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Urea-induced ROS cause endothelial dysfunction in chronic renal failure

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Abbreviations:
CRF
chronic renal failure
ROS
reactive oxygen species
GlcNAc
O-linked-N-acetylglucosamine
UCP-1
uncoupling protein 1
MnTBAP
manganese tetrakis (4-benzoic acid)
porphyrin

ABSTRACT

Objective: The pathogenic events responsible for accelerated atherosclerosis in patients with chronic renal failure (CRF) are poorly understood. Here we investigate the hypothesis that concentrations of urea associated with CRF and increased ROS production in adipocytes might also increase ROS production directly in arterial endothelial cells, causing the same pathophysiologic changes seen with hyperglycemia.
Methods: Primary cultures of human aortic endothelial cells (HAEC) were exposed to 20 mM urea for 48 h. C57BL/6J wild-type mice underwent 5/6 nephrectomy or a sham operation. Randomized groups of 5/6 nephrectomized mice and their controls were also injected i.p. with a SOD/catalase mimetic (MnTBAP) for 15 days starting immediately after the final surgical procedure.
Results: Urea at concentrations seen in CRF induced mitochondrial ROS production in cultured HAEC. Urea-induced ROS caused the activation of endothelial pro-inflammatory pathways through the inhibition of GAPDH, including increased protein kinase C isoforms activity, increased hexosamine pathway activity, and accumulation of intracellular AGEs (advanced glycation end products). Urea-induced ROS directly inactivated the anti-atherosclerosis enzyme PGI_2 synthase and also caused ER stress. Normalization of mitochondrial ROS production prevented each of these effects of urea. In uremic mice, treatment with MnTBAP prevented aortic oxidative stress, PGI_2 synthase activity reduction and increased expression of the pro-inflammatory proteins TNFα, IL-6, VCAM1, Endoglin, and MCP-1.
Conclusions: Taken together, these data show that urea itself, at levels common in patients with CRF, causes endothelial dysfunction and activation of proatherogenic pathways.

1. Introduction

Cardiovascular disease risk is increased up to 30-fold in patients with chronic renal failure (CRF) compared with the general population [1,2], and remains 10 to 20 times higher after stratification for age, sex, and presence of diabetes [1]. Overall, the 5-year mortality rate for patients on dialysis is 60% [2]. Despite the well-established high incidence of atherosclerosis in CRF patients with uremia, the pathogenic events responsible for accelerated atherosclerosis are poorly understood. Although CRF patients have higher levels of general risk factors for cardiovascular disease (CVD) such as hypertension, diabetes, obesity, and lipid abnormalities [3], these traditional cardiovascular risk factors (CVRFs) do not fully account for the high risk of atherosclerosis, CVD, and total mortality in patients with CRF. Growing evidence supports a major role for nontraditional CVRFs in the pathogenesis of accelerated atherogenesis in this population [4]. Several molecules whose levels rise as a consequence of decreased renal function have been associated with CVD in CRF, but mechanistic data demonstrating causality are

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lacking.

Recently, we showed that urea at concentrations seen in CRF can induce reactive oxygen species (ROS) production in cultured 3T3-L1 adipocytes, resulting in O-GlcNAc modification of several downstream insulin signaling effectors with reduction in insulin-stimulated IRS-1 (insulin receptor substrate-1) and Akt phosphorylation and glucose transport [5]. Similarly, uremic mice also displayed increased ROS production, modification of insulin signaling molecules by O-GlcNAc, and increased insulin resistance and levels of insulin resistant adipokines. Moreover, urea infusion into normal rats induced insulin resistance with elevation of the insulin resistance–associated adipokines. As treatment with a SOD/catalase mimetic prevents these urea-induced abnormalities [5], we hypothesized that urea itself causes increased ROS in adipose tissue, which cause systemic insulin resistance.

Endothelial dysfunction has been shown to be the best predictor of subsequent cardiovascular events [6], and evidence of endothelial dysfunction has been detected in uremic patients from early stages of the disease [7]. In an animal model of CRF-atherosclerosis, plasma urea itself was the only significant predictor of aortic plaque area fraction, and the antioxidant N-acetylcysteine prevented accelerated atherosclerosis in uremic apolipoprotein-E knockout mice [8]. This observation suggested to us that the high levels of urea associated with chronic renal failure might also have direct pro-atherogenic effects on vascular cells, in addition to causing systemic insulin resistance. We therefore hypothesized that concentrations of urea associated with chronic renal failure and increased ROS production in adipocytes might also increase mitochondrial ROS production directly in arterial endothelial cells, damaging these cells by activating the same pro-inflammatory pathways and inactivating the same anti-atherosclerosis enzymes caused by diabetes [9,10]. In this study, we demonstrate that urea induces endothelial cell ROS production, which causes the same pathophysiological changes reported to occur with hyperglycemia.

2. Materials and methods

2.1. Cell culture conditions

Confluent primary human aortic endothelial cells (HAECs) from Cambrex, (East Rutherford, NJ) (passages 2–5) were maintained in EBM-2 medium (from Lonza, San Diego, CA) with 0.4% fetal bovine serum and all the supplements. Cells were incubated with either 20 mM urea or with 20 mM mannitol used as osmotic control, for 48 h. The urea used in these experiments was certified to be free of LPS and heavy metals (Sigma Aldrich, St. Louis, MO). In subsequent experiments, cells were infected with UCP-1 adenovirus, MnSOD adenovirus, or control adenovirus at an MOI of 500, 4 h before addition of 20 mM urea containing medium.

2.2. Adenoviral vectors

UCP1and SOD2, (obtained from Open Biosystems) were cloned into the shuttle vector pAd5CMVK-NpA, and adenoviral vectors and empty control virus were prepared by the Gene Transfer Vector Core at the University of Iowa.

2.3. Measurement of ROS generation

Treated cells seeded in a 96-well plate were incubated with 10 μmol/l CM-H2DCFDA (Molecular Probes-Life Technology, Brooklyn, NY) for 45 min at 37 °C, and the intracellular formation of ROS was measured at excitation/emission wavelengths of 485/530 nm using a Wallac 1420 Fluorescent Plate Reader.

2.4. Measurement of NADPH oxidase activity

NADPH oxidase activity was determined using the lucigenin-enhanced chemiluminescence method as previously described [11]. Superoxide production was measured as the rate of relative chemiluminescence (light) units per minute per microgram of protein (RLU/min mg) and expressed as fold increase compared to untreated cells.

2.5. Prostacyclin synthase activity

Activity was measured by determination of 6-keto-PGF-1α, a stable product produced by the nonenzymatic hydration of PGI_2. A competitive immunoassay method (Correlate-EIA) was used for the quantitative determination of 6-keto-PGF-1α, according to the manufacturer's instructions (Assay Designs Enzo Life Sciences Farmingdale, NY). For 6-keto-PGF-1α determination in mouse aortae, the aortae were washed with PBS and incubated at 37 °C for 3 h in 400 μl incubation buffer.

2.6. RT reaction and real-time quantitative PCR

Total RNA from treated cells was extracted using the RNeasy Mini Kit (QIAGEN Milan, IT), following the manufacturer's instructions. The mRNA was reverse transcribed by SuperScript III First Strand Synthesis System (Life Technology, Brooklyn, NY). Experiments were performed in quadruplicate in optical 96-well reaction plates on an iCycler iQ Multicolor Real-Time PCR Detector (Bio-Rad, Hercules, CA) with iQ SYBR green supermix (Bio-Rad). Expression levels of p65NFkB, MCP-1 VCAM1, BiP/GRP78 and XBP1 mRNA were normalized to β-actin levels in the same sample. Melting curves were analyzed to ensure that fluorescence signals solely reflected specific amplicons. PCR conditions were as follows: 7 min at 95 °C and 45 cycles of 30 s at 95 °C and 30 s at 60 °C.

2.7. Determination of proinflammatory protein levels

Commercially available sandwich enzyme-linked immunosorbant assays were used to quantitate TNFα (Pierce, Rockford, IL), IL6 (Linco Research Inc. St. Charles, MO), MCP-1(R&D Systems, Minneapolis, MN) and VCAM1 (BioSource Camarillo, CA).

2.8. NFkB activity

Activity was measured by evaluating the expression of the NFkB-specific target genes (www.nf-kb.org) VCAM1, Endoglin, and VEGI by real time PCR as previously described [12].

2.9. PKC activity

PKC activity assay was performed in according to the manufacturer's instruction using the Protein Kinase C Assay System. (Enzo Life Sciences, Farmingdale, NY).

2.10. Hexosamine pathway activity

Dot Blot was performed using equal amount of cell lysates. Membranes were blotted with Anti-O-linked GlcNAc antibody (Affinity BioReagents Golden, CO). Immunoreactive protein-bound N-acetylglucosamine (GlcNAc) were visualized using an enhanced chemifluorescence kit according to the manufacturer's instructions (BioRad) and quantify using a VersaDoc Gel Imaging System (Bio-Rad) and The Quantity One analytical software.