High-protein-induced glomerular hyperfiltration is independent of the tubuloglomerular feedback mechanism and nitric oxide synthases

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High-protein-induced hyperfiltration has been proposed to be mediated by the TGF mechanism (18, 24). A high protein intake will increase the filtration of amino acids, which, in turn, will increase the amino acid reabsorption in the proximal tubule. Because the reabsorption of most amino acids is sodium dependent [reviewed by Gonska et al. (7)], increased sodium reabsorption will also take place. This will lower the sodium chloride concentration at the macula densa, consequently reducing the degree of TGF signaling, which would be expected to increase from the GFR.

NO is a powerful vasodilator, which is produced by three types of nitric oxide synthase (NOS) isoforms; endothelial- (eNOS), inducible- (iNOS), and neuronal- (nNOS) NOS. In the renal cortex, the isoforms considered most important in the regulation of GFR are eNOS and nNOS. eNOS is abundant in the afferent arterioles, and nNOS is mainly found in the macula densa cells [reviewed by Mount and Power (12)]. NO has been considered to be a possible mediator of hyperfiltration, since the urinary excretion of cyclic guanosine monophosphate is increased during a high-protein intake (8), and unspecific NOS inhibition has been shown to reduce hyperfiltration (25). Furthermore, recent data have indicated a particular role of nNOS in hyperfiltration (27).

In the present study, we developed a model for high-protein-induced hyperfiltration in conscious mice. This technique enables consecutive measurements in the same animal and eliminates the risk of unpredictable effects of anesthesia. Using this model, we investigated two possible mechanisms that could cause high-protein-induced hyperfiltration: a reduced TGF activity due to increased proximal reabsorption and an increased NO production that directly dilates the afferent arteriole.

MATERIALS AND METHODS

All experiments were approved by the regional animal ethics committee in Uppsala and performed in accordance with the National Institutes of Health’s international guidelines for use and care of laboratory animals. Four experimental series were performed in mice.

Series I: Development of a model for high-protein-induced glomerular hyperfiltration in mice. The present model for high-protein-induced hyperfiltration was evaluated by performing time-control GFR experiments and by measuring renal and glomerular hypertrophy. Two groups of wild-type C57BL/6j male mice were used. In both groups, GFR was measured after 10 days on a low-protein diet. In half of the animals, the diet was switched to a high-protein diet (50% casein) for 10 days, followed by a high-protein diet (50% casein) for 10 days. All mice developed high protein-induced hyperfiltration to a similar degree. These results demonstrate that high protein-induced glomerular hyperfiltration is independent of the TGF mechanism and NOS isoforms.

A CHRONIC HIGH-PROTEIN INTAKE is associated with an increase in glomerular filtration rate (GFR) in humans, as well as in animal models (2, 18, 19). Hyperfiltration, in general, has clinical importance, since it has the potential to cause renal damage, which is associated with albuminuria, glomerulosclerosis, and hypertension (4).

The tubuloglomerular feedback (TGF) mechanism is a negative feedback loop operating in the juxtaglomerular apparatus of each nephron, which adjusts the tubular sodium load to match tubular transport capacity (16). If the GFR increases, the macula densa cells will detect an increased tubular sodium chloride concentration, with subsequent activation of the TGF mechanism. The result will be a constriction of the afferent arteriole and a normalized GFR. The signaling from the macula densa cells to the adjacent afferent arterioles involves adenosine, and knockout mice deficient of adenosine A1-receptors (A1AR) completely lack the TGF response (5, 21).

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were embedded in paraffin, cut into 5-μm sections, and stained with periodic acid-Schiff. The glomerular volume was determined as described earlier (22). The entire renal cortical area of one section from the first five animals in each group was photographed. Computer software (Photoshop CS3 Extended; Adobe Systems, San Jose, CA) was then used to manually determine the area of all visible glomeruli (40–98 glomeruli/section). Mean values for the area of the glomeruli were calculated for every section. Assuming that the glomeruli are circular, the mean diameters (d) were calculated from the areas. From the calculated mean diameters, an approximation of the diameter around the waist (D) was calculated using the equation: \( D = (4/\pi) \times d \), as earlier described (3). The glomerular volumes were then calculated using standard formulas.

**Series 2: role of TGF in the development of glomerular hyperfiltration.**

The influence of TGF was studied using female A1AR knockout (A1AR / ) and corresponding wild-type (A1AR / ) mice from the strain developed by Johansson and coworkers (9) and backcrossed by the Jackson Laboratory (Bar Harbor, ME) to a C57BL/6J background. GFR was first measured after 10 days on a low-protein diet. The diet was then switched to high protein, and GFR was measured once again after 10 days.

**Series 3 and 4: role of NOS in the development of glomerular hyperfiltration.** In series 3, the influence of different NOS isoforms was studied using specific NOS knockout mice (eNOS / , nNOS / , and iNOS / ) on a C57BL/6J background and corresponding C57BL/6J wild types bred at the department from homozygous breeding pairs. The animals were regularly genotyped using PCR to verify the integrity of the strains. Male and female mice (3–6 mo old) were used in equal proportions (~50% male/female) in all groups. GFR was first measured after 10 days on a low-protein diet. The diet was then switched to high protein, and GFR was measured once again after 10 days. In series 4, the same procedure was repeated with male wild-type mice that were given an intraperitoneal injection of the unspecific NOS inhibitor N^0-nitro-L-arginine methyl ester (L-NAME; 100 mg/kg) or vehicle (saline) 30 min prior to both GFR measurements.

**Diets.** The animals were given either a low-protein diet or a high-protein diet (8 and 50% casein, respectively; Lantmännen, Kimstad, Sweden). The diets were isocaloric (14 kJ/g) and had a similar electrolyte composition. Before the experiments were commenced, the animals were maintained on the standard diet of the animal facility containing 18.5% protein (R36; Lantmännen).

**Measurement of glomerular filtration rate.** Inulin clearance as a measurement of GFR was determined in conscious animals by a technique modified from the method described by Qi et al. (14). Animals were restrained in a custom-built Plexiglas chamber and were given a bolus injection of [1H]methoxy-inulin (~85 kBq; American Radiolabeled Chemicals, St. Louis, MO, USA) dissolved in 200 μl saline into the tail vein, using a 1-ml syringe fitted with a 27-G needle. The animals were then returned to their cages. Blood samples (~5 μl each) were taken from a small cut at the tail tip into heparinized glass capillaries at 1, 3, 7, 10, 15, ~35, 55, and 75 min. The plasma concentration of inulin was determined by a liquid scintillation counter (Wallac 1409, Wallac Oy, Turku, Finland). Inulin clearance was calculated by dividing the given intravenous dose by the total area under the plasma inulin time curve (AUC0- )

\[ AUC_{0-\infty} = \frac{D}{C_{\text{max}}} \]

\[ \text{AUC}_{0-\infty} \] was estimated using noncompartmental pharmacokinetic data analysis from the summation of the trapezoid areas that are formed by connecting the data points (20). The area of the terminal phase, i.e., from 15 min to infinity (including the residual area after the last measurement), was calculated based on the equation for the slope. The area before the first measurement was calculated using linear extrapolation.

**mRNA expression of NOS isoforms.** The renal cortex from kidneys of wild-type and the different NOS knockout animals on the high-protein-diet was separated and immediately frozen in liquid nitrogen. Total RNA was extracted by using the RNeasy mini kit, and the samples were reverse transcribed using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). NOS expression was quantified with a SYBR Green qPCR kit using a real-time thermocycler (LightCycler, Roche Diagnostics, Mannheim, Germany) and related to the expression of 18S by the ΔCt method. The primer sequences are provided in the supplementary data.

**Statistics.** All values are expressed as means ± SE. The reported ΔGFR following treatment with the high-protein diet was calculated as the mean value of the individual increases in all animals in the group. Multiple comparisons between the genotypes were performed using one-way ANOVA, and the hyperfiltering response was examined using two-way repeated-measures ANOVA. If the ANOVA were significant, Fisher’s protected least significant difference post hoc test was performed. Single comparisons were performed using Student’s t-test. For all comparisons, \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Series 1.** GFR was elevated in wild-type mice treated with the high-protein diet for 10 days, whereas GFR was unchanged in animals maintained on the low-protein diet (Fig. 1A). A representative disappearance curve, showing paired measurements from the two diets is shown in Fig. 1B. The high-protein diet was associated with renal hypertrophy, displayed as an

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**Fig. 1. A:** glomerular filtration rate in wild-type mice given a low protein diet for 10 days, followed by either a high- \( (n = 7) \) or low- \( (n = 6) \) protein diet for 10 days. **B:** representative inulin disappearance curve from one animal on a low- and high-protein diet, respectively. *P < 0.05 vs. low protein, day 10. #P < 0.05 vs. low protein, day 20.
increased kidney weight and glomerular volume, whereas the body weight was unaffected (Fig. 2).

**Series 2.** A1AR−/− mice had a similar GFR and developed a similar hyperfiltration, as their corresponding wild-type controls (Fig. 3). The increase in the A1AR−/− mice was 5.0 ± 1.8 and in the wild-type mice 6.4 ± 2.3 µL·min⁻¹·g body wt⁻¹. A1AR−/− mice had a slightly higher body weight than their wild-type controls (low protein: 33 ± 2 vs. 27 ± 1 g; high protein: 33 ± 2 vs. 28 ± 1 g).

**Series 3.** Animals lacking nNOS, eNOS, or iNOS, developed a similar degree of hyperfiltration as their wild-type controls (Fig. 4 and Table 1). The eNOS−/− displayed a lower GFR compared with the wild types on both diets. The nNOS−/− had a lower GFR on the low-protein diet, but this was not significant on the high-protein diet (P = 0.17). For the iNOS−/−, no differences in GFR was found compared with the wild types. iNOS−/− and eNOS−/− had a slightly lower body weight than their wild-type controls; however, this difference was only significant on the high-protein diet. (Table 1). No difference between the genotypes was found in the renal cortical mRNA expression of the different NOS isoforms (Fig. 5).

**Series 4.** Animals given L-NAME had a lower GFR on both low- and high-protein diets, compared with vehicle-treated mice. The L-NAME-treated animals displayed hyperfiltration on the high-protein diet (Fig. 6A). Although the absolute increase in GFR (GFR on high-protein diet-GFR on low-protein diet) was significantly lower during L-NAME treatment compared with animals only given vehicle (1.2 ± 0.6 vs. 3.0 ± 0.7 µL·min⁻¹·g body wt⁻¹), the relative increase was similar in both groups (Fig. 6B).

**DISCUSSION**

In the present study, we developed a model of high-protein-induced hyperfiltration in conscious mice. The use of conscious animals has several advantages: possible interactions with the anesthetic agent are avoided, and consecutive mea-
Glomerular hyperfiltration is also found during diabetes and is associated with an increased risk of developing microalbuminuria and nephropathy later in the disease (1, 11, 15). This increase in GFR has also been proposed to be dependent on the TGF mechanism (23). According to this theory, glomerular hyperfiltration is secondary to increased proximal sodium-glucose cotransport. The resulting reduction in the sodium chloride load to the macula densa will reduce the TGF response, consequently increasing the GFR. However, recent data have demonstrated preserved hyperfiltration in two models of diabetes in the A1AR antagonist, whose selectivity has been questioned (27). In the present study, we, therefore, used knockout mice for each specific NOS isoform expressed in the kidney. Interestingly, all isoforms in wild-type (n = 7), eNOS−/− (n = 4), nNOS−/− (n = 5), and iNOS−/− (n = 6) nitric oxide synthase knockout mice given the high-protein diet.

Measurements in the same animal are possible. This reduces the number of experimental animals needed and makes statistical comparisons more reliable. The reliability over time was verified using time controls that were only given a low-protein diet during both experimental periods. The dietary protein concentrations used in the present study are similar to the concentrations used in earlier studies on high-protein-induced hyperfiltration, to allow comparisons between the studies. Consequently, when the protocols were commenced, the animals were exposed to a slight protein restriction (18.5 to 8%), which could have acted to reduce GFR. This is supported by the slight, but not significant, decrease in GFR observed in the control group of series 1, in which the animals were exposed to the low-protein diet for a prolonged period. However, GFR in the low-protein group is similar to what we usually see during the standard diet, and GFR in the high-protein group is higher, which indicates that the effects of the relative protein restriction are minor. Renal and glomerular hypertrophy is usually found during a high-protein intake (17), and these effects were also observed in the present study. Consequently, the present model appears robust and is suitable for the study of the physiological mechanisms causing high-protein-induced hyperfiltration.

During a high-protein intake, sodium-dependent amino acid reabsorption will increase in the proximal tubule. In earlier experiments, we have demonstrated that rats fed a high-protein diet, despite their elevated GFR, have a reduced sodium chloride concentration in the early distal tubules, indicating a reduced concentration at the macula densa (18). Thus, the feedback signal to the afferent arteriole is reduced, which may act to increase GFR. According to this explanatory model, the GFR in A1AR−/− lacking the TGF mechanism, is, therefore, expected to be less affected by dietary protein intake. However, in the present study, when these knockout mice were treated with a high-protein diet, they exhibited a similar degree of hyperfiltration as wild-type mice, indicating that hyperfiltration occurs independently of the TGF mechanism.

Table 1. Body weight and increase in glomerular filtration rate in wild-type, eNOS−/−, nNOS−/−, and iNOS−/− mice given a low-protein diet for 10 days, followed by a high-protein diet for 10 days (series 3)

<table>
<thead>
<tr>
<th>Protein diet</th>
<th>Wild-Type</th>
<th>eNOS−/−</th>
<th>nNOS−/−</th>
<th>iNOS−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27 ± 2</td>
<td>29 ± 2</td>
<td>23 ± 1</td>
<td>23 ± 1*</td>
</tr>
<tr>
<td>ΔGFR, μl•min⁻¹•g body wt⁻¹</td>
<td>3.8 ± 0.7</td>
<td>2.6 ± 0.6</td>
<td>3.7 ± 1.4</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
<td>ΔGFR, %</td>
<td>34 ± 7</td>
<td>38 ± 12</td>
<td>44 ± 17</td>
<td>40 ± 8</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. GFR, glomerular filtration rate; NOS, nitric oxide synthase. Wild type: n = 8; endothelial NOS null (eNOS−/−): n = 8; neuronal NOS null (nNOS−/−): n = 6; inducible NOS null (iNOS−/−): n = 9. *P < 0.05 vs. wild-type, same diet.
knockouts developed similar degrees of hyperfiltration. Furthermore, when all NOS activity was inhibited by L-NAME, the relative degree of hyperfiltration was unaffected. The basal GFR during L-NAME treatment was ~45% lower than that of mice only given vehicle, underscoring the importance of NO in maintaining a normal GFR. Because the relative hyperfiltration was similar in knockout mice of all NOS isoforms and also during L-NAME treatment, it seems plausible that high-protein hyperfiltration occurs independently of functional NOS isoforms. Consequently, this interpretation differs from earlier reports (25, 27), arguing for a role of NOS. However, these experiments lack an appropriate control group, since high-protein-fed animals given NOS antagonists were compared with animals only given a low-protein diet. Since NOS obviously has an important role in the control of the basal GFR, conclusions regarding the role of NOS in high-protein-induced hyperfiltration cannot be drawn using this experimental protocol. To test whether the different NOS knockout mice compensate for the loss of their gene by increasing the expression of the other NOS isoforms, the mRNA expression levels on the high-protein diet were analyzed by quantitative RT-PCR. However, no differences were found, suggesting that the other isoforms do not compensate. Consequently, the unaltered expression levels and the observation that hyperfiltration occurred even during L-NAME treatment, indicates that compensatory increases do not explain the conserved hyperfiltration in the NOS knockout mice.

In the present, as well as in several other models of hyperfiltration induced by chronic high-protein intake, an increased glomerular volume is reported. Since neither NOS inhibition nor TGF deficiency affected the degree of hyperfiltration, it is possible that the increased glomerular volume directly contributes to the increased GFR. The vascular endothelial growth factor has been shown to be involved in the development of glomerular hypertrophy during a chronic high protein intake (17) and might be a target for future studies regarding glomerular growth and hyperfiltration. Furthermore, since the present study only deals with the chronic situation of an increased protein intake, it is possible that NO and the TGF mechanism are important for the acute hyperfiltering response to amino acid infusion or a protein-rich meal.

In conclusion, glomerular hyperfiltration induced by a high protein intake occurs independently of the TGF mechanism and NOS isoforms. However, during basal conditions, NO derived from eNOS and nNOS is important for maintaining a normal GFR. Since high-protein-induced glomerular hyperfiltration occurs independently from those important regulatory systems, it is plausible that glomerular growth that increases the filtration area, may directly contribute to the increased GFR.

Perspectives and Significance

The present study was performed using relatively new methodology for GFR measurements, which was shown to be simple and highly useful for assessing the hyperfiltration response to alterations in dietary protein load. None of the investigated factors (i.e., specific NOS isoforms or TGF) were found to significantly contribute to high-protein-induced hyperfiltration. However, the described protocol could be useful in future studies targeting the mechanisms causing hyperfiltration.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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