused control	6	16.96 ± 0.19	29.68 ± 0.84
Perfused control	4	16.39 ± 0.23	30.04 ± 0.72
10 min Ischaemia	4	12.75 ± 0.80*	24.50 ± 0.30[a,b]
10 min Ischaemia + reperfusion	4	9.89 ± 0.22*	20.59 ± 0.822[a,b]

Statistical significance ($p < 0.05$): [a]versus unperfused; [b]versus unsupplemented results.

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supplemented might, therefore, be due to the production of oxygen radicals. In the supplemented group the concentration of antioxidants was probably sufficient in that there were less free radicals to cause cellular damage. In the unsupplemented group the α-tocopherol concentration dropped to very low levels, thus indicating that the scavenging of free radicals was inefficient. In our experimental design we were unable to detect any β-carotene, retinol or retinolpalmitate in the heart. This might be due to an inefficient transfer of β-carotene across the intestine in rats.[17] We assumed that although we did not measure the water-soluble antioxidants (vitamin C, GSH); that these might also play a protective role. Although, what we observed was a partial protection of mitochondrial oxidative function, we feel that by using supplementation with water-soluble antioxidants or increasing the supplementation doses of α-tocopherol and β-carotene, mitochondrial oxidative function might be completely protected during ischaemia/reperfusion.

From this study it can be concluded that the mitochondrial oxidative function can be partially protected against smoke injury by supplementation of rats with α-tocopherol and β-carotene. The vitamin supplement had no significant effect on the LMWI content, and therefore, hydroxyl radical production rate could be unaffected so that the protective effect was not likely by the scavenging of free radicals.

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