Nitric Oxide and Lipid Peroxidation in Childhood Nephrotic Syndrome

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Abstract

Objectives: This study was conducted to find if there is any alteration in the synthesis of endothelial-derived relaxing factor nitric oxide (NO) and in lipid peroxidation in childhood nephrotic syndrome. Both mechanisms are claimed to have a role in the pathogenesis of immunologically-mediated renal injury and both are reported to interact with one another.

Methods: The stable metabolite of NO, urinary nitrite, and the degradation product of lipid peroxide, serum malondialdehyde (MDA) together with serum creatinine, blood urea nitrogen (BUN) and urinary protein in 24 hour urine were estimated in 10 healthy control children (Group I) and in 47 children suffering from different forms of the nephrotic syndrome: Minimal change nephrotic syndrome (MCNS) at first presentation (Group II, 10 cases), in remission (Group III, 10 cases), in relapse (Group IV, 10 cases) and focal segmental glomerulosclerosis (FSGS) (Group V, 8 cases) and membranous glomerulonephritis (MGN) (Group VI, 9 cases).

Results: Our results showed significantly higher levels of urinary nitrite in patients with MCNS versus controls and children with FSGS or MGN. The urinary nitrite level was 3.5 +/- 1.3 umol/L in the controls, 9.2 +/- 1.7 umol/L in Group II (p < 0.001), 7.0 +/- 1.8 umol/L in Group III (p < 0.001), 10.6 +/- 1.6 umol/L in Group IV (p < 0.001), 3.08 +/- 1.3 umol/L in Group V (p NS) and 3.38 +/- 0.9 umol/L in Group VI (p NS). The urinary nitrite levels showed no significant correlation to the 24 hour urinary protein in all patients (r = 0.163, p > 0.05). On the other hand serum MDA showed significantly higher levels in all patient groups (Group II: 2.8 +/- 0.96 umol/L; Group III: 1.61 +/- 0.46 umol/L; Group IV: 2.38 +/- 0.86 umol/L; Group V: 4.06 +/- 1.11 umol/L and Group VI: 4.42 +/- 1.25 umol/L) compared to controls (0.73 +/- 0.29 umol/L) (p < 0.001). The increase was maximum in FSGS and MGN and least in MCNS. There was a strong positive correlation between serum MDA and 24 hour urinary protein (r = 0.584, p < 0.001).

Conclusions:
1) The measurement of urinary nitrite excretion may be useful in differentiating MCNS from FSGS and MGN.
2) NO may play a protective role in cases of MCNS as indicated by the higher urinary nitrite levels.
3) The continuous production of NO in MCNS even in remission may serve to scavenge small fluxes of superoxide radical produced by endothelial cells.
4) Lipid peroxidation may play a major role in the production of proteinuria and in the pathogenesis of the nephrotic syndrome.

INTRODUCTION

There is increasing evidence that the endothelial-derived relaxing factor, NO, is synthesized in the kidney and plays a crucial role in regulation of renal hemodynamics and excretory function\(^1\). NO decreases renal vascular responsiveness to vasoconstrictors, while inhibitors of NO synthesis reduce renal blood flow\(^2\). NO also affects tubular function, as it inhibits sodium transport by cortical tubular cells\(^3\). NO is also claimed to be associated with
proximal tubule epithelial cell injury\(^{(4)}\) and to mediate immunologic injury in experimental glomerulonephritis\(^{(5)}\).

NO is synthesized from L-arginine by two different types of enzymes: i- Constitutive calmodulin-dependent NO synthases (cNOS) which are present in endothelial cells. These synthases are activated by increased intracellular calcium levels and temporarily produce small amounts of NO which play a major role in the regulation of vascular tone and neurotransmission\(^{(6)}\); ii- Inducible NO synthases (iNOS) which are expressed in macrophages, vascular smooth muscle cells, mesangial cells and renal tubular cells. In contrast to cNOS, iNOS are activated by specific cytokines and produce large amounts of NO for prolonged periods\(^{(7)}\). Released NO exerts its effects through binding to iron-containing enzymes leading to activation or inactivation of those enzymes, by increasing cGMP and/or by facilitating transfer of an ADP-ribose group to an accepting molecule, a process called ADP-ribosylation\(^{(8,9)}\).

There is also considerable evidence suggesting that reactive oxygen species (ROS) as superoxide anion (O\(_2^-\)), hydroxyl radical and hydrogen peroxide are implicated in the pathogenesis of ischaemic, toxic and immunologically-mediated renal injury. ROS are generated by both infiltrating blood-borne cells (polymorphonuclear leucocytes and monocytes) and resident glomerular cells mainly mesangial cells\(^{(10)}\). ROS cause lipid peroxidation of cell and organelle membranes and hence disruption of the structural integrity and capacity for cell transport and energy production\(^{(11)}\).

Both NO and superoxide radical are endothelial-derived factors, however, the former is a relaxing factor and the latter is a contracting factor. NO was reported to be a biological scavenger or inactivator of oxygen free radicals\(^{(12,13)}\); however, Beckman et al.\(^{(14)}\) reported that NO can combine with O\(_2^-\) to form an unstable peroxynitrite molecule which further reacts to form highly toxic hydroxyl radicals in the tissues. Peroxynitrite has great relevance to renal diseases through induction of alterations in ion channels and transporter proteins\(^{(15)}\).

Therefore we conducted this study to determine the possible changes in NO production and lipid peroxidation in different forms of childhood nephrotic syndrome, and to find any possible correlation between each parameter and the proteinuria occurring in the disease.

**SUBJECTS AND METHODS**

Forty-seven children and adolescents (age range: 5 - 14 years) suffering from nephrotic syndrome were selected from the nephrology clinic of the New Children Hospital of Cairo University. Ten age-matched control subjects, free of kidney disease were enrolled in the study. All subjects were hospitalized for three days to be kept on the same feeding conditions. These 57 subjects were divided into the following six groups:

Group I: Control children (10 subjects).
Group II: Children suffering from MCNS at first presentation (10 cases).
Group III: Children suffering from MCNS in remission (10 cases).
Group IV: Children suffering from MCNS in relapse (10 cases).
Group V: Children with FSGS (8 cases).
Group VI: Children with MGN (9 cases).

In addition to an accurate history, all patients had a full physical examination, including weight, height and blood pressure measurement.

The diagnosis of MCNS was made in children with recurrent steroid-responsive proteinuria. The diagnosis of FSGS and MGN was based on percutaneous renal biopsy. Nephrotic range proteinuria was defined as a urinary protein level of more than 1g/m²/day. Relapse was defined as proteinuria of more than 1g/m²/day for 3 consecutive days. Remission in response to treatment was achieved when urine specimen was free of protein for 3 consecutive days. None of the children was being treated with immunosuppressive medications other than corticosteroids. Urine and blood samples were obtained at the time of the initial diagnosis of nephrotic syndrome. Additional urine and blood samples were collected from children who went into remission in response to corticosteroid therapy and from children with previously diagnosed nephrotic syndrome during remission or relapse of their disease. Culture of the urine samples was done to exclude samples positive for urease-producing bacteria. Urine samples were frozen within one hour of collection.

The following parameters were estimated:

Colourimetric determination of BUN, serum creatinine, urinary protein/24 hour urine (by conventional methods), urinary nitrite excretion as a marker of NO production and the degradation product of lipid peroxides, serum MDA.

* Estimation of urinary nitrite excretion

Total urinary nitrite and nitrate was quantitated after incubation of urine samples with nitrate reductase (Aspergillus Species-Sigma), NADPH and FAD in a 37°C water bath for 15 minutes to convert nitrate to nitrite. 0.4 ml of the reduced samples were mixed with 0.8 ml of 1% sulfanilamide in 2.5% phosphoric acid and 0.8 ml of 0.5% naphthylethylene diamine hydrochloride in 2.5% phosphoric acid. Absorbance was read at 543 nm and concentrations were determined from a linear standard curve obtained from standards of known concentrations (range 3-100 μmol/L) of sodium nitrite.

* Estimation of serum MDA

This was done using the method of thermal decomposition of lipid peroxide to MDA which reacts with thiobarbituric acid (TBA) to form a coloured product.

RESULTS

- Results are summarized in Table (1).
- There was a significant increase in urinary protein/24 hour in all patient groups compared to controls (p < 0.05).
- There was a highly significant increase in urinary nitrite level in the children suffering from MCNS versus controls (p
< 0.001) with a significant difference between those in remission on one hand and those in relapse or those at first presentation (p < 0.05).

- There was no significant difference between urinary nitrite levels in controls and those with FSGS or MGN (p > 0.05 for each).

- There was no correlation between urinary protein and nitrite levels in nephrotic children (r = 0.163, p > 0.05).

- As regards serum MDA, there was a significant increase in all nephrotic groups compared to controls (p < 0.005 for each) with highest levels in MGN and FSGS which were also significantly higher than levels in groups with MCNS (p < 0.05).

- A positive correlation was found between serum MDA levels and levels of urinary protein/24 hours (r = 0.584, p < 0.001).

Table (1): Levels of BUN (mg/dl), serum creatinine (mg/dl); urinary protein (g/m²/day), urinary nitrite and nitrate (μmol/L) and serum MDA (μmol/L) in the various groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MCNS 1st presentation</th>
<th>MCNS in remission</th>
<th>MCNS in relapse</th>
<th>FSGS</th>
<th>MGN</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/dl)</td>
<td>6.6 ± 2.12</td>
<td>8.1 ± 2.53</td>
<td>7.7 ± 2.99</td>
<td>8.8 ± 3.65</td>
<td>9.5 ± 3.77</td>
<td>9.0 ± 4.2</td>
</tr>
<tr>
<td>Serum Creatinine (mg/dl)</td>
<td>0.51 ± 0.20</td>
<td>0.61 ± 0.29</td>
<td>0.57 ± 0.21</td>
<td>0.63 ± 0.31</td>
<td>0.71 ± 0.33</td>
<td>0.74 ± 0.38</td>
</tr>
<tr>
<td>Urinary Protein (g/m²/day)</td>
<td>0.11 ± 0.003</td>
<td>1.31 ± 0.28*</td>
<td>0.35 ± 0.06*</td>
<td>1.36 ± 0.44*</td>
<td>1.65 ± 0.37*</td>
<td>1.78 ± 0.46*</td>
</tr>
<tr>
<td>Urinary nitrite (μmol/L)</td>
<td>3.5 ± 1.26</td>
<td>9.21 ± 1.66*</td>
<td>7.0 ± 1.82*</td>
<td>10.6 ± 1.55*</td>
<td>3.08 ± 1.27</td>
<td>3.38 ± 0.87</td>
</tr>
<tr>
<td>Serum MDA (μmol/L)</td>
<td>0.73 ± 0.29</td>
<td>2.8 ± 0.96*</td>
<td>1.61 ± 0.46*</td>
<td>2.38 ± 0.86*</td>
<td>4.06 ± 1.11*</td>
<td>4.42 ± 1.25*</td>
</tr>
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(∗: The difference between the mean values of the group and the control group is significant p < 0.05).  
(Levels are expressed as mean ± S.D.)

DISCUSSION

In the present study, urinary nitrite, an indicator of NO production\(^{21}\), showed elevated levels in MCNS children compared to controls with a significant difference between those in remission and those in relapse or at the first presentation. Trachtman et al.\(^{17}\) also reported elevated urinary nitrite levels in all children with MCNS regardless of the activity of the disease or whether the patients were being treated with corticosteroids. They concluded that measurement of increased urinary nitrite is highly diagnostic of MCNS in a child with new onset nephrotic syndrome. A circulating factor such as the lymphokine produced by activated suppressor T cells\(^{22}\) or other cytokines may be responsible for
stimulating NO synthesis in children with MCNS. Drapier et al. reported that tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) are potent inducers of NO synthesis. Markewitz et al. reported that stimulation of the kidney epithelial cells with TNF-α and IFN-γ dramatically increase the levels of iNOS mRNA. Bustos et al. reported higher levels of TNF-α in patients with MCNS in activity than in those in remission. Neuhaus et al. reported that relapse of steroid-sensitive idiopathic nephrotic syndrome is associated with release of IFN-γ. The above may give a possible explanation to the higher urinary nitrite levels in relapse and at the first presentation than the corresponding levels in remission of MCNS.

On the other hand, our results showed no significant difference in urinary nitrite levels between patients with FSGS or MGN and controls. Kovacs et al. reported normal nitrite levels in IgA nephropathy and Trachtman et al. also reported normal levels in FSGS and IgA nephropathy patients. Wada et al. and Matsumoto et al. reported higher levels of interleukin-8 (IL-8), which is a potent inhibitor of NO synthesis, in patients with MGN and IgA nephropathy respectively. Thus, it seems that the difference in cytokine production in different forms of nephrotic syndrome may be the cause of increased NO production in MCNS and not in other forms and that these forms of nephrotic syndrome represent distinct disorders with separate mechanisms for the development of glomerular dysfunction. In accordance with our results, Kovacs et al. and Trachtman et al. concluded that urinary nitrite should not be considered a marker of immune complex glomerular injury.

In the present study, lipid peroxide levels (expressed in terms of serum MDA) were shown to increase significantly in all nephrotic groups with highest levels in FSGS and MGN and least levels in MCNS. Lipid peroxidation results in morphologic lesions and in modification of glomerular permeability to proteins. This was reported to be induced via liberation of platelet-activating factor (PAF) which is the active inducer of vascular protein leakage.

Strong positive correlation between serum MDA levels and proteinuria was observed, a finding that suggests a crucial role of lipid peroxidation in the occurrence of proteinuria in nephrotic patients. On the other hand, the non-significant correlation between urinary nitrite and protein levels suggests that renal NO synthesis does not directly modulate proteinuria in nephrotic individuals. Similar results regarding the latter finding were reported by Trachtman et al. On the contrary, Komers et al. reported that NO might be a candidate for mediating proteinuria.

Omata and Wolfe et al. reported that the administration of superoxide dismutase (superoxide scavenger) and L-arginine (NO precursor) respectively could effectively reduce proteinuria in patients with chronic glomerulonephritis. Rubanyi et al. demonstrated a cytoprotective function of NO by inactivation of oxygen free
radicals produced by human leucocytes. Administration of L-arginine was reported to decrease the infiltration of the kidney by macrophages (important generators of free radicals) in acute puromycin aminonucleoside nephropathy (an analog of MCNS). Kubes and Granger showed that the inhibition of NO production by vascular endothelium leads to a reversible increase in microvascular protein efflux mediated by both leucocyte-dependent and independent mechanisms. They postulated that the inhibition of NO may lead to activation of other inflammatory cells, including platelets, mast cells, or macrophages, which in turn release substances that can increase microvascular permeability. Gaboury et al., also reported that NO prevents platelet and monocyte adhesion, which cause the alterations in vascular integrity, and suggested that the antiadhesion properties are related to its ability to inactivate the superoxide anion.

Thus, we may suggest a protective role of NO in cases of MCNS as indicated by the higher urinary nitrite and lower serum MDA levels versus the other forms of nephrotic syndrome. The continuous production of NO even in MCNS in remission may serve to scavenge small fluxes of superoxide radical produced by endothelial cells. The higher lipid peroxidation and the lower NO production in FSGS and MGN may explain why the prognosis is much poorer in those forms than in MCNS. Measurement of urinary nitrite excretion may be useful to discriminate MCNS from the other two forms of nephrotic syndrome (FSGS and MGN) especially at the onset of the disease instead of resorting to renal biopsy or testing clinical response to steroids. However to verify the above statement, further studies on a large scale of cases in a limited age period should be performed.

REFERENCES


