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Research Paper

Role of glutathione biosynthesis in endothelial dysfunction and fibrosis

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ABSTRACT

Glutathione (GSH) biosynthesis is essential for cellular redox homeostasis and antioxidant defense. The rate-limiting step requires glutamate-cysteine ligase (GCL), which is composed of the catalytic (GCLc) and the modulatory (GCLm) subunits. To evaluate the contribution of GCLc to endothelial function we generated an endothelial-specific *Gclc* haplo-insufficient mouse model (*Gclc e/+* mice). In murine lung endothelial cells (MLEC) derived from these mice we observed a 50% reduction in GCLc levels compared to lung fibroblasts from the same mice. MLEC obtained from haplo-insufficient mice showed significant reduction in GSH levels as well as increased basal and stimulated ROS levels, reduced phosphorylation of eNOS (Ser 1177) and increased eNOS S-glutathionylation, compared to MLEC from wild type (WT) mice. Studies in mesenteric arteries demonstrated impaired endothelium-dependent vasodilation in *Gclc(e/+)* male mice, which was corrected by pre-incubation with GSH-ethyl-ester and BH_4. To study the contribution of endothelial GSH synthesis to renal fibrosis we employed the unilateral ureteral obstruction model in WT and *Gclc(e/+)* mice. We observed that obstructed kidneys from *Gclc(e/+)* mice exhibited increased deposition of fibrotic markers and reduced Nrf2 levels. We conclude that the preservation of endothelial GSH biosynthesis is not only critical for endothelial function but also in anti-fibrotic responses.

1. Introduction

Endothelial dysfunction constitutes a major challenge from both the biological and clinical standpoints as it is fundamentally associated to the vascular damage present in major pathological entities such as hypertension, diabetes, atherosclerosis, and aging [1]. An important body of literature supports a causative link between oxidative distress and impaired vascular function [2,3]. Since the discovery of endothelium-derived relaxing factor (EDRF), later identified as nitric oxide (NO), overwhelming evidence has been mounted regarding the disturbed redox state of endothelial cells and therefore their inability to maintain adequate vasodilation. At the cellular level, antioxidant responses to injury are elicited through multiple pathways. Among these pathways, the endogenous nucleophile glutathione (GSH) is of paramount importance for redox homeostasis, due to its high intracellular concentrations (1–10 mM), its capacity to interact with peroxidases and its potential to act as an electron donor for free radicals [4–6]. GSH synthesis proceeds through two major steps regulated by the enzymes glutamate cysteine ligase (GCL) and glutathione synthetase (GS). GCL is the rate-limiting enzyme in the biosynthesis of GSH. GCL has been the object of intense study regarding the regulation of its expression and activity [7]. GCL is a heterodimeric holoenzyme that includes two regulatory domains, the catalytic (GCLc, 73 kDa) and the modifier (GCLm, 33 kDa) subunits; each of them is the object of distinct regulation [8,9]. The interaction between the two subunits and that of both of them together with their common substrates, cysteine and glutamate, dictates the final efficiency of GSH synthesis [10].

Dysregulation of GSH synthesis has been described in several

Abbreviations: Ach, acetylcholine; ARE, antioxidant response element; BH_4, Tetrahydrobiopterin; DMNQ, 2,3-Dimethoxy-1,4-naphthoquinone; EDRF, Endothelium-derived relaxing factor; eNOS, Endothelial nitric-oxide synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCL, glutamate cysteine ligase; GS, glutathione synthetase; KHS, Krebs-Henseleit solution; MLEC, mouse lung endothelial cells; NA, noradrenaline; NAC, N-acetylcysteine; SDS, sodium dodecyl sulphate; SNP, sodium nitroprusside; UUO, Unilateral ureteral obstruction

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pathological conditions including liver injury [11], diabetes [12], neurological disorders [13], organ fibrosis [14] and cardiovascular disease [15]. Human polymorphisms of GCLc and/or GCLm may also result in decreased GSH synthesis and severe pathological phenotypes [16,17]. Genetic ablation of *Gclm* in mice is associated with impaired vascular reactivity [18], ovarian failure [19], lung inflammation [20] and mitochondrial dysfunction [21]. Of interest, the relationship between alterations in the redox status and fibrogenesis is intimately related to GSH homeostasis [14]. In this regard, it has been demonstrated that TGF-β decreases the expression of GCL [22] and our group reported that a specific miRNA, miR-433 was in part responsible of this repression mechanism [14,23,24]. However, due to the lethal embrionary phenotype of *Gclc* null mice, the specific role of the vascular suppression of GCLc has been difficult to address *in vivo*. In this work, we present data regarding the generation and characterization of conditionally endothelial-deficient *Gclc* mice. We found that endothelial haplo-insufficiency is sufficient to promote endothelial dysfunction in mesenteric beds, leading to eNOS inactivation in endothelial cells of heterozygous mice. In addition, these mice express reduced levels of the master regulator of antioxidant defense, Nrf2 and are prone to renal fibrosis.

2. Material and methods

2.1. Generation of endothelial-specific Gclc(e/+) Knockout Mice

Tie2-Cre transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). We generated endothelial-specific *Gclc* knockout [*Gclc(e/+)*] mice by intercrossing *Gclc(f/f)* mice with Tie2-Cre transgenic mice. The floxed *Gclc* mouse line, described in [25] was provided under agreement by Professors Ying Chen and Vasilis Vasiliou, from the University of Colorado. We conducted all studies on littermates. Animals were handled in agreement with the Guide for the Care and Use of Laboratory Animals contained in Directive 2010/63/EU of the European Parliament.

2.2. PCR analysis

For differentiation of the *Gclc(f) versus Gclc(+)* allele, genomic DNA isolated from tails was analyzed and genotyping was confirmed by PCR analysis using specific primers for DNA amplification (Sigma, St. Louis, MO) as described in [25]. The *Gclc(f)* allele was detected using primers A (GGGTGTTGG GTCGTTTGT) within the NEO gene, and B (CTATAATGTCCTGCACTGGG) within intron 3. The *Gclc(e)* allele was detected using primers C (TAGTGAACGGTGTTAAAGG) within intron 3, and D (TCACTGGATTCT CTCACC) within intron 6. The *Gclc(wt)* allele was detected using primers B and C. Primers (GCGGTCTGGCAGTAAAAACTATC, forward) and (GTGAAACAGC ATTGCTGTCACTT, reverse) were used to detect the Cre transgene.

2.3. Isolation of mouse lung endothelial cells (MLEC) and primary lung fibroblasts

Mice were sacrificed by cervical dislocation and lungs were excised and digested with collagenase from Clostridium histolyticum (Sigma, St. Louis, MO). The mixed population obtained was subjected to positive selection with anti-ICAM-2 (BD Biosciences, San Jose, CA, USA) and anti-IgG-coated magnetic beads (Invitrogen, Carlsbad, CA, USA); the leftover mixed cell population (fibroblast and macrophages) was subjected to negative selection with anti-FCsRII/III (BD Biosciences, San Jose, CA, USA). MLEC were cultured in EGM2 media and grown on a mixture of fibronectin (Sigma, St. Louis, MO), type I collagen (Thermo Scientific, Rockford, IL, USA), and 0.1% gelatin (Sigma, St. Louis, MO)-coated plates. Primary lung fibroblasts were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% Fetal bovine serum supplementation.

2.4. Western blot and densitometry analysis

After treatment, cells were washed with phosphate-buffered saline (PBS) and lysed in 150 µl RIPA lysis buffer containing 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 1% NP-40 and 25 mM Tris–HCl pH 7.6, in the presence of protease (Complete, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Cells were harvested by scraping, samples were clarified by centrifugation at 13,000 rpm for 15 min at 4 °C and protein concentration was measured with the BCA assay (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (40–80 µg) from the total extract were separated by electrophoresis using an acrylamide/bisacrylamide (10%) gel and transferred to a nitrocellulose membrane (GE Healthcare, Germany) at 12 V for 20 min in a semi-dry Trans-Blot Turbo system (Bio-Rad, Hercules, CA). After blocking the membranes in 5% skimmed milk in TBS-T (10 mM), they were incubated with the appropriate primary antibodies, anti-GCLc (generous gift of Dr. Kavanagh's laboratory), anti-GCLm (1:1000; sc-32251, Santa Cruz Biotechnology, CA, USA), anti-αSMA (1:1000; sc-32251, Santa Cruz Biotechnology, CA, USA), anti-β-actin antibody (1:15000; A1978, Sigma, St. Louis, MO), anti-tubulin (1:15000; T9026, Sigma, St. Louis, MO), anti-eNOS (1:1000; 610297, BD Biosciences, San Jose, CA, USA) and anti-p-eNOS (Ser1177) (1:1000; 9571, Cell Signaling Technology, Boston, USA). Secondary antibodies against rabbit and mouse were from LI-COR Biosciences (Lincoln, NE). Densitometry of at least 3 Western blots for each experiment was done using β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:15000; Sigma, St. Louis, MO) as housekeeping controls for the total levels of protein loaded. Phospho eNOS signals were corrected by the levels of total eNOS, after normalization with β-actin. These analyses were represented in bar graphs showing the mean ± SEM of protein respective to control conditions.

2.5. Real-time PCR

Total RNA was isolated with miRNeasy Mini Kit (Qiagen, California, USA). Reverse transcription was performed with 1 µg/µl mRNA sample using iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA). qRT-PCR was carried out with the iQ™SYBR Green Supermix (Bio-Rad, Hercules, CA), using specific primers for mRNA amplification (Sigma, St. Louis, MO). The relative quantification of gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [26]. Using this method, we obtained the fold changes in gene expression normalized to an internal control gene, GAPDH. The sequences of the primers were: mouse GAPDH, forward: 5′-GAGTCAACGGATTTGGTCGT-3′, reverse: 5′-TTGATTTTGGAGG GATCTC G-3′; mouse FN, forward: 5′-AGAGGCTGTGTTGCTGTGAA-3′, reverse: 5′-AA ATCCATCGGGTATCTGGA-3′; mouse Col1α1, forward: 5′-TGG AGAGGAAAGTGGCGGGGAG-3′, reverse: 5′-GCCTCACGGAACCACGAACG-3′; mouse GCLm, forward: 5′-CGGACGACCTGGTGAGAGA-3′, reverse: 5′-CATTGTGTCCCCTAATGCCTT-3′; mouse NRF2, forward: 5′-GTGTTGGG AATGGTCGTGGGGAATG-3′, reverse: 5′-CCAATGCCACGGCCATAGCAGTA GC-3′; mouse α-SMA, forward: 5′-CTGACAGAGGCACCACTGAA-3′, reverse: 5′-CATCTCCA GAGTCCAGCACA-3′.

2.6. GSH assay

Reduced and oxidized forms of GSH were measured in MLEC utilizing the luminescence GSH-Glo and 2GSH/GSSG assays, based on the conversion of a luciferin derivative into luciferin in the presence of GSH (Promega, Wisconsin, USA) according to the manufacturer's instructions. The cells were analyzed on a luminescence plate reader (Glomax, Promega, Wisconsin, USA).

2.7. ROS production

MLEC were plated in a 96-well plate to reach a confluence of 70%.