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EICOSANOID PRODUCTION AND LYMPHATIC RESPONSIVENESS IN HUMAN CIGARETTE SMOKERS COMPARED WITH NON-SMOKERS

H. Sinzinger

Wilhelm Auerswald Atherosclerosis Research Group Vienna (HS,AO), Department of Internal Medicine, Hospital Göttlicher Heiland, Vienna (JK), and Department of Nuclear Medicine, University of Vienna (HS,AO), Austria

ABSTRACT

Leg lymphatic segments were isolated from 10 patients (4 cigarette smokers and 6 non-smokers) undergoing conventional lymphography. Prostaglandin (PG) levels and PG synthesis in the lymphatics and in a variety of body fluids and the effects of eicosanoids on lymphatic contractility were determined. Leg lymphatics from 4 smokers generated less PGI_2 and contained more 8-epi-$PGF_{2\alpha}$ when compared with leg lymphatics in 6 non-smokers. Similarly, levels of 8-epi-$PGF_{2\alpha}$ in smokers compared with non-smokers were higher in plasma (28.6 cf 19.7 pg/ml), leg lymph (146.7 cf 65.3 pg/ml), serum (299.0 cf 204.1 pg/ml), and urine (473.4 cf 241.0 pg/mg creatinine). Lymphatics from smokers also showed a higher contractile response, less ^{14}C-arachidonic acid conversion to PGI_2 and less PGI_2-formation with various stimuli compared with non-smokers. Together these findings suggest that smoking induces oxidation injury, promotes altered (iso-)eicosanoid production and impacts on the function and dysfunction of peripheral lymphatics under normal circumstances and in a variety of clinical disorders.

Smoking, even passive, like other risk factors in the development of atherosclerosis, adversely affects the prostaglandin (PG)-system by increasing proaggregatory (1) and contractile elements (personal observations), while decreasing antiaggregatory (2) compounds. In the blood vascular system, these effects promote hemostatic imbalance favoring thrombosis and/or atherosclerosis. Lipid peroxidation occurs during exposure to free oxygen radicals (3), and the formed 15-hydroxyperoxy-arachidonic acid is a recognized inhibitor of PGI_2-synthase (4). Isoprostanes and in particular 8-epi-$PGF_{2\alpha}$, the most investigated member of the family of compounds (7) are formed during free oxygen radical catalyzed action at the local site (8). To date, however, no information is available on the relationship of cigarette smoking and the (iso-)eicosanoid system to the lymph vascular system. Because both PGI_2 (9) and 8-epi-$PGF_{2\alpha}$ (10) are potent antagonists of lymphatic tone, we examined the formation and conversion of PGI_2 in the presence of 8-epi-$PGF_{2\alpha}$ in human peripheral lymphatics and lymph derived from both smokers and non-smokers. Plasma and serum levels and urinary excretion of these compounds were also determined in these individuals.

MATERIALS AND METHODS

Lymph Vessel Isolation

TABLE 1
8-epi-$PGF_{2\alpha}$ and PGI_2 (or one of its Metabolites)* in Various Compartments

		non-smokers	n	smokers	n	units
lymph vessels	8-epi-$PGF_{2\alpha}$	110.7 ± 5.4	6	287.4 ± 39.6**	4	pg/ml
	PGI_2	4.7 ± 2.2	6	2.0 ± 0.6**	4	pg/ml
lymph fluid	8-epi-$PGF_{2\alpha}$	65.3 ± 15.8	5	146 ± 25.2**	4	pg/ml
	6-oxo-$PGF_{1\alpha}$	18.6 ± 5.5	6	10.2 ± 3.9**	4	pg/ml
plasma	8-epi-$PGF_{2\alpha}$	19.7 ± 3.1	6	28.6 ± 2.8**	4	pg/ml
	6-oxo-$PGF_{1\alpha}$	<1	6	<1	4	pg/ml
serum	8-epi-$PGF_{2\alpha}$	204.1 ± 22.6	6	299.0 ± 26.3**	4	pg/ml
	6-oxo-$PGF_{1\alpha}$	186.4 ± 17.3	6	299.0 ± 26.3**	4	pg/ml
urine	8-epi-$PGF_{2\alpha}$	241.0 ± 36.2	5	473.4 ± 30.8**	4	pg/mg creatinine
	2,3-dinor-6-oxo-$PGF_{1\alpha}$	299.0 ± 26.3	6	196.5 ± 31.7**	4	pg/mg creatinine

*$\bar{x}$±SD; **p<0.01

Leg lymphatic segments were obtained from 4 "smokers" (3 males, 1 female; age 24-53 years) and 6 "non-smokers" (4 males, 2 females; age 15-47 years) undergoing conventional (oil-contrast) lymphography. The reason for lymphography was for diagnosis of malignant lymphatic cell disease. No abnormality of lymphatic vessels was present. None was taking a drug known to affect either oxidation or the PG system during the previous week. Each smoker had used >20 cigarettes/day (range 20-50) for 7-34 years. The protocol was carried out in accordance with the Declaration of Helsinki and written informed consent of each patient was obtained. Tissue samples were rinsed in ice-cold buffer (pH 7.4) and further processed. For 8-epi-$PGF_{2\alpha}$ determination, tissue was weighed, homogenized by Ultraturex and extracted and purified by chromotography. To measure PGI_2-production, the lymphatic segments were incubated after a single wash in ice-cold (0°C; pH 7.4) Tris-HCl-buffer for 3 minutes in 300 µl Tris-HCl-buffer at 37°C. After weighing (mg weight after drying by filter paper) of the lymphatic segments, the incubation buffer was frozen and stored at -70°C until assaying (< 2 weeks).

Radiothinlayer Chromotography (RTLC)

Minced lymphatics were incubated together with 0.50 µCi (56.2 mCi/mmol) ^{14}C-arachidonic acid for 5 minutes at 37 °C in a shaking water bath. After stopping the conversion by acidification (HCl, pH 3), the samples were centrifuged, decanted, and extracted (ethyl acetate; Merck, Darmstadt, Germany). The organic phase was dried under nitrogen. The material together with the respective standards was automatically (Lamag-Linomat, Bonaduz, Switzerland) mounted on a thin-layer chromatography plate (DC-Fertigplatte, Kieselgel 60; Merck, Darmstadt, Germany) and finally measured and quantified using an automatic TLC-analyzer (LB 283; Berthold, Wildbad, Germany).

Lymph Fluid

TABLE 2
Conversion (%) of Exogenous ^{14}C-arachidonic Acid* in Human Leg Lymphatics

Eicosanoid	NS (n=6)	S (n=4)
6-oxo-PGF$_{1\alpha}$	3.71 ± 0.74	2.66 ± 0.29[1]
PGE$_2$	1.34 ± 0.56	1.27 ± 0.62
PGF$_{2\alpha}$	0.78 ± 0.31	0.81 ± 0.46
TXB$_2$	0.11 ± 0.07	0.47 ± 0.12[2]
OH-fatty acids	2.64 ± 0.31	4.87 ± 0.69[2]

*($\bar{x}$±SD); [1]p<0.05; [2]p<0.01;
NS=non-smokers; S=smokers

Leg lymph fluid was obtained using microhematrocrit tubes by capillary action in the presence of 1% EDTA and 20 mg acetylsalicylic acid (ASA)/ml. After centrifugation (4°C, 10 minutes, 1500xg) lymph samples were stored at -70°C until assaying (< 2 weeks).

Contractility

The lymphatic segments were cut into small rings with a circumference of about 2-5 mm. Two wires were fixed to the lumen as described by Johnston and Gordon (11). The lower one was fixed to the bottom of a 5 ml perfusion bath, the upper one connected with an isometric transducer (Harvard Instruments Recorder Pharmacia, Sweden). The lymph vessels were continuously perfused with an oxygenated (95% O_2, 5% CO_2) Krebs-Ringer solution at a temperature of 37°C. Thereafter, the lymphatic segments were placed under 500 mg tension until equilibrium (60-90 minutes). The test substances were then added in various concentrations.

Plasma, Serum, and Lymph Fluid (Iso-)Eicosanoids

Blood was drawn from each patient on the morning of the day of investigation after 24 hours urine sampling. After at least 12 hours overnight fasting, 4.5 ml of blood was anticoagulated with 1% EDTA and 10 mg ASA/ml, and a plasma creatinine level was determined. Processing and measurement of (iso-)eicosanoids were performed as previously described by our group in detail (7,12,13). The inter- and intraassay variation for 8-epi-$PGF_{2\alpha}$ was less than 8.7 and 5.6%, and for PGI_2 (i.e., metabolites) < 6.0 and 3.4%.

Urinary (Iso-)Eicosanoids

Urine was collected over a 24-hour period. The total volume was determined and an aliquot processed for determination of 8-epi-$PGF_{2\alpha}$, 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and creatinine levels (radioimmunology).

Statistical Analysis

Values are presented as mean ± SD; calculation for significance was performed using Student's t-test and ANOVA.

RESULTS

PGI_2-Synthesis

Lymphatics derived from smokers generated significantly (p<0.01) less PGI_2 when compared to lymphatics of non-smokers *(Table 1)*. Although no gender difference was detected, the number of samples was very small. In smokers, RTLC showed a significant lower conversion of exogenous ^{14}C-AA to PGI_2 (p<0.05) but higher conversion to thromboxane (TXB_2) and hydroxy fatty acids *(Table 2)*. Exposure to leukotrienes (LTC_4) generated an increase in PGI_2-production *(Fig. 1)* at 50 ng and above in lymph vessels derived from non-smokers. On the other hand, in smokers only at the highest concentration (100 ng) was a significant PGI_2 response to LTC_4 elicited.

TABLE 3
8-epi-$PGF_{2\alpha}$/6-oxo-$PGF_{1\alpha}$ Ratio in Smokers Compared with Non-smokers

	non-smokers	smokers	unit
lymph vessels	23.55	143.70	pg/ml
lymph fluid	3.51	14.28	pg/ml
plasma	19.73	28.65	pg/ml
serum	1.09	1.71	pg/ml
urine	1.23	3.25	pg/mg creatinine

8-epi-$PGF_{2\alpha}$

Levels of this compound in the lymph vessels of smokers were significantly greater than in the lymph vessels of non-smokers ($p<0.01$). In contrast, PGI_2 were significantly lower in the same lymphatic samples *(Table 2)*.

Lymph Fluid

The concentration of the isoprostane 8-epi-$PGF_{2\alpha}$ in the lymph fluid of smokers was more than twice that of non-smokers, whereas the 6-oxo-$PGF_{1\alpha}$ level was nearly one-half *(Table 1)*.

Lymphatic Contractility

Lymphatics from smokers reacted more vigorously to 8-epi-$PGF_{2\alpha}$ *(Fig. 2)* becoming significant at 100 ng/ml and less intensely to PGI_2 *(Fig. 3)*. Overall, the lymphatic contractile response (data not shown) was more intense in smokers.

Plasma and Serum (Iso-)Eicosanoids

6-oxo-$PGF_{1\alpha}$, the stable metabolite of PGI_2, was in the plasma of both smokers and non-smokers below the detection limit of 1 pg/ml *(Table 1)*. In contrast, plasma levels of 8-epi-$PGF_{2\alpha}$ were significantly higher in smokers as compared with non-smokers *(Table 1)*.

Urinary Excretion

Whereas urinary excretion of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ was lower in smokers, 8-epi-$PGF_{2\alpha}$ excretion was higher when compared with non-smokers *(Table 1)*.

8-epi-$PGF_{2\alpha}$/6-oxo-$PGF_{1\alpha}$-ratio

This ratio in all the examined fluid and tissue samples was significantly greater in smokers as compared with non-smokers *(Table 3)*.

DISCUSSION

Johnston et al (11,14) first called attention to the role of cyclooxygenase and lipoxygenase pathways as regulators of lymphatic vasomotion. We previously demonstrated that human lymphatics convert exogenous arachidonic acid primarily to PGI_2 (15) similar to human arteries (16). PGI_2 is the dominant compound (17) generated by endogenous substrate fatty acids. A substantial amount of 6-oxo-$PGF_{1\alpha}$, the stable metabolite of PGI_2, is present in lymph fluid and a variety of data have shown that compounds in draining lymph such as TXA_2 and PGH_2 are lymphatic contractile agonists

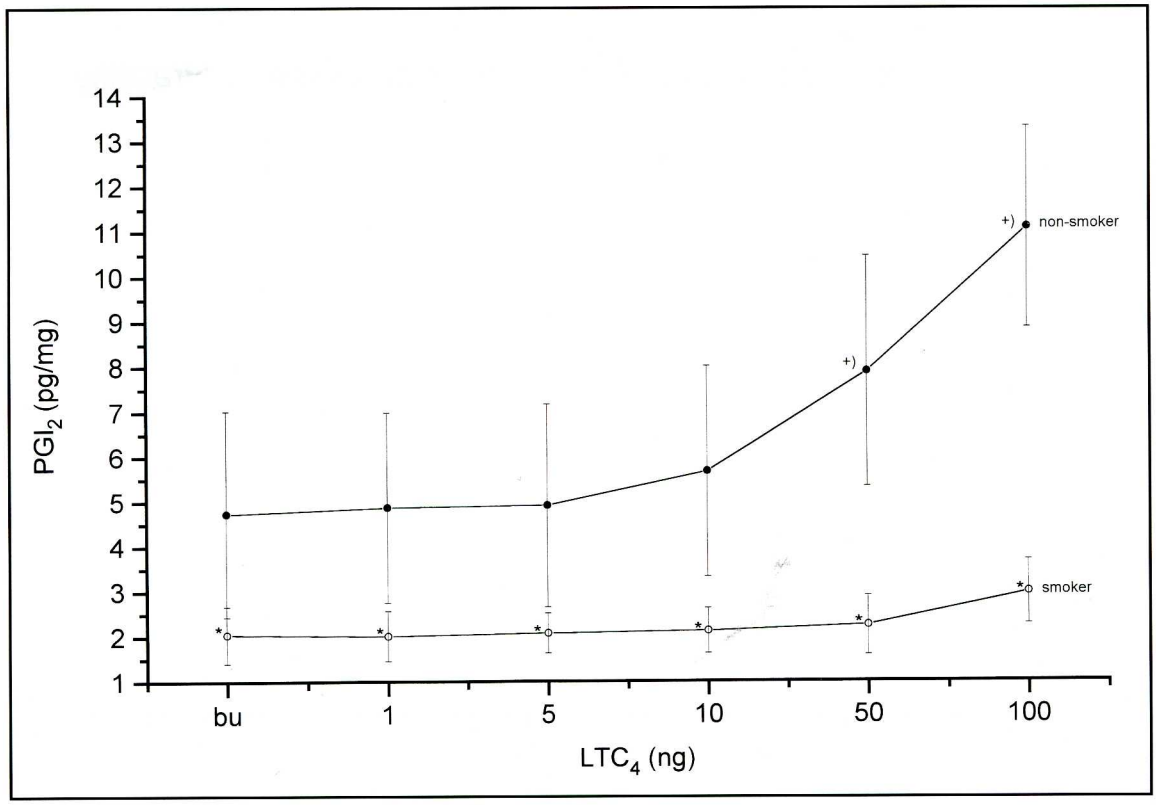

*Fig. 1. Influence of leukotriene C_4 (LTC_4) on PGI_2-synthesis. LTC_4 (ng/ml) generates an increase in PGI_2 but far less in lymphatics derived from cigarette smokers compared with non-smokers (Data in $\bar{x}\pm$ SD). n=4 (smokers) and 6 (non-smokers), respectively; *p<0.01 (vs. other group); $^{+)}$p<0.01 (vs. prevalue); bu=buffer control)*

(9). In a more recent study, we showed that the peripheral lymphatic contractile responses to (iso-)eicosanoids are in the sequence of potency of 8-epi-$PGF_{2\alpha}$ > the thromboxane receptor agonist U-44069 > 8-epi-PGE_2 > $PGF_{2\alpha}$ > 8-epi-PGE_1. 8-epi-$PGF_{2\alpha}$ is a major eicosanoid constituent in lymph. During investigation into the effect of lymph flow on vasomotion of rat lymph microvessels, flow induced lymphatic constriction was abolished by indomethacin, a potent prostaglandin inhibitor (18). These workers concluded that this inhibitory effect was due to the release of endogenous vasoconstrictor prostaglandins (PGH_2, TXA_2). Our findings in the present study support that cigarette smoking not only alters the lymphatic basal and stimulated (19) synthetic profile of prostaglandins (19) but also the PG-content in lymph fluid and the lymphatic response to contractile agonist. Thus, in smokers the lymphatic and lymph concentration of 8-epi-$PGF_{2\alpha}$ increased and also the lymphatic contractile response to this compound was greater. Although the effect was small, it was significant.

Minor amounts of 8-epi-$PGF_{2\alpha}$ may form via cyclooxygenase. Pilot experiments with 2 lymphatic segments incubated with and without aspirin did not, however, reveal a difference in 8-epi-$PGF_{2\alpha}$ concentration. These findings suggest that if there is any COX-1 dependent 8-epi-$PGF_{2\alpha}$ in the lymph vessel, it is very small and probably clinically irrelevant. Moreover, 8-epi-$PGF_{2\alpha}$ is only a minor component of the larger isoprostane family although it is easier to assay and likely a reliable marker for oxidative stress.

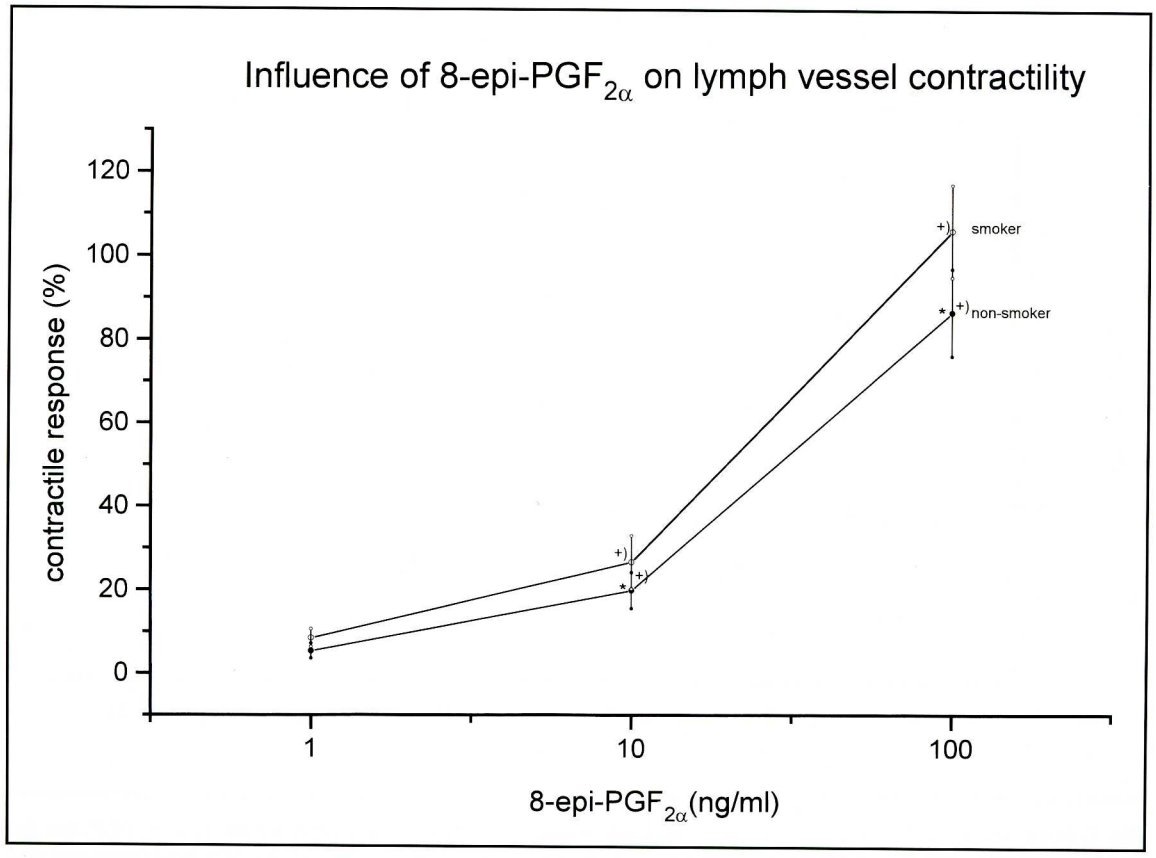

*Fig. 2. Contractile response of peripheral human leg lymphatic suspended in-vitro and exposed to 8-epi-PGF$_{2\alpha}$. The isoprostane dose-dependently contracts lymphatics in smokers more than non-smokers. (Data in $\bar{x} \pm SD$; *p<0.01) (vs. other group); +) vs. prevalue*

Nonetheless, its biological activity when compared with many other isoprostanes still needs to be assessed.

Morrow et al (20) described higher plasma levels of free and esterified F_2-isoprostanes and increased urinary excretion of 8-epi-PGF$_{2\alpha}$ in smokers. Higher 8-epi-PGF$_{2\alpha}$ has also been found in arterial tissue derived from cigarette smokers compared with non-smokers (21). Described as an index for human atherosclerosis, the ratio (8-epi-PGF$_{2\alpha}$ vs. 6-oxo-PGF$_{1\alpha}$) is several fold higher in smokers in all tissues and body fluids thus far examined. A similar relationship was found for lymphatics and fluids in this study.

Taken together, our findings support that the (iso-)eicosanoid synthetic, content and contractility profile is substantially different between cigarette smokers and non-smokers. These data further suggest that cigarette smoking involves oxidative *in vivo* modification of a wide variety of biologically relevant molecules. Information as to ex-smokers and the time period required for biochemical modification is unknown. It seems likely that tobacco use deleteriously affects the lymphatics (iso-)eicosanoid profile as it has already been documented for the cardiovascular system (2,22). This detrimental metabolic effect of cigarette smoking on prostaglandin metabolism and lymphatic dynamics may have relevant implications for a variety of normal and abnormal biological

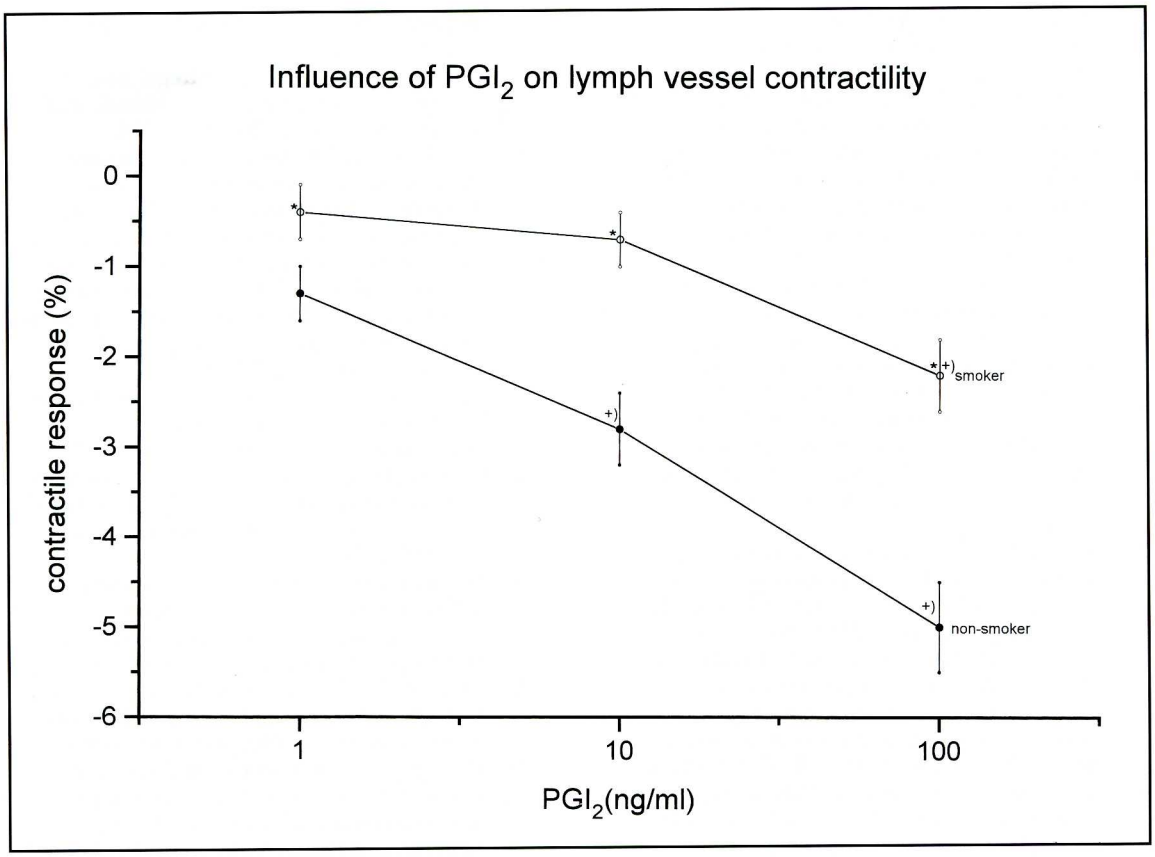

*Fig. 3. Influence of PGI_2 on lymphatic contractility. PGI_2 is less effective in "relaxing" lymphatics in cigarette smokers than in non-smokers. (Data in $\bar{x} \pm SD$; *p<0.01) (vs. other group); +)vs. prevalue*

functions such as inflammation. Because 8-epi-$PGF_{2\alpha}$ has a much longer half life than PGI_2 and is a potent stimulus for lymphatic contraction, alterations of this compound may have special relevance for lymph dynamics. Besides smoking, inflammation, hyperlipoproteinemia, hypertension, diabetes mellitus may also influence regional and systemic lymphatic vasomotion excluding day to day, circadian (6), and seasonal biochemical variations. During lipid peroxidation, large amounts of isoprostanes may form, a finding that may influence transport of chyle.

In conclusion, these findings suggest that cigarette smoking modulates (iso-)eicosanoid metabolism and human lymphatic dynamics. Its full clinical significance still needs to be elucidated.

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Professor Helmut Sinzinger, M.D.
Wilhelm Auerswald Atherosclerosis Research
Group (ASF) Vienna,
Nadlergasse 1, A-1090 Vienna, AUSTRIA
Tel.: +43.1.4082633
Fax: +43.1.4081366
E-mail: Helmut.Sinzinger@akh-wien.ac.at