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Research Paper

Reciprocal regulation of eNOS, H_2S and CO-synthesizing enzymes in human atheroma: Correlation with plaque stability and effects of simvastatin

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ABSTRACT

The molecular and cellular mechanisms underlying plaque destabilization remain obscure. We sought to elucidate the correlation between NO, H_2S and CO-generating enzymes, nitro-oxidative stress and plaque stability in carotid arteries. Carotid atherosclerotic plaques were collected from 62 patients who had undergone endarterectomy due to internal artery stenosis. Following histological evaluation the plaques were divided into stable and unstable ones. To investigate the impact of simvastatin we divided patients with stable plaques, into those receiving and to those not receiving simvastatin. Expression and/or levels of p-eNOS/eNOS, pAkt/*t*-Akt, iNOS, cystathionine beta synthase (CBS), cystathionine gamma lyase (CSE), heme oxygenase-1(HO-1), soluble guanyl cyclase sGCα1, sGCβ1, NOX-4 and HIF-1α were evaluated. Oxidative stress biomarkers malondialdehyde (MDA) and nitrotyrosine (NT) were measured. NT levels were decreased in stable plaques with a concomitant increase of eNOS phosphorylation and expression and Akt activation compared to unstable lesions. An increase in HIF-1α, NOX-4, HO-1, iNOS, CBS and CSE expression was observed only in unstable plaques. 78% of patients under simvastatin were diagnosed with stable plaques whereas 23% of those not receiving simvastatin exhibited unstable plaques. Simvastatin decreased iNOS, HO-1, HIF-1α and CSE whilst it increased eNOS phosphorylation. In conclusion, enhanced eNOS and reduced iNOS and NOX-4 were observed in stable plaques; CBS and CSE positively correlated with plaque vulnerability. Simvastatin, besides its known effect on eNOS upregulation, reduced the HIF-1α and its downstream targets. The observed changes might be useful in developing biomarkers of plaque stability or could be targets for pharmacothepary against plaque vulnerability.

1. Introduction

The presence of atherosclerotic disease in the carotid arteries creates a significant risk for cerebrovascular events, with reported annual ischemic stroke rates ranging from 0.35% to 1.3% in asymptomatic patients with moderate stenosis [1] and from 0.5% to approximately 5% for severe asymptomatic carotid artery stenosis [2]. Around 20% of ischemic strokes appear to originate from carotid plaques [3]. Understanding of atherosclerosis progression and characterization of the role of plaque instability in the pathogenesis of acute ischemic syndromes have been major goals of cardiovascular research during the previous decades. However, the complex molecular and cellular mechanisms underlying plaque destabilization remain largely obscure, and the distinct mechanism through which stabilization of atheroma is achieved is still under investigation [4].

Nitro-oxidative stress, characterized by overproduction of reactive oxygen (ROS) and nitrogen (RNS) species, with a concomitant endothelial dysregulation being manifested through the impairment of nitric oxide (NO) homeostasis, are key factors for plaque formation and instability. Increased expressions of enzymes that promote ROS production, such as NADPH oxidases (NOX), contribute to atherosclerosis. Additionally, upregulation or activation of pro-survival kinases such as protein kinase B (PKB/Akt) or endogenous antioxidant mechanisms, can lead to improved atherosclerotic lesion stability and

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prognosis in various *in vivo* models and in clinical trials. However the elucidation of the actual mechanism of the above findings is still under investigation [5].

In addition to NO, studies have revealed another two gasotransmitters, namely hydrogen sulfide (H_2S) and carbon monoxide (CO), to be vital signaling molecules in vascular cells, contributing to the protection of the cardiovascular system through activation of various antiapoptotic and antioxidant pathways [6]. NO is produced in most of the mammalian tissues and cells by both enzymic [neuronal nitric oxide sythase (nNOS), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS)] and non-enzymic reactions (reduction of nitrite/nitrate from dietary and endogenous sources) [6]. H_2S is generated from cysteine by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulphurtransferase (3-MST) [7–9]. Reduced levels of H_2S have been linked with various cardiovascular disease states that are associated with endothelial dysfunction, including atherosclerosis [10]. Endogenous CO is liberated from heme oxygenases (HO-1 and HO-2) as a result of heme degradation, which along with biliverdin that is rapidly reduced to bilirubin, exhibit antioxidant properties [6]. However, although some information on the role of endogenous NO in atherosclerotic plaques is available [11], the effects of endogenous CO and H_2S on plaque stability remain obscure.

To date, several population-based preventive programs aimed at cardiovascular risk reduction were able to substantially abate cardiovascular morbidity and mortality; most importantly the introduction of statin therapy was able to reduce cardiovascular mortality by over one-third [12]. Besides their hypolipidaemic activity, it is already proven that statins exhibit pleiotropic activities, with anti-inflammatory, antioxidant and anti-thrombotic properties being well established [13]. While it is already shown that statins can improve NO homeostasis through upregulation and activation of eNOS and can induce plaque stabilization in patients, the actual underlying mechanism and their effect on the enzymes that generate H_2S and CO has not been accessed [14].

Considering the translational importance of understanding and targeting the underlying signaling cascade responsible for atheroma stability, we sought to investigate the intraplaquely interplay between NO, H_2S and CO generation enzymes and associate their expression with biomarkers and signaling molecules of nitro-oxidative stress. Moreover, we investigated the effect of simvastatin on plaque stability and unraveled the possible underlying mechanisms of protection.

2. Materials and methods

2.1. Tissue collection

Since 2015, carotid plaques were prospectively collected from 62 random patients, who had internal carotid artery stenosis 70% and underwent carotid endarterectomy. Extent demographic and clinical data, medication, risk factors, and vascular comorbidities were recorded (Table 1). Neurological evaluation of all patients was performed preoperatively in order to classify them as symptomatic (presence of stroke, brain infarcts, transient ischemic attacks and amaurosis fugax) and asymptomatic. Arteriographical evaluation of the carotid bifurcation stenosis was performed in all patients for this study. Degree of luminal stenosis was determined according to North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria [15]. Based on these measurements, stenotic lesions were divided into two groups, namely asymptomatic patients with stable plaques (stable), and symptomatic patients with unstable plaques (unstable). Moreover according to whether patients were under simvastatin administration or not, patients were divided in four subgroups, namely asymptomatic patients with stable plaques under simvastatin therapy (s/st), asymptomatic patients with stable plaques not receiving simvastatin (s/nost), symptomatic patients with unstable plaques under simvastatin therapy (u/st) and symptomatic patients with unstable plaques not receiving simvastatin (u/nost). The present study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The study protocol was approved by the Institutional Ethics Committee and all patients enrolled gave their informed consensus.

Table 1
Clinical and Demographic Data collected from patients, who underwent endoartetectomy.

Patients:	Overall	Simvastatin Therapy	Non simvastatin therapy (non statin)
Demographic data			
No	62	31	31
Mean age (range)	71.05 (55–85)	71	71.06
Male /female	48/14	28/7	20/7
Smoking	40	19	21
(current/past smokers)	26/14	10/9	12/9
Clinical data			
Hypertension	58	31	27
Diabetes	23	15	8
Hyperlipidemia	42	31	11
Ischemic heart disease	38	30	8
Aneurysm	3	2	1
Peripheral arterial occlusive disease	24	18	6
Clinical symptoms	31	9	22
Stroke	12	4	8
TIA	11	1	
Amaurosis fugax	8	4	4
Angiographic carotic stenosis			
< 90%	24	10	14
≥90	38	18	20
Plaque histopathology status			
Unstable	33	11	22
Stable	29	20	9
Medications			
Antiplatelets	57	31	26
ACE inhibitors	30	14	16
b-blockers	28	18	10

2.2. Tissue preparation

All carotid plaque specimens were removed in the operating room and were divided into two parts. One part was fixed immediately in 10% neutral-buffered solution with 4% formaldehyde for 24 h, and embedded in paraffin. The second portion was immediately stored at −80 °C for further analysis of malondialdehyde (MDA), nitrotyrosine (NT), immunohistochemical and immunoblotting analysis.

2.3. Histology

Hematoxylin and eosin staining was performed for histological evaluation of the specimens. Two pathologists, blinded to the clinical data, examined each specimen to assess atheromatous plaque morphology, using the American Heart Association classification of atherosclerotic plaques [16]. According to this classification, carotid plaques were assigned as fibroatherotic (type V) and complicated unstable (intraplaque hemorrhage, ulcer, or thrombus) (type VI).