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Nitric Oxide/*L*-Arginine in Uremia

Sistiana Aiello[a] Marina Noris[a] Giuseppe Remuzzi[a, b]

[a] Mario Negri Institute for Pharmacological Research, Bergamo, and
[b] Division of Nephrology and Dialysis, Azienda Ospedaliera, Ospedali Riuniti di Bergamo, Italy

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Abstract
Nitric oxide (NO), a gaseous free radical derived from *L*-arginine, is a potent modulator of vascular tone and platelet functions. A number of recent studies, both in the experimental model of renal mass reduction (RMR) in rats and in uremic patients, have raised the hypothesis that abnormalities of NO synthetic pathway could have a key role in mediating the complex hemodynamic and hemostatic disorders associated to the progression of renal disease. Thus, kidneys from rats with RMR produce less NO than normal rats and NO generation negatively correlates with markers of renal damage. The abnormality is due to a strong defect of inducible NO synthase (iNOS) content in the kidney. Recent in vitro and in vivo data have raised the possibility that excessive renal synthesis of the potent vasoconstrictor and promitogenic peptide endothelin-1 (ET-1) is a major determinant for progressive iNOS loss in the kidney of RMR rats. In contrast, uremia is associated with excessive systemic NO release, both in experimental model and in human beings. In the systemic circulation of uremic rats, as well as uremic patients, NO is formed in excessive amounts. Possible cause of the increased NO levels is higher release from systemic vessels due to the augmented expression of both iNOS and endothelial NOS. A putative cause for excessive NO production in uremia can be guanidinosuccinate, an uremic toxin that accumulates in the circulation of uremic patients and upregulates NO synthesis from cultured endothelial cells. Upregulation of systemic NO synthesis might be a defense mechanism against hypertension of uremia. On the other hand, more NO available to circulating cells may sustain the bleeding tendency, a well-known complication of uremia.

Nitric Oxide and Nitric Oxide Synthases

Nitric oxide, a *L*-arginine derivative, is involved in a variety of physiological processes, such as vasodilation, neuronal transmission, immune response and platelet function modulation [1]. The synthesis of NO by vascular endothelium is responsible for the vasodilator tone, essential for the regulation of blood pressure. In vivo administration of *L*-arginine analogues such as *L*-NMMA (NG-monomethyl-*L*-arginine), that inhibit NO synthase, induces an increase in blood pressure of about 30 mmHg in rats and rabbits indicating that there is a continuous release of NO [2].

NO was also shown to play a central role in modulating blood clotting, thus contributing to the control of platelet aggregation and adhesion. Addition of human umbilical vein endothelial cells (HUVEC) to a platelet suspension blocked serotonine release and platelet aggregation through generation of NO and this inhibitory effect was reversed by preincubation of HUVEC with *L*-NMMA [3]. In addition to its antiaggregatory function, NO also modulates platelet adhesion: indeed exogenous NO re-

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Giuseppe Remuzzi, MD
Mario Negri Institute for Pharmacological Research,
Via Gavazzeni 11, I–24125 Bergamo (Italy)
Tel. +39 35 319 888, Fax +39 35 319 331, E-Mail gremuzzi@cyberg.it

duced the adhesion of thrombin-activated platelets to the endothelium [4].

NO is derived from the amino acid *L*-arginine in an unusual reaction catalyzed by a family of enzymes termed NO synthases, that convert arginine and oxygen into citrulline and NO [5]. NO synthase isoforms include the neuronal (nNOS, NOS 1), inducible (iNOS, NOS2) and endothelial (ecNOS, NOS3) NO synthases, the genes for which are located on chromosomes 12, 17 and 7, respectively [5]. Neuronal NOS and ecNOS are constitutive enzymes and are inactive until intracellular calcium levels increase [5]. These NO synthases, that can generate small quantities of NO, are activated by calcium-elevating agonists such as acetylcholine, thrombin and hystamine. By contrast, iNOS is a calcium-independent isoform which can be induced by various cytokines and produces huge amounts of NO for long time periods [5]. Originally purified from neurons, vascular endothelium and cytokine-induced macrophages, the three isoforms are now understood to be distributed across a wide spectrum of cell types and tissues. All three enzymes have been localized in the kidney and have been implicated in the control of its function. The constitutive nNOS has been localized strictly to the cells of the macula densa of the rat and mouse kidney by different groups [6]. Thus, a strong NOS catalytic activity (measured by NADPH-diaphorase reaction) was observed in the macula densa of the juxtaglomerular apparatus which colocalized with immunostaining for nNOS [6]. Using reverse transcriptase and polymerase chain reaction (RT-PCR) in individual, microdissected rat nephron segments, however, Terada et al. [7] localized nNOS mRNA in the inner medullary collecting duct and, to a lesser extent, in the glomerulus, inner medullary thin limb, and cortical and outer medullary collecting duct, as well as in parts of the renal vasculature. Messenger RNA for endothelial NOS has been demonstrated by RT-PCR in the glomerulus and afferent and efferent arterioles [8]. In a very recent report the distribution of ecNOS has been studied immunohistochemically in the rat and found in glomerular endothelium; efferent arteriolar endothelium showed stronger staining than afferent [6]. These two forms of NOS are crucially involved in renal pathophysiology and local release of NO serves to control renal blood flow and modulates the activity of tubuloglomerular feedback [9]. Using a juxtamedullary rat nephron preparation, Imig et al. [10] found that NO primarily alters afferent vascular tone, thereby modifying the ability of the preglomerular vasculature to autoregulate glomerular capillary pressure. In vivo micropuncture studies have shown that *L*-NAME infused into the proximal tubule or peritubular capillary augments the tubuloglomerular feedback-mediated decrease in single-nephron glomerular filtration rate (GFR) [9].

The mesangial cell is able to express inducible NOS after cytokine stimulation [11]: in rat mesangial cells iNOS is induced by interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and endotoxin [11]; in human mesangial cells iNOS is induced by IL-1β and interferon-γ in combination [11]; the effect is augmented by TNF-α. Using the PCR technique on mRNA extracted from control and cytokine-stimulated proximal tubular cells, McLay and coworkers [12] have found iNOS product indicating that proximal tubular cells express iNOS in basal condition and respond to immune challenge through the induction of iNOS. Other studies in the rat showed that glomerular mesangium and afferent arteriole, as well as various segments of the nephron, express iNOS under basal conditions and that renal iNOS expression is augmented after immune activation with cytokines or endotoxin [13]. Consistently, immunoperoxidase analysis, with specific antisera to iNOS showed iNOS signal localized to afferent arteriole, collecting ducts, outer medullary tubuli and medullary thick ascending limbs [6] of rat kidney. The steady-state amount of iNOS mRNA and protein in normal rat kidney is highest in tubuli of the outer medulla and in vitro studies on rat renal homogenates showed that the specific activity of NOS in the medulla was three times that of the cortex [14]. The significance of the high expression of iNOS in normal kidney has not been completely elucidated. It has been proposed that NO production by iNOS in medullary thick ascending limbs serves to maintain and regulate medullary blood flow and oxygenation [9]. There is also evidence that NO induces natriuresis by antagonizing the effect of angiotensin II on sodium reabsorption in proximal tubuli [9] and by directly inhibiting renal tubular Na+/K+ ATPase [15].

Role of Nitric Oxide in Pathophysiology of Chronic Renal Failure

Renal Nitric Oxide

It has been suggested that abnormalities of NO synthetic pathway could have a key role in causing and/or mediating the complex hemodynamic disorders associated to the progression of renal disease. Most of the studies have been focused on the experimental model of chronic renal failure (CFR) obtained in rats by extensive

renal mass reduction (RMR). These animals develop systemic hypertension and exhibit a decrease in both GFR and renal blood flow (RBF), despite an initial adaptive increase in single-nephron GFR and plasma flow per nephron [16]. Animals with RMR also develop severe proteinuria and structural changes in the kidney, including glomerulosclerosis, which eventually lead to renal insufficiency [16]. Like uremic patients, RMR rats have a bleeding tendency that manifests itself by a prolonged bleeding time [17].

A very recent study from our group showed that in RMR rats renal generation of NO, measured as ex vivo conversion of ^{3}H *L*-arginine to ^{3}H *L*-citrulline, was markedly lower than normal [18]. The abnormality was already evident seven days after surgery and progressively worsened with time, in close correlation with signs of renal injury, such as glomerulosclerosis and tubular damage. Consistent with these results, urinary excretion of NO_2^-/NO_3^-were significantly reduced in rats with RMR as compared to control animals [18]. These findings indicate that the RMR model is characterized by low renal NO production.

Kidney is a major source of arginine for the body, the largest fraction of renal arginine synthesis is in the proximal tubule and it occurs at the expense of filtrered citrulline [19]. Chan et al. [20] studied the activity of the arginine synthetase complex in rats with advanced CFR and found that it was markedly reduced. On the other hand in a more recent study, Boudy and coworkers showed that total renal arginine synthesis and plasma levels are not diminished in the remnant kidney model in contrast with a significant reduction in GFR [21]. This is possibly due to a combination of three factors: hypertrophy of proximal tubule in remnant nephrons; hyperfiltration which increases the amount of citrulline filtered per nephron and increase in plasma citrulline concentration which enhances arginine synthesis because of the concentration dependency of this synthesis in the kidney. Thus, a defective substrate availability cannot be considered as a main cause of impaired NO synthesis in RMR kidney.

By using two histochemical approaches locating either NOS catalytic activity (NADPH-diaphorase) or NOS isoenzyme expression (immunoperoxidase), we have gone deeper into the cellular basis and the biochemical nature of this abnormality in NO synthesis. NADPH-diaphorase documented a progressive loss of renal NOS activity in RMR rats in all the structures examined including glomeruli (except macula densa), proximal tubules and collecting ducts [18]. A strong, progressive decrease of iNOS immunostaining was also observed, colocalizing with the loss of NADPH-diaphorase signal, indicating that reduced NOS activity in RMR was likely due to a decrease of iNOS enzyme content [18]. In contrast to iNOS, we found that ecNOS staining was rather comparable in RMR and control kidneys. However, in RMR rats the ecNOS signal was reduced in glomeruli affected by slcerosis, possibly because of loss of functional endothelium [18].

NO in the renal vasculature and macula densa appears to control renal blood flow and tubuloglomerular feedback [9]. Besides its vasodilatory properties, NO inhibits mesangial cell proliferation [22] and extracellular matrix synthesis.

Diminished renal NO formation could therefore play some role in the hemodynamic and nonhemodynamic abnormalities reported in the model of extreme renal ablation that precede and eventually lead to the development of glomerulosclerosis and renal scarring. Actually, in rats with ablation of renal mass dietary supplementation with the NO precursor *L*-arginine for six weeks reduced glomeruloscerosis and this beneficial effect appeared to act through a reduction in glomerular capillary pressure and efferent arteriolar resistance [23]. These findings were confirmed by another study, which found that oral supplementation of *L*-arginine at a much lower dose had a protective effect on urinary protein excretion and renal function in RMR rats [24]. In these studies the efficacy of *L*-arginine was attributed, at least in part, to an increased formation of NO. However, whether the favorable effect of *L*-arginine was due to the increased NO release or depended by *L*-arginine per se has not been convincingly documented. Thus, there are data that both an acute oral protein load and intravenous amino acid infusion increase GFR and renal plasma flow in experimental animals and humans, possibly due to increase in vasoactive hormones [25, 26]. To address whether enhancing NO availability were beneficial on the process of progressive renal dysfunction in the ablation model, we chronically administered a NO-donor, molsidomine, to RMR rats. NO-donor treatment reduced proteinuria, slowed the rate of renal disease progression and prolonged survival [27]. This protective effect could be documented either by giving the drug at the time of surgery or later when rats were already proteinuric and hypertensive.

What are the possible causes of iNOS progressive loss and reduced NO formation in CRF? In glomeruli soon after surgical ablation of renal mass inflammatory mediators, such as platelet-derived growth factor (PDGF) [28]

and transforming growth factor β (TGF β) [29], are formed in excessive amounts. PDGF and TGF β are both potent inhibitors of NO synthesis and dose-dependently block IL-1β-induced iNOS mRNA in rat mesangial cells [30]. It is therefore conceivable that an exaggerated concentration of PDGF and TGF β in glomeruli of rats with RMR downregulates glomerular iNOS. However, recent in vitro [31, 32] and in vivo data have raised the possibility that excessive renal synthesis of the potent vasoconstrictor and promitogenic peptide endothelin-1 (ET-1) is a major determinant for progressive iNOS loss in the kidney of RMR rats. Within the kidney, ET-1, the first peptide of the endothelin family to be discovered, is constitutively synthesized and released by both glomerular and tubular cells [33–35]. In rats with RMR, despite no increase in plasma ET-1 levels, renal prepro-ET-1 gene expression [36] and urinary excretion of the peptide increased time dependently, the latter correlating with signs of renal injury [36]. Studies on location of prepro-ET-1 mRNA by in situ hybridization have shown that in RMR rats ET-1 mRNA increases in cortical tubules as early as 14 days after surgical ablation, in concomitance with the onset of proteinuria. At 1 month tubular ET-1 expression further increased in RMR rats and the signal was apparent also in glomeruli [37]. By analyzing serial sections of renal tissue from RMR rats studied longitudinally after surgical ablation, we have observed a colocalization between intense ET-1 mRNA signal and loss of iNOS immunoreactivity on the same cortical tubules (unpublished data). This observation is of particular interest since recent data are available that ET-1 strongly inhibits cytokine-induced NO formation in cultured mesangial [31] and epithelial [32] cells by blocking iNOS transcription. This action of ET-1 is likely mediated by interaction with subtype A receptor, indeed the ET_A receptor antagonist BQ123 can overcome the inhibitory effect of the peptide on iNOS expression and NO release [31, 32]. Thus, it is conceivable that the early increase of ET-1 in cortical tubules in RMR rats may cause a block of iNOS transcription in these structures thus leading to a progressive loss of iNOS protein and NO production.

Evidence that ET-1 effectively plays a role in the process of progressive renal injury in chronic renal disease are now available from studies in which selective pharmacological manipulation of ET-1 receptors have been performed. Administration of a selective ET_A receptor antagonist, FR 139317, to RMR rats normalized blood pressure, reduced proteinuria and ameliorated renal function and histology [38]. Treated animals showed very low average frequency of segmental glomerulosclerosis and a suppressed renal expression of c-*fos* proto-oncogene, an early marker for cell proliferation which was upregulated in untreated remnant kidney animals. Interestingly, a very recent study from our laboratory showed that 1 month treatment with FR 139317 increased ex vivo renal NO generation in RMR rats as compared to untreated RMR animals (unpublished data). These data provide in vivo evidence that increased renal ET-1 release in RMR rats, besides being per se noxious, could also be responsible, at least in part, for the defective renal NO availability.

Systemic Nitric Oxide

Since 1990 the possibility that the bleeding tendency in uremia was associated with excessive systemic formation of NO has been investigated, both in experimental animals and in humans. Bleeding tendency, whose skin bleeding time is still now the best predictive marker, is a well-known complication of uremia, both in experimental models and in human beings, due to a defective primary hemostasis [39].

Nitric oxide interferes with primary hemostasis by multiple pathways; it counteracts vessel injury-induced vasoconstriction [40, 41], inhibits platelet adhesion to damaged endothelium [42] and interferes with the process of platelet–platelet interaction by activating soluble guanylate cyclase that increases platelet cyclic GMP [43]. Several reports have also documented that platelets are not only targets for NO released by other cells such as neutrophils [1], monocytes [1] and endothelial cells [1], but they are themselves also capable of producing NO which acts as an autocrine modulator to limit platelet activation [43, 44]. The in vivo counterpart of the antiplatelet activity of NO is the prolongation of skin bleeding time observed in healthy volunteers given NO by inhalation [45].

Plasma concentrations of the stable NO metabolites, nitrites and nitrates were higher than normal in RMR rats [18] indicating, as opposite to the kidney, an increased systemic NO production. Excessive formation of NO at systemic level derives from vessels as documented by increased NO synthase activity and higher expression of both ecNOS and iNOS isoenzymes in the aorta of uremic animals [18]. It is of interest that aortic ecNOS increased early during the course of the disease, while iNOS rose considerably later, when animals developed uremia. Causes of high vascular ecNOS expression in RMR, as in other experimental models of hypertension, very likely include changes in shear stress, since endothelial cells appear to release NO in response to changes in blood flow, and ecNOS mRNA and the corresponding protein

are induced in endothelial cells after exposure to fluid shear in a cone-and-plate apparatus [46]. A similar mechanism operates in hypertension where pulsatile stretch and distension of the arterial wall favor NO release, as indicated by recent findings that cyclic strain induces the expression of ecNOS transcripts and protein levels in cultured endothelial cells [47]. Besides ecNOS an increased expression of iNOS was found in systemic vessels of RMR rats which was not confined to the endothelium but was also detectable in smooth muscle.

N-monomethyl-*L*-arginine, a competitive inhibitor of NO synthesis, normalized the prolonged bleeding time of uremic rats and increased ex vivo platelet adhesion [17] which supports a causal role of excessive NO synthesis on bleeding tendency in these models. Consistently, in the same model we have documented that the shortening effect on bleeding time of conjugate estrogens, elective drugs in the treatment of symptomatic bleeding of uremics, was associated to a complete normalization of plasma concentrations of NO metabolites and vascular expression of NOS isoenzymes [48]. Once more, administration of the NO precursor *L*-arginine, completely abolished the hemostatic effect of conjugated estrogens in uremic rats [49]. Altogether the above experimental data suggest that uremic bleeding is associated with excessive synthesis of NO and that correction of the hemostatic defect can be achieved by normalization of NO levels.

Experimental findings were confirmed by human studies, thus platelet rich plasma (PRP) from chronic hemodialyzed patients with prolonged bleeding time, studied before a regular hemodialysis (HD) session, generated more NO than PRP from healthy controls either in basal conditions or after exposure to collagen [44]. The same trend was observed when NO synthesis was evaluated as a function of cyclic GMP formed in control and uremic platelets. Very recently Dr. Madore and coworkers [50] and Dr. Matsumoto [51] confirmed in vivo that the synthesis of NO is increased in chronic hemodialyzed (HD) patients. They found that the concentration of NO in the exhaled air [51] and the total NO output [51] were higher in HD patients before a dialysis session than in healthy controls. Consistently predialysis plasma nitrites/nitrates concentrations were also higher in HD patients [50, 51].

Plasma from uremic patients, unlike normal plasma, potently induced in vitro NO synthesis in cultured human umbilical vein endothelial cells [44] as well as in human microvascular endothelial cells exposed to uremic plasma [52] which suggests that substances accumulate in the plasma of uremic patients that are capable of upregulating NO synthesis.

But which are these NO-releasing substances? Cytokines such as TNFα and IL-1β are possible candidates since both are potent inducers of the inducible isoform of NO synthase [53] and circulate in increased amounts in the blood of patients with chronic renal failure either undialyzed or on maintenance HD [54–56]. IL-1β and TNFα are generated in vivo by circulating monocytes during dialysis with complement activating membranes [44, 54, 55]. Increased cytokines production may also be triggered by intact endotoxin, endotoxin fragments and other bacterial toxins that may cross the dialysis membranes [57], and also by acetate-containing dialysate [56]. Thus, in HD conditions that induce massive release of cytokines NO synthesis is upregulated. Actually, Yokokawa and coworkers found that uremics may occasionally have an increase in plasma levels of nitrites/nitrates during HD [58, 59], similarly Rysz and coworkers [60], by using a NO sensor microprobe provided evidence of in vivo NO release in uremic blood during HD with cuprophan.

On the other hand data are also available that NO synthesis does not increase, but rather decreases during the course of a dialysis session [50, 51]. This would indicate that, in optimal HD condition that induces no or minimal cytokine activation, HD corrects the exaggerated NO synthesis, possibly by removing from uremic plasma some dialyzable NO-releasing substances.

In 1993 Drs. Thomas and Ramwell [61] suggested that other substances, apart from *L*-arginine are involved in the generation of NO. They concentrated on guanidino compounds related to arginine, such as guanidinosuccinate (GSA), guanidino acetate, guanidino propionate, guanidine and methylguanidine which accumulate in plasma of uremics in micromolar concentrations [62, 63] and are shown to be potential toxins [64]. Most of the endogenous guanidines had no effect on isolated rat aortas, used as biological indicator of NO formation [61, 65]. By contrast GSA did actively relax isolated rat aorta in a dose-dependent fashion [61]. The vasodilatory effect of GSA was predominantly mediated by the endothelium [61], and was associated with increased cyclic GMP formation in rat vessels. Both the vasodilation and cyclic GMP formation were attenuated by NO inhibitors such as *L*-NMMA and hemoglobin [61]. Altogether the above data suggested, although indirectly, that GSA could induce generation of NO by rat vessels. By reasoning on that, we recently undertook a study [66] with the aim to investigate whether GSA actually is able to induce NO release by vascular endothelial cells. Human endothelial cells were exposed in vitro to increasing amounts of the GSA (from 0.1 μ*M* to 50 μ*M*) and NO release in the supernatant was assayed by measuring the

concentration of nitrites/nitrates. We found that GSA induced a dose-dependent increase in NO release which was abrogated by treating cells with either diphenyleniodonium or *L*-NMMA, two specific inhibitors of NO synthase. Of note the concentration of GSA that induced maximal NO release in vitro (10 μ*M*) is very close to those we found in the plasma of uremic patients before dialysis (15.5 ± 7.2 μ*M*). A lower, although still significant stimulation was observed at 1 μ*M*, a concentration similar to that found in postdialysis while 0.1 μ*M* GSA (concentrations found in normal plasma) has no effect on NO synthesis.

The effect of GSA of stimulating NO release provides a biological explanation of the old data of early 1970 showing that among uremic toxins GSA was the only one that consistently inhibited platelet function to such a degree that it was defined the 'x factor' in uremic bleeding [67].

Conclusion

Alterations in NO synthetic pathway do play a crucial role in the physiology and pathology of the uremic condition. In the kidney, with the progressive development of renal insufficiency, less and less NO is formed locally, apparently as a consequence of a time-dependent progressive reduction in the amount of the iNOS molecule expressed in the kidney. In this setting a marked reduction of NO, in the face of continuous local generation of vasoconstrictor and promitogenic substances such as ET-1, may well contribute to intraglomerular capillary hypertension and cell proliferation.

By contrast, uremia causes the release of abnormally large amounts on NO into the systemic circulation, likely as the consequence of upregulation of both ecNOS and iNOS. While the increase in vascular ecNOS occurs early in response to progressive renal function deterioration, upregulation of iNOS can only be detected in the advanced phases of the disease and is not confined to the smooth muscle cells, but also involves the vascular endothelium.

A putative source of NO in uremia can be guanidinosuccinate, the uremic toxin that accumulates in the circulation of uremic patients and induces an excessive synthesis of NO from vascular endothelium [66]. Increased NO release might be an early defense mechanism against hypertension of uremia. On the other hand, more NO available to circulating cells may sustain the bleeding tendency, a well-known complication of uremia in experimental animals and humans.

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