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Towards biochemical marker of endothelial dysfunction; identification by Raman spectroscopy

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In the present work we propose the spectroscopic approach to identify biochemical alterations in endothelial dysfunction. The method is based on the quantification of the ratio of phenylalanine (Phe) to tyrosine (Tyr) contents in the endothelium. The synthesis of Tyr from Phe requires the presence of tetrahydrobiopterin (BH4) as a cofactor of phenylalanine hydroxylase (PAH). Limitation of BH4 availability in endothelium is a hallmark endothelial nitric oxide synthase (eNOS) dysfunction that may also lead to PAH dysfunction and a fall in Tyr contents. Using Raman spectra, the ratio of marker bands of Tyr to Phe was calculated and the pathological state of endothelium detected. We provide evidence that Phe/Tyr ratio analysis by Raman spectroscopy discriminate endothelial dysfunction in ApoE/LDLR^{-/-} mice as compared to control mice.

Introduction

Atherosclerosis, the major cause of death in industrialized societies [1, 2] is understood as a chronic inflammatory vascular inflammation triggered and promoted by endothelial dysfunction [3]. Indeed, the endocrine/paracrine/autocrine function of vascular endothelium plays a fundamental role in the regulation of cardiovascular system that includes the regulation of inflammatory, immunological and thrombotic processes, vascular tone, blood flow, vascular wall permeability and structure, as well as various metabolic processes. The evidence accumulated that endothelial dysfunction, that is associated with the impairment of activity of vasoprotective mediators, leads to *atherothrombosis* and is even considered as an independent prognostic factor of cardiovascular risk [4, 5]. Accordingly, endothelial pharmacotherapy, can be regarded as a new approach in preventing *atherothrombosis* and other cardiovascular diseases [1, 4, 6].

Nitric oxide (NO) represent a major endothelial mediator involved the regulation of cardiovascular homeostasis [7] and in endothelium it is produced by the endothelial nitric oxide synthase (eNOS). NO affords anti-atherogenic effects, inhibits platelets and leukocytes activity, as well as vascular smooth muscle proliferation. In particular, NO deficiency represent a biochemical hallmark of endothelial dysfunction [8] and is frequently linked to the impaired availability of BH4.

In fact, the enzymatic synthesis of NO by eNOS requires the activity of tetrahydrobiopterin (BH4) as a cofactor (Fig. 1). In healthy endothelium the eNOS catalyses the formation of NO by coupling an oxidation for the amino acid L-arginine with the reduction of the molecular oxygen. As a byproduct, L-citrulline is produced. When availability of BH4 is limited, eNOS is

uncoupled and superoxide $O_2^{-\cdot}$ is produced instead of NO, increasing this way an oxidative stress [8, 9]. Importantly, BH4 deficiency as well as the reduction of NO bioavailability seem to be a common feature of endothelial dysfunction not only in atherosclerosis but also in many other cardiovascular or metabolic diseases including diabetes and hypertension.

Fig. 1 A scheme depicting the involvement of BH4 as a cofactor in NOSes and PAH in endothelium – the conceptual framework for the spectroscopic-based assay of Phe:Tyr ratio as a diagnostic marker for endothelial dysfunction. (NOS-nitric oxide synthase; eNOS- endothelial NOS; nNOS-neuronal NOS; iNOS- inducible NOS; L-arg- L-arginine; L-cit- L-citrulline; PAH- phenylalanine hydroxylase; Phe-phenylalanine; Tyr-tyrosine) [9].

Interestingly, BH4 is also a cofactor of various other enzymes [9] including PAH that is also present in endothelium but its role is largely unknown. Anyhow., limitation of BH4 availability in endothelium that represent a hallmark of eNOS dysfunction may also lead to PAH dysfunction, resulting in the alteration of Phe:Tyr ratio in the endothelium. Since using Raman spectra, the ratio of marker bands of Tyr to Phe can be calculated, in the present work we aimed to provide evidence that Phe:Tyr ratio analysis by Raman spectroscopy may discriminate endothelial dysfunction in endothelium in ApoE/LDLR^{-/-} mice that spontaneously develop

atherosclerosis (ApoE/LDLR$^{-/-}$) as compared with healthy endothelium in control mice (C57BL/6J) [10]. Additionally, we also tested whether the Phe:Tyr ratio will be modified in ApoE/LDLR$^{-/-}$ mice treated with 1-methylnicotinamide (MNA) known to have vasoprotective activity and to reverse endothelial dysfunction [11, 12]

Experimental

The samples were resected from a thoracic fragment of the aorta taken from the ApoE/LDLR$^{-/-}$ mice (n=4, 3-4 samples for each mice, called here ApoE/LDLR), ApoE/LDLR$^{-/-}$ mice treated with MNA (100mg/kg of body mass) for 2 month (n=4, 1 sample for each mouse, called here ApoE/LDLR MNA) and C57/BL/6J mice (n=6, 2 samples for each mouse, called here C57) at the age of 6 months. After resection, the samples were fixed for 10 min in 4% buffered formalin (Merck), then embedded in the OCT medium (Thermo) and frozen at −80 °C. The 5 μm thick cross-section slides were put on the calcium fluoride substrate. The experimental procedure used in the present study was approved by the local Animal Research Committee.

Raman imaging were done with a Confocal Raman Imaging system WITec alpha 300 with the application of 100× air objective (Olympus, MPlan, NA=0.9). The laser excitation wavelength of 532 nm, laser power of ca. 10 mW and integration times of 0.2 s per spectrum were used. Images of 140 × 140 pixels and 25 by 25 μm edge length were recorded. For spectra from every image the preprocessing was applied – cosmic rays were removed and background was subtracted.

Results and discussion

The protocol of analysis, described in details below, was focused on the separation of the endothelium area from other parts of the vessel, and consequently determine the representative spectra, ascribed to the endothelium. The main difficulties were in identification of the signals coming from the subendothelial space and media of the vessels as opposed the signals from the endothelium.

The spectra were analyzed with chemometric tools (Fig. 2). To assign the spectra to the endothelium, the K-means cluster analysis (KCA) (for 15 class) was performed for each obtained Raman hyperspectral data set in CytoSpec 2.00.01 [13]. Then, for every image, 30 randomly chosen spectra from the most inner layer i.e. endothelium were isolated and then averaged as a single spectrum. The averaged spectra obtained in this way were used for hierarchical cluster analysis (HCA) performed in OPUS7.0. The process were done using the Ward's algorithm [14] and vector normalization in two following ranges: 1040-990 cm^{-1} and 871-824 cm^{-1}. The integration ratios and the statistical ANOVA test together with the Tukey test were calculated in OriginPro 9.0 for the significance level of 0.05 [15]. It should be underlined that the Raman intensities (ant their ratios, consequently) are not absolute measures of the

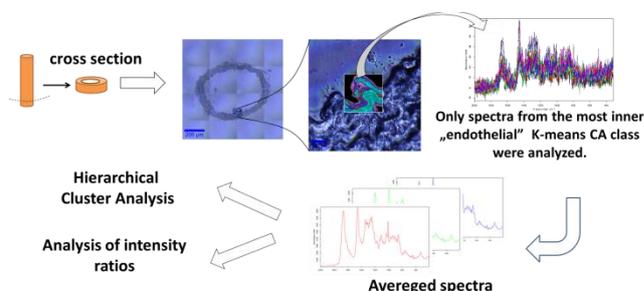content of the respective components and may have different scattering cross-sections.

Fig. 2 The scheme of the protocol for sample preparation, measurement and analysis.

General characteristics of the average spectra extracted from endothelium

The average spectra obtained from single mean spectra coming from: ApoE/LDLR, ApoE/LDLR MNA and C57 (control) mice are compared in
Fig. 3. The single mean average spectra were extracted from the most inner layer of the vessel wall (presumably the endothelium) of the each sample, according to the protocol in Fig. 2.
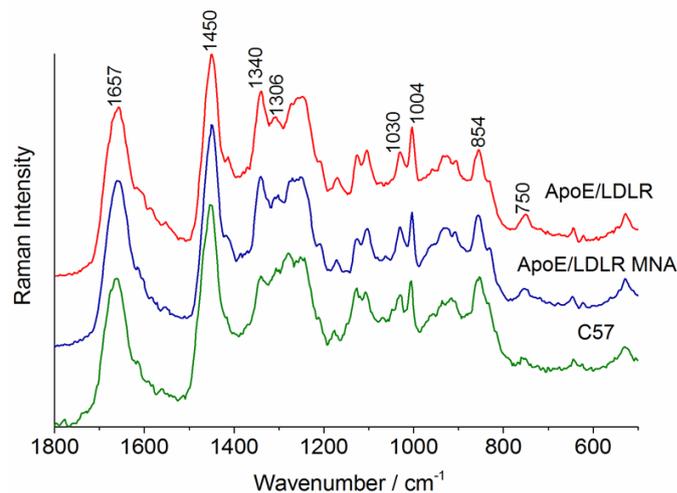

Fig. 3 The comparison of the average spectra obtained from the endothelium of atherosclerotic (ApoE/LDLR), atherosclerotic treated with MNA (ApoE/LDLR MNA) and control (C57) mice.

The spectra (Fig. 3) are dominated by a typical protein profile. The main difference in the spectra between analyzed models is seen in the range of the amide III bands i.e. 1340 cm^{-1}, 1306 cm^{-1} and 1250 cm^{-1}. Nevertheless, this range is characteristic also for collagen (bands at 1306 cm^{-1}, 1340 cm^{-1}) [16, 17] as well as for elastin [3]. We need to stress that even the endothelial layer was defined, some overlapping with signals coming from the deeper parts of tissue cannot be excluded.
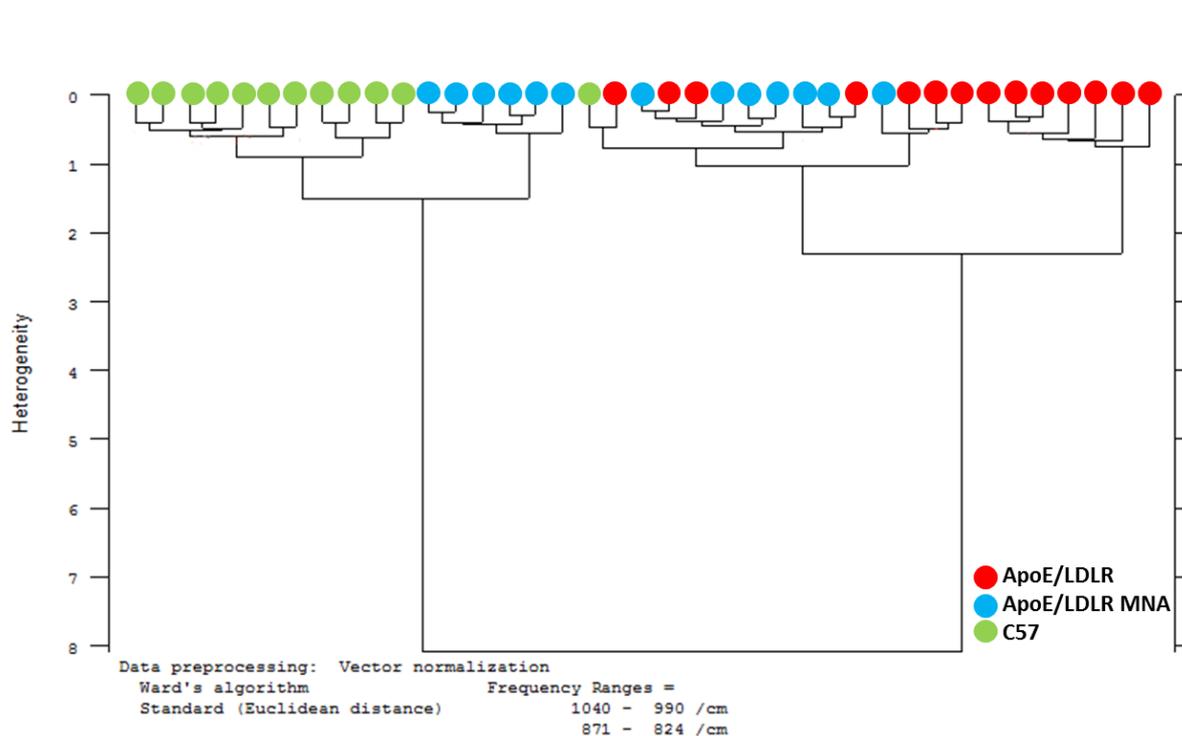
Fig. 4 The result of hierarchical cluster analysis for the average endothelium spectra obtained in three experimental groups: ApoE/LDR, ApoE/LDR MNA and C57 mice.

In the further analysis, we focused on the small differences between analyzed models in relative intensities of Phe and Tyr band at 1004 cm^{-1} and 854 cm^{-1}, respectively. These changes were of small magnitude so we decide to apply the chemometric tools what is described below.

Although, the profiles of spectra are dominated by protein contribution but the bands around 1306 cm^{-1} could be ascribed also to lipids. However, the lack of other marker bands e.g. 1455/1446 cm^{-1}, 1093 cm^{-1}, 1072 cm^{-1} and 892 cm^{-1} [18] disenables to definitely indicate about presence of lipids.

HCA analysis of spectra collected from ex vivo aortic endothelium in mice

As it was mentioned above, the differences in the relative intensity of the marker bands attributed to Phe (1004 cm^{-1}) and Tyr (854 cm^{-1}) [19] were observed for the atherosclerotic, MNA-treated and control mice. In order to investigate it in a detail, the HCA was performed (Fig. 4).

The HCA was carried out in the following regions: 1040-990 cm^{-1} and 871 – 824 cm^{-1}, that covers bands characteristic for Phe and Tyr, respectively. The distinction between the endothelium from atherosclerotic mice (red dots) and control one (green dots) is clearly seen and the value of heterogeneity is significantly high. However, the spectra from endothelium of atherosclerotic mice treated with MNA (blue dots) are divided into two groups, ApoE/LDLR and C57 samples, but grouped together within both classes.

The relationship between phenylalanine and tyrosine in the studied models

To confirm the HCA result, the bands' intensity ratios were calculated for Phe and Tyr bands in the 1014-991 cm^{-1} and 872-823 cm^{-1} regions, respectively. Both ratios were related to the whole amount of proteins, based on the amid I band in the 1710-1624 cm^{-1} range (Fig. 5a and b).

The intensity ratios for Phe and Tyr marker bands vs. amid I band are statistically different for control (C57) and atherosclerotic (ApoE/LDLR) endothelium samples. According to the presented results, the amount of Phe is higher for ApoE/LDLR than for C57 samples whilst for Tyr the ratio is inversed: the ApoE/LDLR samples are characterized by higher amount of Tyr than C57 samples

Interestingly, MNA treatment of ApoE/LDLR mice reversed partially the changes in intensity ratios and were definitely higher for AmidI/Phe ratio and lower for AmidI/Tyr than for untreated ApoE/LDLR samples. These observations however, gives information only about tendency because the results are not statistically different.

This result is in agreement with the one obtained by using the HCA where the difference between the spectra of the endothelium from the control and atherosclerotic mice is clearly seen, whilst for ApoE/LDLR MNA not so.
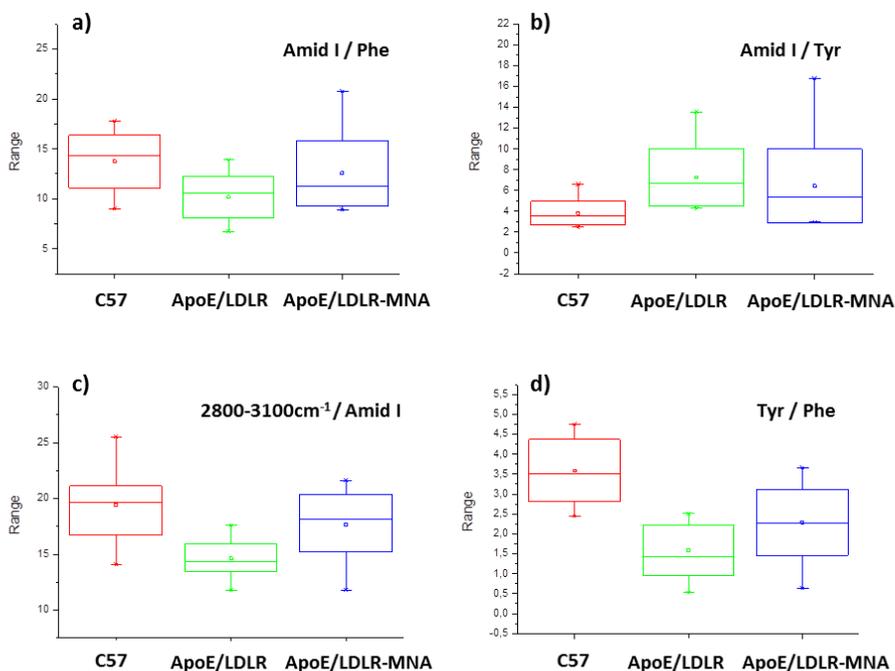
Fig. 5 The integration ratio of the bands a) amid I vs Phe, b) amid I vs Tyr, c) 2800-3100cm^{-1} vs amid I, d) Tyr vs Phe, for ApoE/LDLR, ApoE/LDLR MNA and C57 experimental groups .

The analysis described above is based on the amid I mode as the representative band for calculations of the amount of all proteins in a sample. It is known, however, that lipids bands are present in this spectral region also and could disturb the results (e.g. the cholesterol band which could be expected at 1674 cm^{-1} [3]). Nevertheless, the idea of semi-quantitative analysis based on the amid I band seem to be acceptable because the lipids' content in the region of amid I band is definitely lower than proteins. To be sure that our results are correct we decide to confirm it by the following steps.

The attempt of defining the difference in the lipid content between the models was done. It was based on the integration ratio between the C-H stretching band (2800-3100 cm^{-1}) where the impact from lipids is the most visible, and amid I band, that is originated both from lipids, but this influence is significantly lower than in the higher spectral region. According to our results (Fig. 5c), the endothelium of ApoE/LDLR mice are less lipidic compared to C57 animals and the difference is statistically important for ApoE/LDLR vs. C57. The results obtained for ApoE/LDLR mice treated with MNA give values of intensity ratios higher than for ApoE/LDLR mice but still lower than for healthy C57 samples.

Based on this analysis it was decided that impact of lipid bands in the amid I region is insignificant. However, taking into consideration the discussion, we compare the intensity ratio for Tyr vs. Phe (Fig. 5d) as a final proof that impact of lipids on the amid I band could be neglected and our results were obtained according to the correct protocol. It is clearly seen that the value of Tyr:Phe ratio is significantly lower for ApoE/LDLR tissues than for the control samples and these changes were partially reversed by MNA treatment.

Our results seem in agreement with the knowledge about the impact of BH4 deficiency on the endothelium, where synthesis of Tyr from Phe is depending on PAH, which requires BH4 as a cofactor [9]. According to previous works [8,9], the amount of BH4 is lower in atherosclerotic/pathological endothelial tissues. As a result, the ratio of tyrosine (Tyr) to phenylalanine (Phe) contents observed in the spectra collected from the endothelium of atherosclerotic mice is significantly lower. In our experiments, this is illustrated as a considerable decrease of the ratio of intensity of Tyr:Phe marker bands. The obtained results confirm that it is possible to observe the differences in the relative content of Phe and Tyr in the endothelium of atherosclerotic and control (healthy) mice.

Despite abundant literature on the role of PAH in a context of phenylketonuria and on PAH biochemistry and function in various cell types and tissues, there are limited data on PAH function in endothelium [20]. Even more, to our knowledge, there is no data on the reciprocal relationship between PAH and NOS that could confirm our Raman-based evidence that a decreased Tyr:Phe ratio is linked to PAH deficiency and could well be used as a biomarker of endothelial dysfunction associated with impaired BH4 availability and NOS deficiency in various cardiovascular disease.

Conclusions

We propose the spectroscopic approach to identify biochemical marker of endothelial dysfunction that is commonly linked to the impairment of BH4 activity in endothelium. We provide evidence that Phe:Tyr ratio analysis that can be regarded as a measure of impaired PAH activity linked to BH4 deficiency, discriminate endothelial dysfunction in ApoE/LDLR$^{-/-}$ mice as compared to control mice. We also demonstrated that MNA endowed with vasoprotective activity [11] known to reverse endothelial dysfunction [12] tended to improve Phe:Tyr ratio in endothelium. These results suggest the improvement of endothelial function in MNA-treated ApoE/LDLR$^{-/-}$ mice.

Raman spectroscopy, especially Raman imaging, is tested broadly as a diagnostic tool. The fact that this technique allows for *in situ* analysis, without any extraction of an analyte, enables performing measurements in the conditions close to physiological. We suggest that Phe:Tyr ratio measured by Raman spectroscopy proposed here as a marker of endothelial dysfunction may prove useful to determine endothelial biochemical status in various circumstances [10] e.g. in *ex vivo* human vessels before vascular grafts surgery.

Acknowledgements

Notes and references

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