Research article/Raziskovalni prispevek

EFFECT OF SUBCHRONIC EXPOSURE TO MAINSTREAM CIGARETTE SMOKE ON ENDOTHELIUM-DEPENDENT VASODILATION IN RAT ARTERIES

VPLIV SUBKRONIČNE IZPOSTAVLJENOSTI CIGARETNEMU DIMU NA OD ENDOTELIJA ODVISNO VAZODILATACIJO ARTERIJ PODGANE

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Key words: *smoking; vascular reactivity; arterial rings; nitric oxide (NO); endothelium-derived hyperpolarizing factor (EDHF)*

Abstract – Background. *Cigarette smoking is reported to impair endothelium-dependent vasodilation. The aim of the present study was to assess the effect of 30-day exposure to mainstream cigarette smoke on vascular reactivity of rat abdominal aorta, carotid, renal and mesenteric artery. Separately, the NO-mediated and the EDHF-mediated, endothelium-dependent vascular relaxations were determined.*

Methods. *Two groups of »Whistar Kyoto« rats were exposed to mainstream cigarette smoke (2 hours/day, 5 days/week for 30 days) and to fresh conditioned air, respectively. Rats were sacrificed on the second day after the last exposition to cigarette smoke. Vascular reactivity studies were performed on isolated, endothelium-intact, phenylephrine-preconstricted rat artery rings. Cumulative concentration-relaxation curves to acetylcholine (ACh) were obtained in the absence and presence of the endothelial NO synthase (eNOS) inhibitor N$^{\omega}$nitro L-arginine (L-NA) and the cyclo-oxygenase (COX) inhibitor diclofenac, respectively. After washing period of 1 hour, vessels were exposed either to the intracellular superoxide scavenger tiron, to the cytochrome P450 (CYP) inhibitor miconazole or the Na-K-ATPase inhibitor ouabain before being preconstricted with phenylephrine and determining the concentration-response curve to ACh.*

Results. *ACh induced concentration-dependent relaxations. In none of the vessels investigated did we observe a significant difference in the relaxations obtained in arteries from control rats and rats exposed to cigarettee smoke. Although smoking is known to cause an increase in oxidative stress, treatment of the vessels with tiron did not affect the NO-mediated relaxations. To evaluate the contribution of EDHF to endothelium-dependent vasodilation rings were preincubated with L-NA. The EDHF-mediated relaxations were significantly attenuated compared to the NO-mediated relaxations in renal and mesenteric artery and almost completely abolished in aorta and carotid artery in both groups of rats. Comparing the EDHF-mediated relaxations in arterial preparati-*

Ključne besede: *kajenje; žilna reaktivnost; žilni obročki; dušikov oksid (NO); endotelijski hiperpolarizirajoči dejavnik (EDHF)*

Izvleček – Izhodišča. *Znano je, da kajenje dolgoročno okvari arterijsko steno ter povzroča aterosklerozo. Številne raziskave so pokazale, da je ena od zgodnjih sprememb okvara endotelijske funkcije, ki naj bi nastala kot posledica spremenjenega razmerja med endotelijskimi vazodilatatorji, kot so dušikov oksid (NO), prostaciklin, endotelijski hiperpolarizirajoči faktor (EDHF), in vazokonstriktorji, kot so endothelin-1, produkti ciklooksigenaze (COX) in superoksidni anioni. Vendar pa natančni mehanizmi, ki vodijo do sprememb endotelija, še niso poznani; prav tako ni še povsem raziskano, kako hitro omenjene spremembe nastanejo. Prav zato je bil naš namen raziskati, če subkronična izpostavljenost cigaretnemu dimu (30 dni) že okvari endotelijsko funkcijo nekaterih arterij podgane. Posebej smo želeli ovrednotiti vpliv cigaretnega dima na endotelijsko vazodilatacijo, ki jo posreduje NO, ter endotelijsko vazodilatacijo, ki jo posreduje EDHF.*

Metode. *Aktivno kajenje smo simulirali tako, da smo podgane vrste »Whistar Kyoto« izpostavili neposrednemu cigaretnemu dimu (2 uri/dan, 5 dni/teden, 30 dni). Da bi se izognili zaznavanju akutnih sprememb, smo od zadnje izpostavljenosti cigaretnemu dimu do izvajanja meritev žilne reaktivnosti počakali dva dni. Po žrtvovanju živali smo izolirali abdominalno aorto, karotidno, mezenterično in ledvično arterijo. Žilno reaktivnost smo preučevali v kopeli za izolirane organe. Obročke izoliranih žil z ohranjenim endotelijem obeh skupin podgan smo aktivno skrčili z dodatkom noradrenalina (30 nM do 300 nM) ter nato z dodajanjem naraščajočih koncentracij acetilholina (ACh, 1 nM–10 μM) izzvali relaksacijo obročkov; po izpiranju smo obročke inkubirali z dodatkom znotrajceličnega »lovilca prostih radikalov«, tirona (1 mM, 40 minut) ter ponovili osnovni protokol izvajanja relaksacije. Za vrednotenje vpliva EDHF na od endotelija odvisno vazodilatacijo smo obročke inkubirali z dodatkom inhibitorjev endotelijske sintaze NO (eNOS), N$^{\omega}$nitro L-arginina (L-NA; 300 μM), in COX-a, diklofenaka (10 μM), ter izzvali krivuljo odvisnosti relaksacije od koncentracije ACh; za karakterizacijo vazodilatacije, ki jo posreduje EDHF, smo del*

ons from the control group with those from smoke-treated rats, we found no significant differences in ACh-induced relaxations. The inclusion of both, miconazole and ouabain in organ chambers induced a rightward shift in the ACh-induced, EDHF-mediated relaxations in renal and mesenteric arteries what confirmed the involvement of CYP and Na/K ATPase in EDHF-mediated relaxations. Again, no differences were registered between the vessels of treated and untreated group.

Conclusions. *The results of the present study show that 30 days of transient (subchronic) exposure to cigarette smoke did not induce detectable changes in global endothelial function, or in specific NO and EDHF-mediated relaxations in the rat aorta or in carotid, mesenteric and renal arteries.*

obročkov predhodno inkubirali z inhibitorjem citokroma P450 (CYP), mikonazolom (3 μM; 20 min), oziroma z inhibitorjem Na-K-črpalke (Na-K-ATPaza; 20 min), ouabainom (100 μM).

Rezultati. *ACh je izzval od koncentracije odvisno relaksacijo na obročkih vseh žil. Od EDHF odvisne relaksacije so bile v primerjavi z od NO odvisnimi relaksacijami izrazito slabše pri vseh žilah iz obeh skupin živali (sl. 1). Med tretirano in kontrolno skupino živali nismo zasledili statistično značilnih razlik tako v relaksaciji, ki jo posreduje NO, kot tudi v relaksaciji, ki jo posreduje EDHF (sl. 1). Čeprav je znano, da kajenje poveča »oksidativni stres« v celici, predhodni dodatek tirona v kopel ni imel vpliva na od NO odvisno relaksacijo v skupini tretiranih živali (sl. 2). Tako dodatek mikonazola kot tudi dodatek ouabaina v kopel je na obročkih ledvične in mezenterične arterije povzročil premik krivulje z ACh izzvane relaksacije, ki jo posreduje EDHF, v desno (sl. 3), kar kaže na vpletenost CYP in Na-K-ATPaze pri vazodilataciji, ki jo posreduje EDHF. Ponovno nismo zasledili značilnih razlik med kontrolno in tretirano skupino živali.*

Zaključki. *Rezultati študije so pokazali, da prehodna 30-dnevna (subkronična) aktivna izpostavljenost cigaretnemu dimu ni povzročila značilnih globalnih sprememb funkcije endotelija; specifični, tako od NO kot tudi od EDHF odvisna relaksacija abdominalne aorte, karotidne, mezenterične in ledvične arterije podgane se po 30 dneh aktivnega kajenja nista spremenili.*

Introduction

Cigarette smoking is strongly associated with artery disease and atherosclerosis. Numerous studies have shown that smoking leads to endothelial dysfunction, signaled by impaired endothelium-dependent vasorelaxation (1–3).

Endothelium actively contributes to the regulation of vascular tone by releasing a variety of endothelium-derived autacoids, vasodilators such as nitric oxide (NO), prostacycline, endothelium-derived hyperpolarizing factor (EDHF) and vasoconstrictor factors, such as endothelin-1, cyclooxygenase (COX)-derived vasoconstrictor products, and superoxide anions.

Cigarette smoke has been suggested to induce an imbalance in the generation of vasodilator and vasoconstrictor mediators which in turn can have profound implications on vascular gene expression and the development of cardiovascular disorders. However, the exact mechanisms involved are not completely understood. Some studies have suggested that cigarette smoke increases the production of endothelin-1 (4, 5), while others suggest that impaired agonist-induced relaxation can be attributed to a decrease in the activity of the endothelial NO synthase (eNOS) (6) or rather to a decrease in the bioavailability of NO as a consequence of it being scavenged by oxygen-derived free radicals. Ironically, cigarette smoking has also been reported to increase eNOS gene expression and activity (7, 8) although it is unclear whether this eNOS produces only NO or whether the enzyme is uncoupled and, as a consequence also generates O_2^- (9). There are however other physiologically important vascular sources of oxygen-derived free radicals that interact with and decrease the bioavailability of NO, such as the NADPH oxidase and CYP enzymes (10). Thus, the lower bioavailability of NO could be a consequence of increased degradation of NO by oxygen-derived free radicals. Indeed, there is increasing evidence that smoking enhances oxidative stress by increasing the generation of reactive oxygen species (11, 12) which results in decreased NO bioactivity. Along with these changes go also the findings that free radicals scavenging (antioxidant potential) is impaired in smokers (13, 14). On the other hand, it has been shown that increasing the antioxidant potential by oral vitamin C restored the coronary microcirculatory function in young smokers (15).

One of the recently discovered endothelium-derived vasodilator is endothelium-derived hyperpolarizing factor (EDHF) which could explain the existence of an NO/PGI_2-independent component of endothelium-dependent relaxation. The EDHF produced by coronary and renal arteries from humans, pigs, cows, dogs, rats and rabbits displays characteristics similar to those of a cytochrome P450 (CYP)-derived metabolite of arachidonic acid (16). The arachidonic acid metabolites in question are the epoxyeicosatrienoic acids (EETs) generated by endothelial CYP epoxygenases. These substances elicit the hyperpolarization and relaxation of vascular smooth muscle cells by activating calcium-dependent K^+ (K^+_{Ca}) channels as well as the Na-K-ATPase (17).

The exact physiological role of EDHF in the regulation of vascular tone still needs to be elucidated. Even less is known about the impact of disease on EDHF-mediated vasodilation. Of the few studies which determined the role of EDHF in pathological states some reported a diminished EDHF-mediated vascular response (18–20) while others showed an enhanced EDHF-mediated vascular relaxation in diabetic rats (21). Such observations led to the suggestion that EDHF may act as a backup vasodilator in circumstances with a pronounced »endothelial dysfunction« in which the bioavailability of NO is impaired. Cigarette smoking undoubtedly is such a circumstance but it has not been identified yet whether cigarette smoking also exerts some effects on EDHF-mediated relaxation.

Since cigarette smoking represents an atherogenic potential and finally leads to the development of cardiovascular disease, it seems reasonable to further investigate the effect of smoking on cardiovascular function. Most of the studies in-

vestigated either acute effects of smoke (22, 23) or changes induced after chronic exposure to smoke (3, 24). Only a few studies dealt with the problem of subchronic administration of cigarette smoke.

Thus, the study was aimed to determine whether or not subchronic application of cigarette smoke influences endothelial function in rat arteries, particularly the endothelium-dependent vasodilation. Separately we wanted to assess the NO-mediated and the EDHF-mediated vasodilation in arteries of normal rats and in arteries of rats that have been subchronically exposed to cigarette smoke (for 30 days). Furthermore, we wanted to elucidate whether free radicals scavenging improves vasorelaxation in cigarette smoke treated rats and to investigate whether the mechanisms of endothelium-dependent vasodilation, particularly the EDHF-mediated are disarranged in cigarette smoke-treated rats.

Methods

Animals

The study was performed on »Whistar Kyoto« rats divided into two groups of six. One group of rats (n = 6) was exposed to mainstream smoke (2R4F, 450 µg/l, 2 hours/day, 5 days/week for 30 days). The control group was exposed to fresh conditioned air (2 hours/day, 5 days/week for 30 days). On the second day after the last exposition to cigarette smoke, the rats were anesthetized with inhalation anesthetics isofluran and sacrificed. Their abdominal aorta, carotid, mesenteric and renal arteries were harvested and cut into 3–4 mm segments for organ chamber experiments and for free radicals measurement.

Vascular reactivity studies

Vascular reactivity studies were performed on rings of isolated, endothelium-intact rat abdominal aorta, carotid, mesenteric and renal artery. Arteries were isolated and cut into 3–4 mm long rings which were mounted between rigid support and force transducers (Hugo Sachs Elektronik-Harvard Apparatus, Germany) for isometric force measurement. Rings were immersed in organ baths (10 ml) containing oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution (pH 7.4, 37 °C) of the following composition: NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.6 mM, K_2HPO_4 1.2 mM, $NaHCO_3$ 25 mM, glucose 12 mM. Following equilibration for approximately 40 minutes under a resting passive tension of 0.5 g to 1.5 g (depending on the type of the vessel), artery rings were repeatedly contracted with KCl-rich (80 mM)-Krebs solution to determine the maximum contractile force. After washing rings were preconstricted to 80% of their maximal contractile force by phenylephrine (30 nM to 300 nM) and cumulative concentration-relaxation curves to acetylcholine (ACh, 1 nM–10 µM) were obtained in the absence and presence of the eNOS inhibitor N$^{\omega}$nitro L-arginine (L-NA; 300 µM) and the cyclo-oxygenase inhibitor diclofenac (10 µM). After washing period of 1 hour and the reestablishment of a baseline tension, vessels were exposed either to the intracellular superoxide scavenger tiron (4.5-dihydroxy-1.3-benzene-disulphonic acid, 1 mM for 40 minutes), to the CYP inhibitor miconazole (3 µM, rings were incubated with L-NA) or the Na-K-ATPase inhibitor ouabain (100 µM) for 20 minutes before being preconstricted with phenylephrine and determining the concentration-response curve to ACh.

Materials

All drugs were purchased from Sigma (Deisenhofen, Germany).

Data evaluation and statistical analysis

Vascular relaxations are expressed as percentage of the maximal contractions obtained with phenylephrine. Data are presented as means ± SEM of 6 experiments. Statistical analysis was performed using Student's t test for paired data or one-way analysis of variance (ANOVA for repeated measurements) where appropriate. Values of $p < 0.05$ were considered to be statistically significant. R_{max} represents the maximal relaxation (expressed as percentage of the phenylephrine-induced contraction) recorded in response to the cumulative addition of the agonist (ACh). The potency of ACh is expressed as pD_2 (-log EC_{50}) value which was calculated by non-linear regression of the concentration-relaxation curves to ACh using the Prism Graphpad.

Results

Effect of smoking on the NO-mediated relaxations of rat arteries

In phenylephrine-precontracted, endothelium-intact rings of rat aorta, carotid, renal and mesenteric arteries, ACh elicited concentration-dependent relaxations (Figure 1). R_{max} and pD_2 values were: 92.6 ± 1.6 and 7.04 ± 0.11 for the aorta, 80.9 ± 7.7 and 6.55 ± 0.12 for the carotid artery, 83.3 ± 8.9 and 7.08 ± 0.04 for the renal artery and 93.5 ± 4.5 and 6.96 ± 0.04 for the mesenteric artery. In none of the vessels investigated did we observe a significant difference in the relaxations obtained in arteries from control rats and rats exposed to cigarette smoke (Figure 1). R_{max} and pD_2 values for arteries from the smoke-exposed animals were: 84.9 ± 5.4 and 7.38 ± 0.10 for the aorta, 75.3 ± 6.9 and 6.32 ± 0.13 for the carotid artery, 7.05 ± 0.08 and 86.4 ± 4.1 for the renal artery and 96.6 ± 1.6 and 7.02 ± 0.04 for mesenteric arteries. These values did not differ significantly from those obtained using tissue from control animals (for the pD_2 and R_{max} values see above).

Effect of smoking on the EDHF-mediated relaxation of rat arteries

To evaluate the contribution of EDHF to endothelium-dependent vasodilation rings were preincubated with L-NA and diclofenac. Combined eNOS and COX inhibition significantly diminished the ACh-induced relaxation (Figure 1). As expected, the relaxations were almost completely abolished in aorta (R_{max}: 12.4 ± 4.62 and pD_2: 6.34 ± 0.41) and carotid artery (R_{max}: 14.28 ± 6.01 and pD_2: 6.04 ± 0.36) whereas relaxations still remained in renal (R_{max}: 58.7 ± 9.6 and pD_2: 6.61 ± 0.07) and mesenteric artery (R_{max}: 77.6 ± 5.6 and pD_2: 6.67 ± 0.06). This part of relaxation could be attributed to EDHF. Comparing EDHF-mediated relaxations in smoke-treated group of rats to the control group we did not find any significant differences in ACh-induced relaxations (Figure 1) (R_{max} and pD_2 values for the treated group were: aorta: 22.3 ± 5.34 and 6.3 ± 0.23, carotid artery: 1.95 ± 1.36 [pD_2 value not measurable]; renal artery: 41.4 ± 9.4 and 6.46 ± 0.07 and mesenteric artery: 84.0 ± 3.4 and 6.67 ± 0.06; for the values for the untreated group see above). Only in renal artery there was a trend for a rightward shift in the concentration-response curve to ACh which was not significant.

Effect of superoxide scavenging on the NO-mediated relaxations of rat arteries

To evaluate the effect of smoking on the production of reactive oxygen species (ROS), arterial rings were preincubated with the superoxide scavenger tiron for 40 minutes. The inclusion of tiron in the organ bath did not significantly affect the ACh-induced relaxations of any vessels studied from

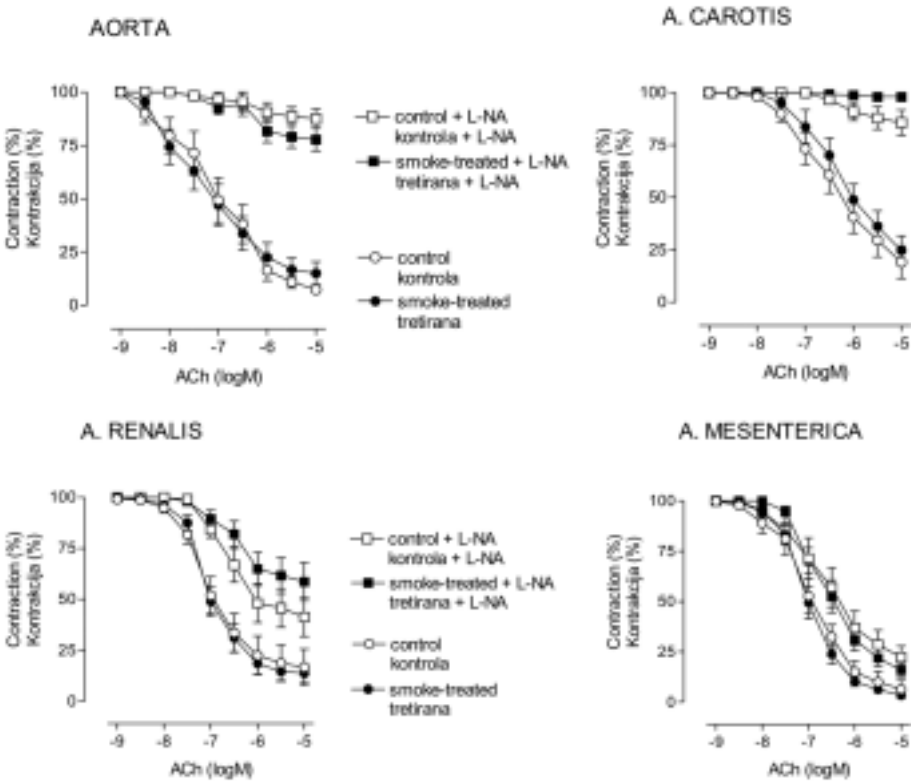


Figure 1. *Effect of cigarette smoking on NO- and EDHF-mediated vasorelaxation, induced by acetylcholine in rat aorta, carotid, renal and mesenteric artery.*

Concentration-response curves to ACh (1 nM–10 μM), obtained in rings of rat arteries from the cigarette smoke-treated group (closed symbols) and from the control group (open symbols). Rings were precontricted with phenylephrine (0,01 to 0,5 μM) in the absence (circles) and presence (squares) of L-NA (300 μM). Results are presented as mean ± SEM of six experiments. Relaxations were significantly ($p < 0.05$) attenuated when rings were pretreated with L-NA.

Sl. 1. *Vpliv (subkroničnega) kajenja na od NO in od EDHF odvisno vazodilatacijo, izzvano z acetilholinom, aorte, karotidne, mezenterične in ledvične arterije podgane.*

Prikazuje krivulje odvisnosti relaksacije od koncentracije ACh (1 nM–10 μM), na žilnih obročkih arterij podgane iz skupin tretiranih (polni simboli) in kontrolnih (prazni simboli) živali. Obročke smo prekontrahirali z noradrenalinom (0,01–0,5 μM) brez (krogci) in v prisotnosti (kvadratki) inhibitorja eNOS, L-NA (300 μM). Prikazani rezultati so srednje vrednosti ± standardna napaka; n = 6.

either of the animal groups. R_{max} and pD_2 values for the different vessels were: 87.5 ± 5.54 and 7.0 ± 0.05 for the aorta, 81.1 ± 7.09 and 6.43 ± 0.12 for carotid arteries, 85.9 ± 6.62 and 7.0 ± 0.05 for renal arteries and 93.2 ± 0.05 and 7.2 ± 3.4 for mesenteric arteries from control rats. In arterial preparations from cigarette smoke-treated rats R_{max} and pD_2 values were: 81.15 ± 3.1 and 6.95 ± 0.05 for the aorta, 77.69 ± 7.97 and 6.41 ± 0.12 and for the carotid, 91.6 ± 2.7 and 6.92 ± 0.07 for the renal and 95.36 ± 1.88 and 6.97 ± 0.04 for the mesenteric artery (Figure 2).

Characterization of the EDHF-mediated relaxations

To determine the nature of EDHF (whether it is CYP 2C9 metabolite), the CYP 2C9 inhibitor miconazole was applied to the bath. Miconazole significantly attenuated the ACh-induced relaxations and shifted the concentration-response curve to ACh to the right in all vessels but no differences were recorded between the treated (R_{max} and pD_2 values for selective vessels were: aorta: 97.26 ± 1.94 and 6.56 ± 0.72, respectively; renal artery: 69.8 ± 5.1 and 6.02 ± 0.08: mesenteric artery: 25.4 ± 5.1 and 6.17 ± 0.09) and the control group (R_{max} and pD_2 values: aorta: 94.53 ± 2.93 and 6.15 ± 0.45; renal artery: 69.6 ± 8.1 and 6.04 ± 0.08 and mesenteric artery: 27.3 ± 7.7 and 6.06 ± 0.04) (Figure 3). Since the NO/prostacyclin-independent relaxations were negligible in aorta and carotid artery preincubation of these vessels with miconazole is irrelevant.

To estimate the involvement of the Na/K-ATPase in the ACh-induced relaxations rings were preincubated with the specific inhibitor of the Na/K-ATPase ouabain in the absence and presence of L-NA, respectively. The inclusion of ouabain in the bath significantly attenuated both, the NO- and the EDHF-mediated relaxations in both groups of animals but the effect of ouabain was more pronounced when rings were pretreated with L-NA (EDHF-mediated relaxations) (Figure 3). There were no differences in the response to the inhibition of the Na/K-ATPase between the treated and untreated group (Figure 3). Interestingly, the relaxations were almost completely abolished in L-NA pretreated renal artery (Figure 3C) (R_{max} and pD_2 values were: control group: 10.1 ± 5.2 and 5.78 ± 0.11, respectively; treated group: 6.60 ± 3.5 and 4.74 ± 0.10) whereas theey were still present in L-NA pretreated mesenteric artery (Figure 3D) (R_{max} and pD_2 values were: control group: 41.3 ± 8.7 and 5.82 ± 0.08, respectively; treated group: 30.9 ± 8.0 and 5.77 ± 0.11).

Discussion

The results of the present study demonstrate that subchronic administration (30 days) of cigarette smoke does not affect the endothelium-dependent, either NO- or EDHF-mediated vasorelaxation in rat arteries. We did not find any differences in NO- or EDHF-mediated relaxations between cigarette-smoke treated rats and unreated rats. Also, the inclusion of the free radicals scavenger tiron did not influence the ACh-induced relaxations in any group. Moreover the inhibition of either the cytochrom P450 or the Na/K-ATPase did not confirm any changes between the treated and untretaed group.

The results contradict most published data and also our hypothesis since many studies showed that cigarette smoking impaires endothelial function in various vascular beds in different animal species as well as in humans (2, 3, 15, 25). However, there are still controversial data about the effect of smoking on endothelial function. Most of the vascular reactivity studies performed on rat arteries (mainly on aorta and coronary vessels) showed an impairment of endothelium-dependent vascular relaxation (26). The study of Kurahashi even (12) reported that in the presence of L-NAME, nicotine induced contractions in rat coronary artery. Similarly, the contraction of canine basilar artery was enhanced in the presence of nicotine (27).

Contrary to the observations of smoking-induced endothelial dysfunction, the study of Nene (28) showed that cigarette smoking increased the endothelium-dependent vasorelaxation in rat carotid artery. In this study, rats were exposed to six cigarettes (Kentucky, 1R4F) per day for 50 days. One of the explanation of enhanced endothelium-dependent vasorela-

xation could be a temporary elevated exogenously derived NO from cigarettes in plasma (29).
Furthermore, it has been reported that acute smoking induces only temporary impairment of vascular function (23) and we may imply that potentially acute changes were abrogated by an efficant mechanism.
In accordance with our findings is the study of Li (30) where no changes, induced by nicotine in rat tail artery, were found. The difference to our study is that rats were exposed to nicotine only and not to the full cigarette-smoke. The discrepancies of the findings of different studies may relate to different protocols used, to different cigarettes and tar and nicotine contents or to different times of exposure to cigarette smoke.
We could only speculate why we could not confirm the hypothesis that subchronic application of cigarette smoke impairs the endothelium-mediated vascular relaxation in rat arteries. In our study we checked the vascular reactivity of many different vessels in rat: aorta (abdominal), carotid, renal and mesenteric artery, so the possibility that cigarette smoking compromises only certain types of vessels could practicaly be ruled out. No changes were observed eighter in the NO-mediated or in the EDHF-mediated relaxations between smoke-treated rats and controls. Also, the application of free radicals scavenger, tiron, did not induce any changes in vascular reactivity. Namely, it has been reported that smoking increases the level of free radicals in plasma (31). We could imply that the generation of free radicals is probably not increased after 30 days of transient exposure to mainstream cigarette smoke or an efficient mechanism is switched on to sufficiently scavange the generated free radicals.
The most probable explanation why smoking did not cause any impairment of endothelial function in rat vessels seems to be the time of exposure to cigarette smoke. It could be possible that 30 days of exposure are not sufficient to induce changes in

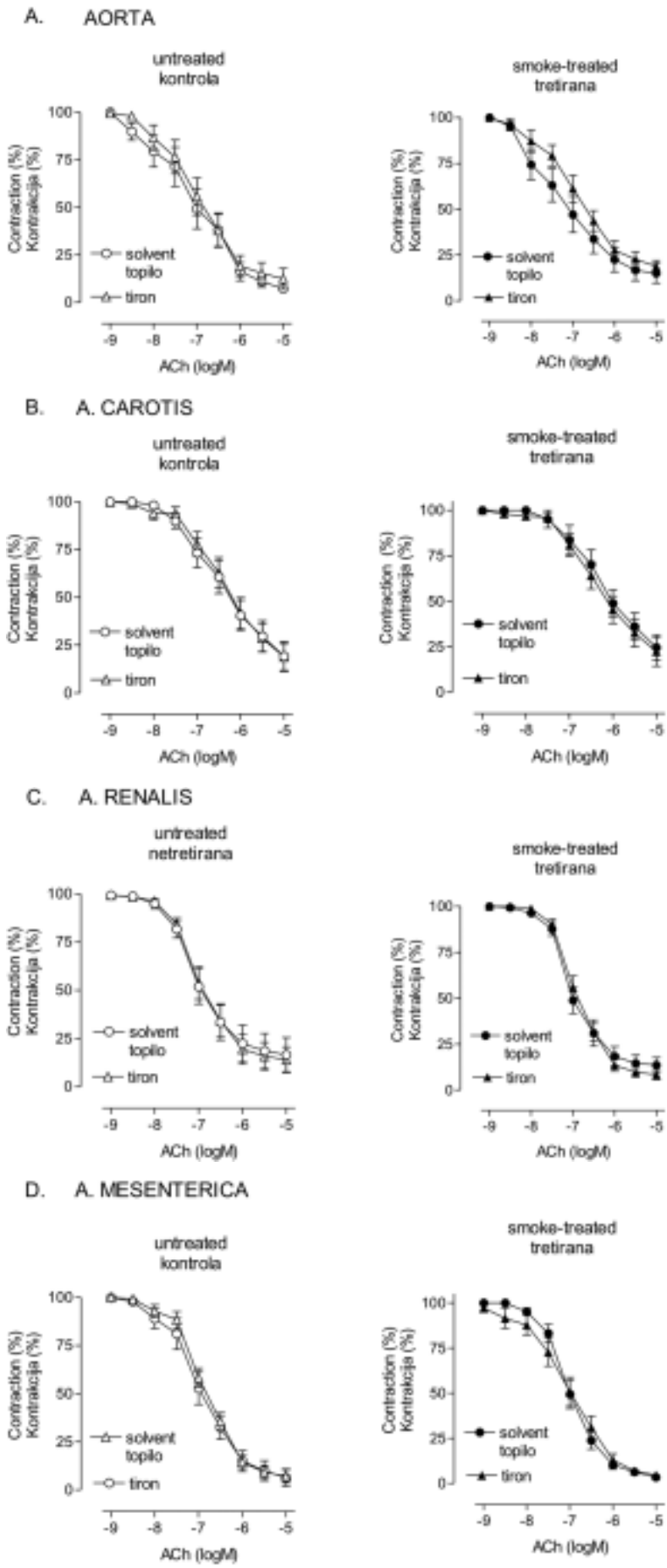
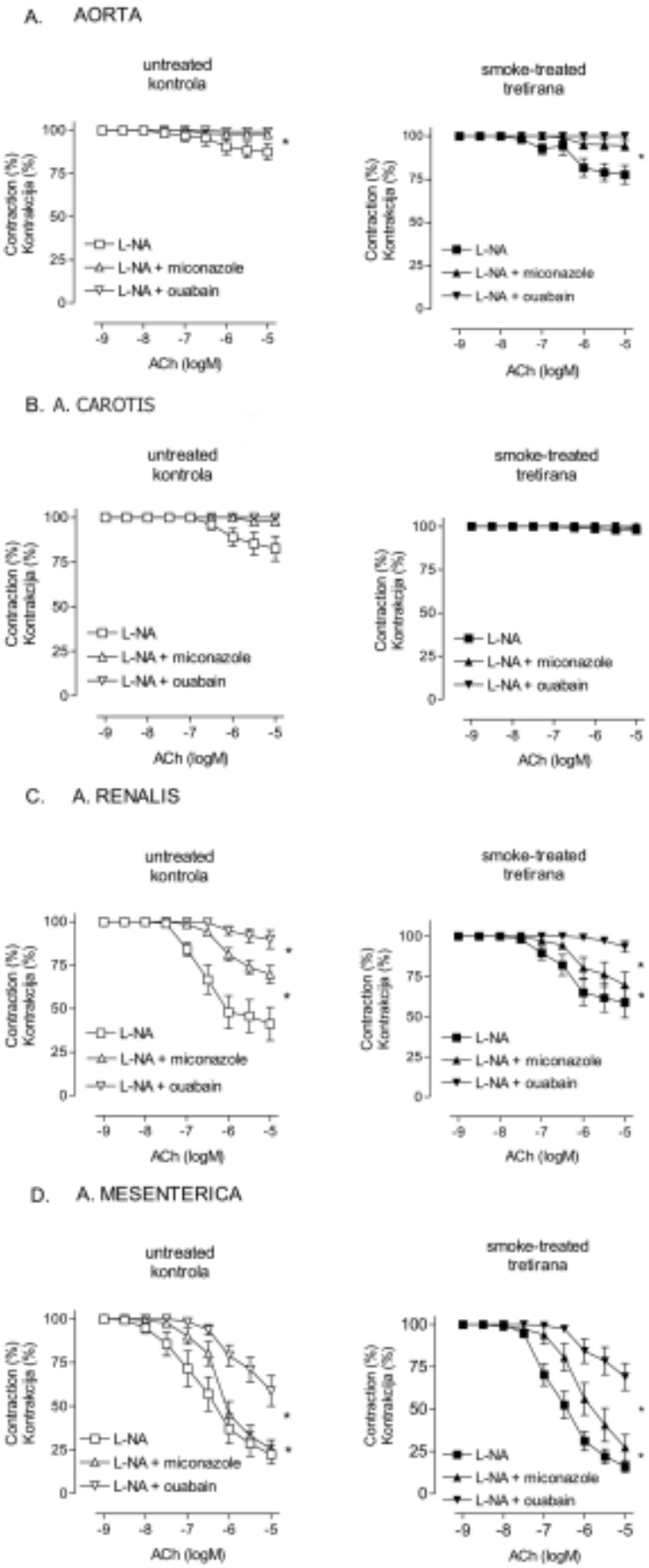


Figure 2. *Effect of tiron, a superoxide scavenger, on NO-mediated relaxations in rat arteries from cigarette-smoke-treated and control group. A. aorta, B. carotid artery, C. renal artery, D. mesenteric artery.*

Concentration-response curves to ACh (1 nM–10 μM), obtained in phenylephrine (0.01–0.5 μM)-preconsticted rings of rat arteries from the cigarette smoke-treated group (closed symbols) and from the control group (open symbols). Rings were preconstricted with phenylephrine (0.01–0.5 μM) in the absence (solvent; circles) and presence (triangles) of the superoxide scavenger tiron (1 mM). Results are presented as mean ± SEM, n = 6.

Sl. 2. *Vpliv tirona, »lovilca prostih radikalov«, na od NO odvisno vazodilatacijo arterij podgane iz tretirane in kontrolne skupine živali. A. aorta, B. karotidna arterija, C. ledvična arterija, D. mezenterična arterija.*

Prikazuje krivulje odvisnosti relaksacije od koncentracije ACh (1 nM–10 μM), na žilnih obročkih arterij podgane iz skupin tretiranih (polni simboli) in kontrolnih (prazni simboli) živali. Obročke smo prekontrahirali z noradrenalinom (0,01–0,5 μM) brez (topilo; krogci) in v prisotnosti (trikotniki) »lovilca prostih radikalov« tirona (1 mM). Prikazani rezultati so srednje vrednosti ± standardna napaka; n = 6.



endothelial function. Along with this assumption goes also the study of Li (30) where rats were daily exposed to cigarette smoke for 14 days and no changes were observed. Another possibility could be insufficient concentration of toxic substances in cigarette smoke (2R4F, 450 µg/l) that reached the systemic circulation. As already mentioned, it could be that pottentially acute changes induced by smoking were only temporary (23). Namely, our experiments were performed on the second day after cessation of smoke exposure so it could also be possible that if smoking had induced some acute changes the effects had already vanished in this time. In this case we were not able to register the acute effects of smoking. On the other hand, 30 days of exposure to smoke were not sufficient for chronic changes to occur.

Apart from the effects of smoke on endothelial function some other interesting findings of our study should be stressed. The maximal ACh-induced relaxations were significantly smaller when rings were treated with L-NA. These results imply that EDHF appears to play only a minor role in the regulation of vascular tone under physiological conditions. As we did not find any differences between treated and control animals, it is difficult to comment on its role in pathological circumstances. The NO/PGI_2-independent relaxations were more pronounced in smaller vessels (renal and mesenteric artery) than in larger ones (aorta and carotid artery) what is in accordance with the general belief that the contribution of EDHF to the endothelium-dependent relaxations increases as the vessel size decreases (32). EDHF-mediated relaxations were attenuated by miconazole and ouabain in mesenteric artery and almost completely abrogated by ouabain in renal artery. These observations support the hypothesis that EDHF is a metabolite of CYP epoxygenase and that its effect involves the Na-K-ATPase. The fact that ouabain also diminished the NO-dependent relaxation in all vessels studied (data not shown) confirmes the suggestion that part of the NO-mediated relaxation is mediated by the Na/K-ATPase (33).

Figure 3. *Effect of the CYP 2C inhibitor miconazole or the Na/K-ATPase inhibitor ouabain on the EDHF-mediated relaxations in rat (A) aorta, (B) carotid, (C) renal and (D) mesenteric artery.*

Arterial rings from the smoke-treated group (closed symbols) and from the control group (open symbols) were incubated with L-NA (300 µM), preconstricted with phenylephrine (0.01–0.5 µM) and concentration-response curves to ACh (1 nM–10 µM) were performed in the absence and presence of the CYP 2C inhibitor miconazole (3 µM) or the Na/K-ATPase inhibitor ouabain (1 mM), respectively. Results are presented as mean ± SEM of six experiments. * $p < 0.05$.

Sl. 3. *Vpliv inhibitorja CYP, mikonazola, in inhibitorja Na/K-ATPaze, ouabaina, na vazodilatacijo, ki jo posreduje EDHF, (A) aorte, (B) karotidne, (C) ledvične in (D) mezenterične arterije podgane pri skupini tretiranih in kontrolnih živali.*

Obročke arterij iz skupine tretiranih (polni simboli) in kontrolnih (prazni simboli) živali smo inkubirali z L-NA (300 µM), prekontrahirali z noradrenalinom (0,01–0,5 µM) in izzvali krivulje odvisnosti relaksacije od koncentracije ACh (1 nM–10 µM); relaksacijo smo izzvali brez in v prisotnosti CYP 2C inhibitorja, mikonazola (3 µM) oziroma inhibitorja Na/K-ATPaze, ouabaina (1 mM). Prikazani rezultati so srednje vrednosti ± standardna napaka; n = 6. * $p < 0,05$.

Conclusion

In conclusion the results of the present study show that 30-days of exposure to cigarette smoke did not induce recordable changes in endothelial function in rat abdominal aorta, carotid, mesenteric and renal artery. Most probably, 30 days of cigarette smoking are not sufficient to induce chronic changes; accute changes could not be excluded because of the experimental protocol.

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