Endothelial Cell Dysfunction—Can One Outsmart Oxidative Stress by Direct Interaction with the Pathological Oxidized or Heme-Free Soluble Guanylyl-Cyclase?


It is well known that the rate of CV death is excessive in renal patients (1), but the underlying causes have not been completely resolved. Endothelial dysfunction, however, is thought to play a central role. One indicator of endothelial dysfunction, endothelial cell-dependent vasodilatation, was found by some (2,3), but not all, investigators to be impaired. Endothelial cells produce the vasodilator and vasoprotective molecule nitric oxide (NO), but in pathologic states the bioavailability of NO is reduced because the endothelial isoform of NO synthase (eNOS or NOSIII) is dysfunctional and generates reactive oxidant species—probably a phylogenetically ancient host defense reaction (4). Oxidative stress has been well documented in renal disease (5); specifically, nitrotyrosine, a footprint of oxidative stress, has been demonstrated in the vasculature (6).

To understand the rationale that led to the development of the compound 4-[(4-carboxybutyl)[2-[[(4-phenethyl-benzyl)oxy]phenylethyl]amino)methyl[bensoic]acid (BAY 58-2667), some introductory comments may be useful. This novel compound targets a signaling cascade that goes awry under conditions of oxidative stress, which is present in many forms of endothelial dysfunction. The cascade then produces oxidant species that promote tissue damage and, to make matter worse, reduces the production of NO.

The cascade comprises the sequential operation of the following enzymes (7):

- nitric oxide (NO) synthase (NOSIII)
- soluble guanyl cyclase (sGC), producing cyclic guanosine monophosphate (cGMP)
- cyclic GMP activated protein kinase (cGK)

The three NO synthase isoforms have a heme prosthetic group and require several cofactors: oxygen, NADPH, flavin mononucleotide, flavin adenine dinucleotide, calmodulin, and tetrahydrobiopterin (BH4). The NO synthases oxidize the guanidine nitrogen of L-arginine to L-hydroxy-arginine and subsequently to the gas NO and citrulline. In the presence of the above cofactors, the enzyme operates as an NO synthase; in the absence of BH4 (so-called “uncoupling”) it generates the highly reactive oxidant superoxide, which is further converted into hydroxyperoxide, hydroxyl radicals, and, by interaction with NO, into peroxynitrite.

The receptor for NO is the hemoprotein guanyl cyclase. Of the different membrane-bound or soluble cytosolic isoforms, the soluble isoform (sGC) is of interest in the following context. The heterodimer consists of α and β subunits with a ferrous (divalent iron Fe²⁺) heme prosthetic group. The iron is liganded to histidin 105 of the β subgroup. When NO binds to the Fe²⁺, the bond with histidine 105 is disrupted, which thus reverses the inhibition of the catalytic center and promotes the synthesis of cGMP. This mechanism explains why many known regulators of sGC were found to interact with the heme group as activators or inhibitors. Oxidation of the divalent Fe²⁺ in the heme molecule inhibits the activation of sGC by NO (8).

The development of BAY 58-2667 had also benefited from recent insights into the mechanisms underlying nitrate tolerance (9). Since the seminal observation of Stewart (10), it has been known that nitroglycerin loses efficacy as time goes by in patients with angina pectoris. Although part of the phenomenon (pseudotolerance) is explained by compensatory retention of fluid and sympathetic activation in response to the vasodilating effect of nitroglycerin, two phenomena have been found to account for true tolerance: reduced liberation of NO from nitroglycerin (11) (of somewhat controversial in vivo relevance) and development of oxidative stress as a conse-
quence of increased superoxide and peroxynitrite formation (9,11). Such oxidative stress (12) presumably explains why meta-analyses found increased cardiac morbidity with chronic use of nitrates (13). Reactive oxidant species oxidize the crucial cofactor of sGC, BH₄; depletion of BH₄ renders NOSIII dysfunctional ("uncoupling") so that it releases superoxide instead of NO. Reactive oxidant species also scavenge NO and reduce the activity of sGC that contains heme, which acts as a NO receptor (14). Furthermore, superoxide and peroxynitrite also adversely affect intracellular cGMP downstream signaling (15) as indicated by diminished phosphorylation of the vasodilator stimulated phosphoprotein (VASP). Antioxidants restore nitroglycerin sensitivity and VASP phosphorylation.

In view of these adverse effects of oxidative stress, it was of considerable interest that the recently developed BAY 58-2667 is an NO-independent sGC activator that is active even under conditions of nitrate tolerance, a state of known oxidative stress (16). The outcome of the present study is compatible with the concept that BAY 58-2267 selectively activates sGC in a presumably NO-insensitive oxidized and/or heme-free state. The attraction of such a compound is that it would selectively target diseased vessels as present in cardiovascular and renal disease.

How did the authors arrive at this conclusion?

• Protoporphyrin IX is known to bind to the heme pocket of sGC. With a standard recombinant preparation of sGC, the authors showed that BAY 58-2267, similar to protoporphyrin IX, activated sGC concentration dependently, even when the recombinant sGC was heme-free within the limitations of the methodology. Additional experiments suggested that both BAY 58-2267 and protoporphyrin IX bound either to an unoccupied heme pocket or a heme pocket after displacing weakly bound oxidized heme.

• Because multiple isoforms of sGC are known, the potential relevance of BAY 58-2267 for vascular disease was examined. In endothelial cell cultures the authors demonstrated under basal conditions an increase of cGMP upon administration of the compound without any effect on cGMP breakdown by phosphodiesterases, which suggests increased production of cGMP and excludes effects on cGMP catabolism. To imitate oxidative stress, these cells were also incubated with a peroxynitrite donor. This maneuver decreased NO-stimulated sGC activity after stimulation with NO and diminished cGMP concentrations. Paradoxically, the response to BAY 58-2267 was even increased by pretreatment with peroxynitrite.

• Apart from these results in acute studies, chronic studies showed that, in addition, prolonged exposure of endothelial cells to BAY 58-2267 caused an increase in the protein level of the heme-binding β1 subunit of sGC. The latter effect was not seen with known NO donors or NO sensitizers. The authors offered the plausible working hypothesis that BAY 58-2667 stabilized the β1 subunit of sGC, reducing its degradation under basal conditions. In addition, it also prevented its degradation via the proteasome pathway after exposure of sGC to oxidants such as methylene blue, presumably by stabilizing the conformation of the enzyme protein.

• To provide a functional readout the authors then proceeded to study isolated precontracted blood vessels (rat aortic ring preparations) in the presence or absence of peroxynitrite. The result was relaxation in response to administration of acetylcholine or of BAY 58-2667. Predictably, peroxynitrite impaired the maximal response to acetylcholine. An NO donor produced complete relaxation in the untreated vessel, but the potency was strikingly decreased after pretreatment with peroxynitrite. In contrast, pretreatment with the oxidant peroxynitrite strikingly increased the potency of BAY 58-2667.

• To prove the in vivo relevance of this finding the authors studied models of vascular disease; as a model of hypertension: the spontaneously hypertensive rat (SHR)

—as models of atherosclerosis: Watanabe heritable hyperlipidemic rabbits and ApoE⁻ ⁻/⁻ mice

—as models of diabetic vessel disease: eg, mesocolon arteries from patients with type 2 diabetes

• BAY 58-2667 improved relaxation and increased cGMP levels in the aortas of SHR.

• In saphenous artery rings of Watanabe rabbits, BAY 58-2667 inhibited the contraction in response to phenylephrin more potently than in wild-type rabbits, while the conventional
NO donor nitroprusside was similarly effective in the Watanabe and wild-type rabbits. This finding suggests that the effect of BAY 58-2667 is potentiated by oxidative stress. Analogous findings were obtained in precontracted aortas of ApoE/−/− mice on high-fat diet.

- Mesocolon arteries obtained during bypass surgery from diabetic patients were more potently relaxed by BAY 58-2667 than arteries of nondiabetic patients.
- Finally, the authors showed in vivo that such vasorelaxation translated into blood pressure lowering; blood pressure decreased after oral administration of BAY 58-2667 in SHR and transgenic renin rats [TG(mRen2)27 rats] treated with N-nitro-L-arginine-methylester to inhibit NO synthase. At the end of the study the rats had also lower levels of B-type natriuretic peptide, creatinine, urea, and renin.

The potential importance of this observation resides in the fact that this novel drug selectively targets a state of the modified sGC that is prevalent under disease conditions as shown in several animal models and in human disease. This selectivity of action is explained by the fact that BAY 58-2667 targets the oxidized heme-free sGC. This novel drug may open the door for more selective and sophisticated diagnostic procedures as well as interventions in cardiovascular disease, and potentially renal failure as the authors rightly state. It is certainly a long way from preclinical studies to clinical use—but the perspectives are promising.

References
The Antisenescence Protein Klotho Is Necessary for FGF23-Induced Phosphaturia

Klotho Converts Canonical FGF Receptor into a Specific Receptor for FGF23.


It was almost one decade ago (1) that the *klotho* gene had been identified, but it does not fail to still spring surprises. Mice with a defect of *klotho* gene expression secondary to an insertional mutation disrupting the 5' region develop a phenotype that reproduces not only individual features of human aging, but a complex spectrum of premature senescence: short lifespan, growth retardation, infertility, premature thymic involution, arteriosclerosis, skin atrophy, muscle atrophy, osteoporosis, pulmonary emphysema, ectopic calcification, motor neuron degeneration, cognition impairment, hearing disorder, and others (1). Conversely, overexpression of *klotho* extended the lifespan of mice (2). These observations suggest that the *klotho* gene acts as an aging suppressor gene. The link of the protein to the lifespan of mice led to the somewhat strange name “klotho.” Klotho was a Greek goddess, one of the three Parca, who was thought to spin the thread of life from her distaff onto her spindle. This mythology led Johannes Brahms to his famous romantic “Gesang der Parzen” (Schicksalslied; song of destiny).

The *klotho* gene is not only a determinant of premature senescence in mice. In humans as well, features related to senescence such as lifespan, osteoporosis, stroke, coronary disease are correlated to polymorphisms of the *klotho* gene (3,4). The *klotho* gene codes for a 130-kDa single-pass transmembrane protein with a short 10-amino acid cytoplasmic domain and a large extracellular domain that is shed and secreted into the blood (2,5). Although the Klotho protein is expressed in several tissues, the quantitatively most important site is the distal convoluted tubule in the kidney (1,6), but it is not yet certain whether Klotho acts in the kidney in a paracrine or endocrine fashion (in this case it would be another renal hormone).

How does *klotho* prolong lifespan? The *klotho* mouse with deficient *klotho* (*klotho*^−/−) is hypoglycemic and extremely sensitive to insulin (7), whereas transgenic mice that overexpress *klotho* are insulin-resistant. Insulin resistance caused by genetic manipulation of insulin receptors or postreceptor steps is known to be associated with lifespan extension in a long list of species from *Caenorhabditis elegans* or *Drosophila* to mammalians such as the mouse (8).

A second feature of the Klotho mouse is increased oxidative stress (9). But in the context of the kidney, the relation of Klotho to mineral and vitamin D metabolism is of greatest interest. The FGF23 gene was originally identified as the gene mutated in autosomal dominant hypophosphatemic rickets (10,11). FGF23 codes for a phosphaturic hormone (11) that also accounts for other forms of hypophosphatemia (12) and is synthesized mainly by osteoblastic cells (13). The striking observation has been made that *klotho* knockout (1) and FGF23 knockout mice (14,15) share a great number of features such as short lifespan, growth retardation, hypogonadism, premature thymic involution, skin atrophy, muscle atrophy, arteriosclerosis, osteoporosis, pulmonary emphysema, soft tissue calcification, and, of particular interest in the context of the above study, hyperphosphatemia and increased 1,25(OH)₂D₃ (14–16). FGF23 inhibits not
only phosphate reabsorption in the proximal tubule, but also regulates the activities of the key enzymes controlling vitamin D metabolism in the kidney, ie, CYP27B1 (1α-hydroxylase) and CYP24A1 (24-hydroxylase) (17). FGF23 deficiency in contrast increases renal phosphate reabsorption and increases 1,25(OH)2 D3 (14,15). Simultaneous knockout of FGF23 plus 1α-hydroxylase abrogated hyperphosphatemia and tissue calcification and rescued many premature aging-like features of the FGF23 knockout mice (18). This observation suggests an important role of active vitamin D in the genesis of the FGF23 knockout phenotype (6,19). Previous studies have shown that FGF23 not only inhibits phosphate reabsorption via the sodium phosphate transporter (NaPi2a) in the brush border membranes of the proximal tubule, but also shares common signalling pathways with Klotho (20). The Klotho protein binds to multiple FGF receptors. Furthermore, the Klotho-FGF receptor complex binds FGF23 with higher affinity than the FGF receptor or Klotho alone, which suggests that FGF23 requires Klotho to activate FGF signalling, thus explaining why klotho knockout mice develop many features of the phenotype observed in FGF23 mice.

The above paper (21) now carries this issue one step further by clarifying in considerable detail the signalling pathways involved. In a nutshell they showed that the Klotho protein is required to convert a FGF receptor that interacts broadly with many FGF isoforms (a canonical receptor) into a receptor highly specific for FGF23. The authors discovered that injection of FGF23 into mice triggered early events in the kidney but not in other organs: phosphorylation of extracellular signal-regulated kinase (ERK) within the ultrashort period of 10 minutes; upregulation of the expression of early growth-responsive gene 1 (Egr1) was seen and used as a readout within 1 hour.

The authors now pursued several strategies. First, in homogenates of mouse kidney they tried to identify the molecule(s) directly binding to FGF23. To this end the homogenate was run through FGF23-Sepharose; matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry revealed that the protein bound was Klotho. When klotho was transiently expressed in Chinese hamster ovary (CHO) or other cells, high and low affinity binding sites for FGF23 emerged and the cells responded by phosphorylating ERK and upregulating Egr-1. As was already known, klotho knockout mice had elevated serum phosphate, 1,25(OH)2 D3, and calcium, as do FGF knockout mice. The authors now showed that—presumably in response to hyperphosphatemia—FGF23 concentrations were massively increased (ie, several thousand-fold) in the klotho knockout mice. This observation resembles the increased FGF23 concentration that is seen in uremic patients in response to phosphate retention (12). To strengthen the evidence, the authors used a complementary approach, and to this end they first produced a monoclonal antibody against the extracellular portion of mouse Klotho protein. The monoclonal antibody blocked the FGF23-induced increase in Egr-1 promoter activity as a readout in cells expressing Klotho. To prove that the monoclonal antibody against Klotho abrogated the interaction between FGF23 and Klotho in vivo, the monoclonal antibody was injected into wild-type mice. The vitamin D–regulating enzymes in the kidney changed in the direction seen in FGF23 knockouts (14,15): the mRNA for CYP27B1 increased and the mRNA for CYP24A1 decreased; in parallel with an increased abundance of the sodium–phosphate cotransporter (NaPi2a) in the renal brush border membranes, serum phosphate increased—possibly the result of increased 1,25(OH)2 D3—serum calcium increased as well. Thus, blocking Klotho with the monoclonal antibody reproduced the main features of the FGF23 knockout mouse, which indicates that interaction between Klotho and FGF23 is necessary for FGF23 to express its activity in the kidney.

The study thus identified another player in the genesis of the disturbed mineral metabolism of renal disease. The unfolding evidence indicates, in retrospect, that the simplifying assumption of the past, ie, that the disturbance of the mineral metabolism in renal failure was the result of abnormal phosphate and calcium concentration and balance (22), requires revision and sophistication in the light of findings obtained with the powerful methodologies available today.
References


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