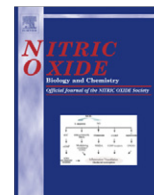
Nitric Oxide 27 (2012) 176–184

Contents lists available at SciVerse ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox



Cigarette smoke and LDL cooperate in reducing nitric oxide bioavailability in endothelial cells via effects on both eNOS and NADPH oxidase

Yvonne Steffen [a,*], Gregory Vuillaume [b], Katrin Stolle [a], Karin Roewer [a], Michael Lietz [a], Jutta Schueller [a], Stefan Lebrun [b], Thomas Wallerath [a]

[a] *Philip Morris International R&D, Philip Morris Research Laboratories GmbH, Cologne, Germany*
[b] *Philip Morris International R&D, Philip Morris Products SA, Neuchatel, Switzerland*

ARTICLE INFO

Article history:
Received 29 November 2011
Revised 12 June 2012
Available online 3 July 2012

Keywords:
Nitric oxide
Cigarette smoke
Oxidative stress
Endothelial cell
LDL

ABSTRACT

The ubiquitous free radical nitric oxide (NO) plays an important role in many biological processes, including the regulation of both vascular tone and inflammatory response; however, its role in the effects of cigarette smoke exposure on atherosclerosis remains unclear. Our aim was to study the mechanisms of NO regulation in endothelial cells in response to cigarette smoke exposure in vitro. Using human umbilical vein endothelial cells (HUVEC), we have demonstrated that combining non-toxic concentrations of cigarette smoke bubbled through PBS (smoke-bubbled PBS [sbPBS]) with native LDL (nLDL) significantly reduces the amount of bioavailable NO. The effect is comparable to that seen with oxidized LDL (oxLDL), but has not been seen with sbPBS or nLDL alone. Mechanistic investigations showed that the combination of sbPBS + nLDL did not reduce the amount of endothelial nitric oxide synthase (eNOS), but did inhibit its enzymatic activity. Concomitantly, both sbPBS + nLDL and oxLDL significantly increased the production of reactive oxygen species (ROS) in the form of superoxide anions ($\cdot O_2^-$) and peroxynitrite ($ONOO^-$) in HUVEC. Selective inhibition of NADPH oxidase prevented this response. Incubation of sbPBS + nLDL revealed the formation of 7-ketocholesterol (7-KC) and 7-hydroxycholesterol, which are indicators for oxidative modification of LDL. This could explain the reported increase in circulatory levels of oxLDL in smokers.

Our results suggest that reduction of functional NO in response to a combination of sbPBS + nLDL is secondary to both reduction of eNOS activity and stimulation of NADPH oxidase activity. Because sbPBS alone showed no effect on eNOS activity or ROS formation, nLDL should be included in cigarette-smoke-related mechanistic in vitro experiments on endothelial cells to be more reflective of the clinical situation.

© 2012 Elsevier Inc. All rights reserved.

Introduction

Cigarette smoking, hypercholesterolemia, hypertension and diabetes mellitus are traditional risk factors for coronary heart disease, and have all been associated with impairments in endothelial function. Smoking is associated with both acute and chronic effects on endothelial cell (EC) function in humans [1,2]. It is assumed that the effects of smoking on the vasculature are mediated by smoke constituents that are 'spilled over' from the lung into the circulatory system [3]. More than 5300 individual constituents [4] have been identified in cigarette smoke; however, their individual contributions and potential mechanisms of action are largely unknown. Cigarette smoke has also been shown to enhance oxidative stress in the endothelium and decrease the release or activity of nitric oxide (NO) in vascular ECs [5].

Risk factor modification, particularly elevated concentrations of low-density lipoprotein (LDL) cholesterol, improves endothelial function. In clinical studies, hypercholesterolemia is predominantly associated with a concomitant increase in levels of LDL cholesterol. Despite the association of high levels of LDL (>200 mg/dl) [6] with the development of atherosclerosis, native LDL (nLDL) does not appear to affect endothelial nitric oxide synthase (eNOS) expression or eNOS mRNA stability, whereas oxidized LDL (oxLDL) has been shown to both up-regulate and down-regulate eNOS mRNA in vitro, depending on its concentration [7–10]. At the same

Abbreviations: AT II, Angiotensin II; DAF-2DA, diamino-fluorescein-2-diacetate; DHE, dihydroethidine; EC, endothelial cell; eNOS, endothelial nitric-oxide synthase(s); HUVEC, human umbilical vein endothelial cells; LC–MS, liquid chromatography–mass spectrometry; L-NAME, G-nitro-L-arginine-methyl ester; nLDL, native low density lipoprotein; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase(s); iNOS, inducible nitric-oxide synthase; NOX, NADPH Oxidase; $\cdot O_2^-$, superoxide anion; oxLDL, oxidized LDL; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; SDS–PAGE, sodiumdodecyl–polyacrylamide gel electrophoresis.

* Corresponding author. Fax: +41 (0) 32 888 77 13.
E-mail address: yvonne.steffen@pmi.com (Y. Steffen).

1089-8603/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.niox.2012.06.006

time, enhanced serum levels of oxLDL are predictive for endothelial dysfunction and coronary heart disease [11]. Oxidative stress-dependent reactive oxygen species (ROS) have been implicated in the formation of oxLDL, while oxLDL itself stimulates vascular oxygen radical formation in a positive feedback loop [11].

Endothelial dysfunction itself is an early event in the development of atherosclerosis and is characterized by a reduction in the bioavailability of NO. Cardiovascular risk factors such as aging, hypercholesterolemia, hypertension, and exposure to cigarette smoke are known to increase endothelial dysfunction. Acute smoking causes endothelial dysfunction through impairment of NO production, or increased oxidative stress, but the exact mechanism remains to be elucidated. Impairment of NO production and an increase in oxidative stress by a large number of free radicals known to exist in smoke [12] are some of the possible mechanisms responsible for acute endothelial dysfunction observed after smoking one cigarette [13]. Models of endothelial dysfunction in experimental animals, along with clinical data, have provided evidence that NO bioavailability is reduced by increased production of ROS in the vessel wall during the development of atherosclerosis [14,15]. Both ECs and smooth muscle cells (SMC) are capable of producing NO in the vascular wall, though ECs are likely to be the most important source for bioactive NO [16]. When produced from healthy ECs, NO functions to reduce inflammatory cell adhesion and infiltration and platelet activation/aggregation in addition to playing a key role in vasodilatation [17]. The development of atherosclerosis is accompanied by an increase in oxidative stress within vascular tissue involving several enzymes, including NADPH oxidase [18,19]. Superoxide anions ($\cdot O_2^-$) produced by NADPH oxidase may react with NO, thereby generating peroxynitrite ($ONOO^-$) [20]. Peroxynitrite, in turn, has been shown to uncouple eNOS, the enzyme responsible for the constitutive production of NO in healthy ECs. Uncoupling of eNOS results in a further increase in $\cdot O_2^-$ and a reduction in bioactive NO [21]. In addition, reduced levels of eNOS expression have been seen consistently in human atherosclerotic tissue [22,23].

The purpose of this study was to examine the mechanistic relationship between the pro-atherosclerotic effects of cigarette smoke and LDL on NO production. To this end, we conducted studies on the individual and combined effects of cigarette smoke and LDL exposure on the synthesis and metabolism of bioactive NO from human umbilical vein endothelial cells (HUVEC) in vitro.

Materials and methods

Materials

All chemicals, radiochemicals, and enzymes were obtained from Amaxa, Vasopharm Biotech GmbH, Cayman, Sigma Chemical Co., Deisenhofen, Amersham, Freiburg and Roche, Mannheim, respectively, at the highest purity grade available.

Cell culture

Unless specified otherwise, cell culture reagents and chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany). Penicillin/streptomycin, amphotericin-B, gentamycin, hydrocortisone, and fetal calf serum were purchased from PAA Laboratories GmbH (Coelbe, Germany).

Smoke-bubbled PBS (sbPBS) generation

University of Kentucky 3R4F standard reference cigarettes were smoked on a peristaltic pump-smoke machine (Heinrich BorgwaldtRM1/G, Hamburg, Germany) 3R4F in conformity with ISO standard 3308 [24]. sbPBS was generated by bubbling 30 puffs of mainstream cigarette smoke (1 puff = 33 cm^3 of smoke; 1 cigarette = 10 puffs) through 18 ml PBS. The resulting sbPBS solution contained the smoke of three cigarettes (1.7 puffs/ml [p/ml]). To obtain the required concentrations for cell exposure, freshly prepared sbPBS was diluted in Dulbecco's modified Eagle medium (DMEM) containing 0.5% fetal calf serum (FCS). Experiments were performed with 0.03 p/ml sbPBS unless stated otherwise.

Treatment of endothelial cells

HUVEC were purchased from Promocell (Heidelberg, Germany) and cultured according to the supplier's instructions. HUVEC were used at passages 2–4.

All cultures were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in 75 cm^2 culture flasks. For all experiments, HUVEC were seeded in 6-well plates, grown until confluency, and starved in medium with 0.5% FCS for 24 h before further treatment. In a pilot study to evaluate the experimental set-up for these studies, we determined cell viability using MTT and cell titer glo assays after incubation with sbPBS (0.0075–0.09 p/ml), nLDL (50–200 μg/ml), and oxLDL (50–200 μg/ml) for 24 h (data not shown). Concentrations of oxLDL > 150 μg/ml or sbPBS > 0.03 p/ml were cytotoxic in our experimental set-up and therefore not used in the studies reported here. HUVEC monolayers were incubated with PBS as solvent control, sbPBS (0.03 p/ml), LDL (100 μg/ml), sbPBS (0.03 p/ml) + nLDL (100 μg/ml), oxLDL (100 μg/ml), and sbPBS (0.03 p/ml) + oxLDL (100 μg/ml) for 24 h. 1 μg/ml Angiotensin II (AT II) was used as a positive control for $\cdot O_2^-$ generation via NADPH oxidase.

In some experiments, cells were pretreated for 30 min with NADPH oxidase inhibitors apocynin (100 μmol/l) or gp91 docking sequence-tat peptide (gp91ds-tat; 50 μmol/l). Total protein content was determined by a commercial Lowry protein kit (BioRad, Heidelberg, Germany) according to the manufacturer's instructions.

Isolation and modification of human nLDL

Human nLDL was freshly isolated from serum of male nonsmokers obtained from Clinpharm, Cologne according to Kleinveld et al. [25].

Oxidation of nLDL with sbPBS

nLDL was diluted on the basis of its total protein content to a final concentration of 100 μg/ml and subjected to lipid peroxidation at 37 °C by treatment with sbPBS (0.03 p/ml) for 24 h.

Lipid extraction

For analysis of oxysterols, lipids were extracted from 1 ml sbPBS treated nLDL (100 μg) using the methodology developed by Bligh and Dyer with chloroform/methanol (1/2, v/v) [26] containing 0.001% butylated hydroxytoluene as antioxidant and 5α-cholestane (100 μg/ml) as internal standard. To reduce hydroperoxy-lipids (hydroperoxy-sterols and hydroperoxy-fatty acids) in the lipid extracts, the lipid extracts were reconstituted in methanol and analyzed immediately via LC–MS × MS.

Quantification of oxysterols by liquid chromatography–tandem-mass spectrometry (LC-MS/MS)

The amount and content of oxysterols in nLDL and oxLDL were analyzed using LC–MS/MS system, as described by McDonald et al. [27], using Agilent 1200 liquid chromatography (Agilent Technolo-

Download English Version:

https://daneshyari.com/en/article/2000876

Download Persian Version:

https://daneshyari.com/article/2000876

Daneshyari.com