The urea decomposition product cyanate promotes endothelial dysfunction

Dalia El-Gamal[1], Shailaja P. Rao[2], Michael Holzer[1], Seth Hallström[3], Johannes Haybaeck[4], Martin Gauster[5], Christian Wadsack[6], Andrijana Kozina[2], Saša Frank[2], Rudolf Schicho[1], Rufina Schuligoi[1], Akos Heinemann[1] and Gunther Marsche[1]

[1]Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Graz, Austria; [2]Institute of Molecular Biology and Biochemistry, Medical University of Graz, Graz, Austria; [3]Institute of Physiological Chemistry, Medical University of Graz, Graz, Austria; [4]Institute of Pathology, Medical University of Graz, Graz, Austria; [5]Institute of Cell Biology, Histology and Embryology, Medical University of Graz, Graz, Austria and [6]Department of Obstetrics and Gynecology, Medical University of Graz, Graz, Austria

The dramatic cardiovascular mortality of patients with chronic kidney disease is attributable in a significant proportion to endothelial dysfunction. Cyanate, a reactive species in equilibrium with urea, is formed in excess in chronic kidney disease. Cyanate is thought to have a causal role in promoting cardiovascular disease, but the underlying mechanisms remain unclear. Immunohistochemical analysis performed in the present study revealed that carbamylated epitopes associate mainly with endothelial cells in human atherosclerotic lesions. Cyanate treatment of human coronary artery endothelial cells reduced expression of endothelial nitric oxide synthase, and increased tissue factor and plasminogen activator inhibitor-1 expression. In mice, administration of cyanate, promoting protein carbamylation at levels observed in uremic patients, attenuated arterial vasorelaxation of aortic rings in response to acetylcholine without affecting the sodium nitroprusside–induced relaxation. Total endothelial nitric oxide synthase and nitric oxide production were significantly reduced in aortic tissue of cyanate-treated mice. This coincided with a marked increase of tissue factor and plasminogen activator inhibitor-1 protein levels in aortas of cyanate-treated mice. Thus, cyanate compromises endothelial functionality *in vitro* and *in vivo*. This may contribute to the dramatic cardiovascular risk of patients suffering from chronic kidney disease.

Kidney International advance online publication, 18 June 2014; doi:10.1038/ki.2014.218

KEYWORDS: cyanate; endothelial nitric oxide synthase; plasminogen activator inhibitor-1; renal disease; tissue factor

Correspondence: *Gunther Marsche, Institute of Experimental and Clinical Pharmacology, Medical University of Graz, Universitätsplatz 4, 8010 Graz, Austria. E-mail: gunther.marsche@medunigraz.at*

Received 1 October 2013; revised 24 April 2014; accepted 1 May 2014

Cardiac mortality of end-stage renal disease patients is several fold increased in comparison with the general population.[1,2] However, the precise nature of the inflammatory and/or oxidative pathways involved remains unclear. As renal failure progresses, compounds accumulate in blood and tissues due to a decline in renal function. Several studies have postulated that chronic renal failure–associated atherosclerosis and endothelial dysfunction result from accumulation of certain 'uremic factors,' the identities of which are still a matter of debate.[3] A number of retention solutes may contribute to vascular damage in uremia, including urea, complement peptides, cytokines, phosphate, oxalate, para-cresol[4,5] but may also originate from colonic microbial metabolism.[6] Several lines of evidence suggest that breakdown or oxidative modification of retained uremic solutes may potentiate their pathogenicity. While urea itself is innoxious, many molecules can be carbamylated through cyanate, a reactive decomposition product of urea. Due to the reactive nature of cyanate, its formation is best assessed by measuring serum levels of protein-bound homocitrulline (HCit), a footprint of cyanate formation and protein carbamylation.[7–10] Of particular interest, plasma protein-bound HCit levels were recently demonstrated to predict increased cardiovascular risk in patients with kidney failure.[10,11] Importantly, protein carbamylation also predicts cardiovascular risk in non-uremic subjects, given that cyanate is also generated via peroxidase-catalyzed oxidation of thiocyanate.[7,9] Urea levels may increase up to 100 mmol/l in patients with renal failure and about 0.8% of urea autodecompose into cyanate.[9,12–15] In addition, myeloperoxidase-catalyzed oxidation of thiocyanate and myeloperoxidase-induced accelerated decomposition of urea may generate locally high cyanate levels.[7,9] Given that leukocyte-derived myeloperoxidase associates with endothelial cells, markedly increased formation of cyanate in close vicinity to endothelial cells is anticipated.

A key step and one of the earliest changes in the development of atherosclerosis is endothelial dysfunction, which is triggered—at least in part—by reactive metabolites leading to initiation and/or perpetuation of the inflammatory

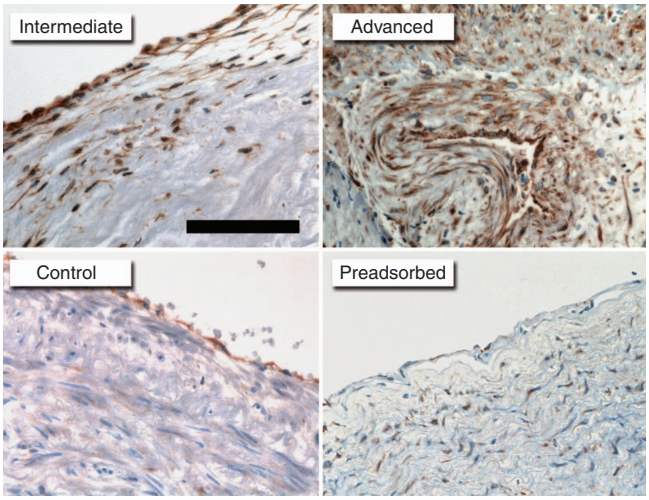

Figure 1 | Immunostaining for carbamylated proteins in human atherosclerotic tissue. Reddish-brown immunostaining indicates accumulation of carbamylated epitopes detected with an anti-homocitrulline (HCit) antibody mainly within or around the endothelial lining of human control tissue and intermediate atherosclerotic lesions. Intense staining was observed in all regions of advanced atherosclerotic lesions. Preincubation of the anti-HCit antibody with carbamylated albumin (preadsorbed) almost completely abolished staining. Bar = 100 μm.

response. Here we assessed whether cyanate affects anti-atherogenic properties of endothelial cells and whether this mechanism is relevant under *in vivo* conditions.

RESULTS

Carbamylated epitopes are mainly associated with endothelial cells in human atherosclerotic lesions

Augmented decomposition of urea/thiocyanate by myeloperoxidase is expected to generate high concentrations of cyanate in close vicinity to endothelial cells. In line with this assumption, we observed that HCit-containing epitopes (carbamylated epitopes) are mainly associated with endothelial cells in intermediate human atherosclerotic lesions, whereas intense staining in several regions of atherosclerotic tissue was observed in advanced lesions (Figure 1). Control tissue showed weak staining mainly associated with endothelial cells. Interestingly, the immunohistochemical analysis was performed in atherosclerotic tissue of non-uremic subjects, as tissue of uremic subjects was not available to us, suggesting that endothelial cells may be exposed to increased levels of cyanate also under conditions of normal urea levels. Preincubation of the anti-HCit antibody with carbamylated albumin almost completely abolished staining (Figure 1), and incubation with control non-immune IgG revealed no staining (Supplementary Figure S1 online)

Cyanate decreases endothelial nitric oxide synthase protein expression in human coronary artery endothelial cells

A key atheroprotective function of the vascular endothelium is the production of the vasorelaxing compound nitric oxide (NO). Treatment of human coronary artery endothelial cells

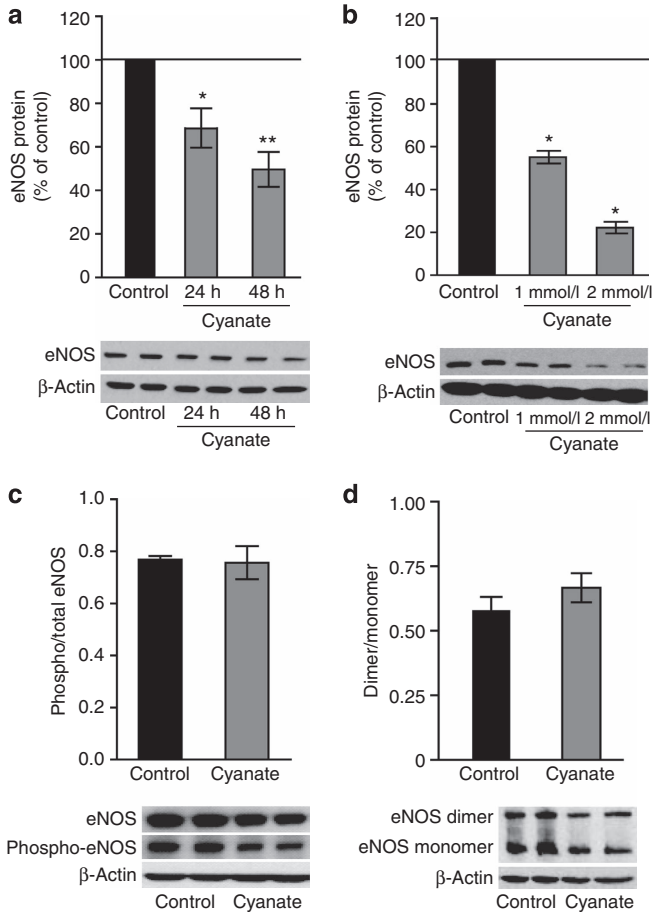

Figure 2 | Cyanate alters endothelial nitric oxide synthase (eNOS) protein in endothelial cells. Human coronary artery endothelial cells (HCAECs) were treated with sodium cyanate (1 or 2 mmol/l) for 24 h or 48 h. Cyanate elicits a (**a**) time-dependent and (**b**) dose-dependent decrease in eNOS protein expression. (**c**) Decreased phospho-eNOS in lysates from cyanate-treated cells (1 mmol/l, 48 h) with quantification of phospho/total eNOS levels. (**d**) eNOS dimerization with quantification of eNOS dimer/monomer levels in lysates from cyanate-treated cells (1 mmol/l, 48 h). Representative western blots from HCAECs are shown. β-Actin was used to normalize the data for equal protein loading, and quantification of bands was done using ImageJ software. Data are expressed as mean ± s.e.m. ($n = 3$–4). Statistical analysis was performed by one-way analysis of variance (ANOVA) for more than two groups and with Student's *t*-test for two groups. Significance was accepted at $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

(HCAECs) with cyanate markedly decreased endothelial nitric oxide synthase (eNOS) protein expression in a time-dependent and dose-dependent manner (Figure 2a and b) but did not affect cell viability (Supplementary Figure S2 online). Activity of eNOS is regulated by phosphorylation and dimerization. Exposure of HCAECs to cyanate significantly decreased total eNOS and phospho-eNOS in a similar fashion (Figure 2c). Dimer/monomer ratio of eNOS was not altered in endothelial cells upon cyanate exposure (Figure 2d).

Cyanate decreases eNOS protein expression on the mRNA level

Cyanate might decrease eNOS protein levels via the ubiquitin–proteasome pathway. However, experiments