Nitric Oxide Increases Albumin Permeability of Isolated Rat Glomeruli via a Phosphorylation-Dependent Mechanism

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Abstract. Nitric oxide (NO) has been implicated in the induction of proteinuria in acute inflammatory glomerulonephritis and in the increased vascular permeability seen in various other disease conditions. The complicated interactions of NO with other factors in vivo hinder analysis of the mechanisms involved. By use of a recently introduced method for measuring albumin permeability (P_a) in isolated glomeruli, the question of whether NO has a direct effect on the permeability barrier of glomerular tufts was examined and the potential mechanisms were explored. Exposure of isolated glomeruli to three NO donors, s-nitroso-N-acetyl-penicillamine (SNAP), (Z)-1-[(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate), and sodium nitroprusside, all increased the P_a. This action of NO was time- and concentration-dependent and could be mimicked by 8-bromoguanosine 3', 5'-cyclic monophosphate. Western blot analysis of the proteins from NO donor-treated glomeruli revealed an increase of phosphotyrosine levels of proteins of molecular mass about 120 and 70 kD. The demonstration that pretreatment of glomeruli with the tyrosine kinase inhibitor, genistein, could largely prevent the effect of SNAP and DETA-NONOate confirmed the crucial role of tyrosine phosphorylation in the NO-induced increase of P_a. Furthermore, the tyrosine phosphatase inhibitor, phenylarsine oxide (PAO), could mimic the action of NO on P_a. NO-enhanced tyrosine phosphorylation was further confirmed by immunofluorescence staining, where positive cells in SNAP- and PAO-treated glomeruli were much more frequent than that in controls. By use of dual-label staining in combination with podocyte specific marker, nephrin, it was observed that most of the phosphorylated positive cells corresponded to podocytes. These results suggest that NO impairs the glomerular permeability barrier through a tyrosine phosphorylation-dependent mechanism.

Nitric oxide (NO) is an indispensable molecule with a variety of biologic functions. It has been shown to regulate multiple cellular functions, including smooth muscle cell relaxation, neurotransmission, and macrophage-induced cytotoxicity, as well as cell proliferation and apoptosis (1–3). In the kidney, the presence of NO under physiologic conditions was confirmed by microdialysis studies (4). Low concentrations of NO, released by glomerular endothelial cells, play a pivotal role in regulating microvascular hemodynamics, in concert with antagonistic vasoactive substances such as angiotensin II and endothelin (5). Under pathologic conditions, NO generation in the glomeruli is markedly enhanced because of the induction of NO synthase expression in intrinsic glomerular cells and infiltrating, activated macrophages (5). In acute inflammation in glomeruli, enhanced production of NO was closely related to the induction of proteinuria. Blocking NO synthase expression with the specific inhibitor L-NMMA profoundly attenuated proteinuria in the anti-Thy 1 antibody-induced model of glomerulonephritis (6), which indicates that NO was involved in the disturbance of the glomerular permeability barrier, leading to proteinuria.

Direct induction of vascular and epithelial hyperpermeability by NO has been reported elsewhere (7–9). However, information concerning the effect of vasoactive substances on glomerular permeability is still incomplete. The complicated interactions of NO with other relevant factors make it extremely difficult to analyze its actions and mechanisms in detail in vivo. In the present work, we examine whether NO has a direct effect on the glomerular capillary permeability barrier, using the method for measuring albumin permeability (P_a) in isolated glomeruli developed by Savin et al. (10). Furthermore, we investigated the potential mechanisms of NO-mediated glomerular hyperpermeability.

Materials and Methods

Reagents
S-nitroso-N-acetyl-penicillamine (SNAP), sodium nitroprusside (SNP), (Z)-1-[(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate), 8-bromoguanosine 3', 5'-cyclic monophosphate (8-Br-cGMP), phenylarsine oxide (PAO), genistein, protamine sulfate, bovine serum albumin (BSA), aprotinin, pepstatin, sodium orthovanadate, phenylmethylsulfonyl fluoride, and poly-L-lysine were obtained from Sigma (St. Louis, MO). Dulbecco’s modified Eagle medium (DMEM) was ordered from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan).

Experimental Animals
Normal male Sprague-Dawley rats (170 to 250 g body weight) with free access to Purina chow and water were used in all experiments.
**Isolation of Glomeruli**

Sprague-Dawley rats were anesthetized with ether and kidneys were immediately removed. The renal capsules were removed, and the outer 1 to 2 mm of the renal cortex was excised and cut into fine fragments. The glomeruli were then isolated in DMEM that contained 6% BSA by standard sieving techniques as described elsewhere (10–13). At each step, the tissue was abundantly rinsed in DMEM that contained 6% BSA. The PH of the medium was adjusted to 7.4 before use. The whole procedure for isolation of glomeruli was carried out within 30 min at room temperature.

**Incubation Medium and Washing Medium**

The incubation medium was identical to the isolation medium (see above) except for the addition of the agents to be tested. The washing medium was DMEM that contained 1% BSA. The PH of each medium was adjusted to 7.4 before use.

**Measurement of Glomerular Volume Change**

Glomeruli, incubated in 6% BSA medium with or without various test agents, were allowed to adhere to an observation chamber coated with poly-L-lysine (1 mg/ml) for 5 to 10 s. Unattached glomeruli were removed by gentle washing with fresh isolation medium. Adherent glomeruli were viewed in a microscope, and then the image was captured for their initial images by computer. All selected glomeruli were free of Bowman’s capsules. After an initial period of observation, the medium was replaced with washing medium containing 1% BSA. The change of medium from 6% to 1% BSA produced an oncotic gradient across the glomerular capillary wall and resulted in an influx of fluid and an increase in glomerular capillary volume. Volume changes in the glomeruli subsequent to the applied oncotic gradient occurred within 5 s and were maintained for at least several minutes under both control and experimental conditions. Repeat images were obtained 2 to 3 min after a change of medium. The area of each glomerulus was automatically measured by use of National Institutes of Health software. The initial and final volumes of each glomerulus were calculated from the area (S) by use of the formula $V = 3/4\pi(S/\pi)^{3/2}$. Volume change ($\Delta V$) was calculated as $\Delta V = (V_{\text{final}} - V_{\text{initial}})/V_{\text{initial}} \times 100$. At least ten glomeruli from three or more rats were studied in each experiment.

**Calculation of Albumin Reflection Coefficient ($\sigma_a$) and $P_a$**

The calculation of $\sigma_a$ and $P_a$ was done on the basis of the details reported by Savin et al. (10–13). Because the increase in $\Delta V$ is proportional to the oncotic gradient across the capillary wall, $\sigma_a$, the ratio of $\Delta V$ of experimental to control glomeruli in response to an identical oncotic gradient can be calculated as follows: $\sigma_a = \Delta V_{\text{experimental}}/\Delta V_{\text{control}}$. $P_a$ was calculated from $\sigma_a$ and defined as $P_a = 1 - \sigma_a$. When the reflection coefficient $\sigma_a$ is 0, albumin moves at the same rate as water, and $P_a$ is 1.0. When $\sigma_a$ is 1.0, albumin cannot cross the membrane and $P_a$ is 0.

**Western Blot Analysis**

Samples of isolated rat glomeruli were lysed in RIPA buffer (50 μM Tris-HCL [pH 7.5], 150 μM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% sodium dodecyl sulfate) that contained 25 μg/ml aprotinin, 2 mM sodium orthovanadate, 25 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 50 mM sodium fluoride for 30 min on ice. Lysates were clarified by centrifugation at 15,500 × g for 15 min at 4°C. The protein concentration of the supernatant was measured with a Bio-Rad Protein Assay Kit. The supernatant was mixed with 2× sample buffer (114 mM Tris [pH 6.8], 9% glycerol, 2.7% sodium dodecyl sulfate, 0.02% bromphenol blue, and 4.5% mercaptoethanol), boiled for 5 min, subjected to 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked by phosphate-buffered saline (PBS)–0.1% Tween 20 supplemented with 3% BSA for 1 h at room temperature. The membrane was probed with a horseradish peroxidase–labeled anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5000 for 1 h at room temperature. After extensive washing with three changes of PBS–0.1% Tween 20, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system, followed by exposure to Kodak x-ray film (Eastman Kodak, Rochester, NY).

**Immunocytochemistry**

Isolated rat glomeruli were preincubated with either 400 μM SNAP for 1 h or 1 μM PAO for 15 min or left as untreated controls. The medium was removed, and then the isolated rat glomeruli were rinsed in PBS and fixed in 3% paraformaldehyde for 20 min at 4°C. Free aldehyde groups were blocked with ammonium chloride (50 mM in PBS, 20 min, 4°C). Isolated glomeruli were then permeabilized with 1% Triton X-100. After blocking the nonspecific binding with fetal calf serum (FCS) (20 min, 20°C), glomerular suspension was incubated with mouse monoclonal anti-phosphotyrosine (PY20) antibody (Santa Cruz Biotechnology) diluted 1:100 in 1% FCS-PBS for 1 h at 37°C. Then glomerular suspension was incubated for 1 h at 37°C with a 1:100 dilution of FITC-conjugated rabbit anti-mouse-IgG (Santa Cruz Biotechnology) in PBS that contained 1% FCS. For double immunostaining with anti-PY20 antibody and a podocyte specific marker, glomerular suspension was at first incubated with mouse monoclonal anti-PY20 antibody, followed by the incubation with FITC-anti-mouse IgG, as described above. Then glomerular suspension was incubated with rabbit anti-rat nephrin antibody (14), diluted 1:200 in 1% FCS-PBS for 1 h at 37°C, washed with PBS, and then incubated with tetramethyl rhodamine B isothiocyanate–conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark) before final washing. Glomerular specimens treated were mounted on the slide glass and examined by use of confocal laser scanning microscope (MRC1024, Bio-Rad). Controls with irrelevant secondary or primary antibodies did not exhibit significant immunostaining.

**Cell Viability**

Excessive NO is well known to be cytotoxic. Therefore, it is critical to rule out the possibility of impairment of cell viability by NO donors as well as other experimental agents, which would compromise the glomerular integrity and result in a false increase in $P_a$. For this purpose, the MTT assay was performed. Incubation of isolated rat glomeruli with SNP (500 μM), SNAP (1 mM), CGMP (500 μM), and PAO (10 μM) for 4 h produced no significant difference in the activity of mitochondria enzyme compared with the untreated control.

**Statistical Analyses**

Average $P_a$ was calculated from 10 glomeruli randomly selected from each group under each experimental condition. Each experiment was repeated at least three times. Values were compared by use of the unpaired t test. All values were expressed as mean ± SEM, and $P < 0.05$ was considered to be significant.
Results

NO Increased the P_a in Isolated Rat Glomeruli

Glomeruli were incubated for 3 h at 37°C with three structurally unrelated NO donors, SNAP (400 μM), DETA-NONOate (400 μM), and SNP (1 mM), and the P_a was then measured. As shown in Figure 1, these three different NO donors all caused a significant increase in P_a (0.55 ± 0.06, 0.51 ± 0.09, and 0.43 ± 0.05, respectively) compared with the results in control experiments (0 ± 0.06). As a representative, SNAP was chosen for the following experiments.

Incubation of glomeruli with 400 μM SNAP caused a time-dependent increase of P_a. As shown in Figure 2A, there was a slight increase in P_a after 30 min (0.12 ± 0.08), compared with controls (0 ± 0.05). A significant increase in P_a was evident after only 1 h of incubation (0.26 ± 0.13), and the peak (0.45 ± 0.03) was reached at 3 h.

Glomeruli were incubated for 3 h at 37°C with different concentrations of SNAP, and P_a was estimated. As shown in Figure 2B, SNAP increased P_a in a concentration-dependent manner. At the concentration of 150 μM it already significantly enhanced P_a, and the maximal increase was seen at a concentration 450 μM.

To demonstrate that the action of SNAP on P_a was really due to its released NO, rather than the other metabolite products, we have compared the ability of freshly prepared and the old SNAP medium (preincubated with assay medium at 4°C over 24 h) in inducing glomerular permeability. Because SNAP spontaneously generates NO in aqueous media and has a half-life of only 5 h, little NO would be left in old SNAP medium. As expected, treatment of glomeruli with freshly prepared 400 μM SNAP medium for 3 h enhanced P_a (0.49 ± 0.06), whereas old 400 μM SNAP medium had little effect (0.03 ± 0.04), compared with the untreated control (0 ± 0.07).

cGMP Mimicked the Effect of SNAP on P_a

Most of the actions of NO are mediated via a cGMP signaling pathway (1–3). It was reasonable to speculate that cGMP might also mediate the enhancing effect of NO on glomerular permeability. To test this, glomeruli were incubated with different concentrations of SNAP or left untreated as controls for 3 h. All results were expressed as mean ± SEM (n = 3). *P < 0.05 versus control, **P < 0.01 versus control.

NO Impairs the Glomerular Permeability Barrier through a Tyrosine Phosphorylation-Dependent Mechanism

Many studies have reported that the endothelial and epithelial barrier functions correlates with tyrosine phosphorylation.
Therefore, we asked whether phosphorylation was also involved in the NO-induced disruption of the glomerular permeability barrier. Treatment of isolated rat glomeruli with SNP (1 mM), DETA-NONOate (400 μM), and SNAP (400 μM) for 30 min all caused an increase of the phosphotyrosine levels of the proteins in the molecular mass at 120 and 70 kD (Figure 4). The increase in tyrosine phosphorylation after SNAP treatment was time-dependent, reached a maximum at 30 min, and persisted for at least 2 h (Figure 5). In addition, a dephosphorylation of protein in the molecular mass at ~40 kD (Figure 5) was observed.

To further document that NO perturbs the glomerular permeability barrier via mechanisms that involve tyrosine phosphorylation, we examined whether tyrosine kinase inhibitor genistein could reverse the hyperpermeability induced by NO donors. As shown in Figure 6A, genistein (100 μM) itself did not alter the P_a (−0.02 ± 0.07). However, preincubation with genistein for 1 h significantly prevented the SNAP-elicited increment of P_a. This effect of genistein was concentration-dependent; 10 μM genistein has already caused a 60% inhibition of the SNAP-induced increase of P_a, and 100 μM genistein almost completely reversed the effect of SNAP. The hyperpermeability elicited by another NO donor, DETA-NONOate, could also be significantly blocked by genistein (Figure 6B). These observations suggest a close correlation between NO-induced glomerular hyperpermeability and protein tyrosine phosphorylation.

The crucial role of tyrosine phosphorylation in the NO-induced increase of P_a was further confirmed by the observation that pretreatment of glomeruli with the tyrosine phosphatase inhibitor, PAO, could mimic the action of SNAP on the albumin permeability. The phosphatase inhibitor caused a significant increase of permeability in a time- and concentration-dependent fashion (Figure 7). This effect of PAO occurred rapidly and was most pronounced at 2 h (Figure 7A). A significant increase of P_a could be observed at a concentration as low as 0.1 μM (Figure 7B). Enhancement of tyrosine phosphorylation in isolated rat glomeruli was also involved in the NO-induced disruption of the glomerular permeability barrier. Treatment of isolated rat glomeruli with SNP (1 mM), DETA-NONOate (400 μM), and SNAP (400 μM) for 30 min all caused an increase of the phosphotyrosine levels of the proteins in the molecular mass at ~120 and 70 kD (Figure 4). The increase in tyrosine phosphorylation after SNAP treatment was time-dependent, reached a maximum at 30 min, and persisted for at least 2 h (Figure 5). In addition, a dephosphorylation of protein in the molecular mass at ~40 kD (Figure 5) was observed.

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phosphorylation in isolated rat glomeruli by PAO was confirmed by Western blot (Figure 8). Treatment of isolated rat glomeruli with PAO caused time- and dose-dependent increases in the tyrosine phosphorylation. With 1 μM PAO, the maximal increase was detected at 30 min after incubation and was maintained for at least 2 h (Figure 8A). The major bands that were phosphorylated were of mass at ~110 and 70 kD, similar to that induced by NO donors. A dephosphorylation of protein in the molecular mass at ~40 kD (Figure 8) was also observed, as shown in Figure 5.

To further demonstrate the effect of NO on tyrosine phosphorylation in glomeruli, we carried out immunofluorescence staining with anti-PY20 IgG on isolated rat glomeruli using a confocal laser scanning microscope. In the control group, a low level of staining for phosphotyrosine was detected. In contrast, after treatment of glomeruli with SNAP (400 μM) for 1 h or PAO (1 μM) for 15 min, an obvious increase of phosphotyrosine staining could be observed. The phosphotyrosine positive cells in SNAP- and PAO-treated glomeruli were much more frequent than that in controls (Figure 9). To clarify the property of the glomerular cells expressing enhanced tyrosine phosphorylation, we have done dual-label staining (Figure 10) with anti-PY20 antibody (FITC labeled, green), in combination with a glomerular epithelial specific marker, anti-rat nephrin antibody (tetramethyl rhodamine B isothiocyanate–labeled, red). Computer-aided superimposition of the two fluorescence images revealed that most of the positively phosphorylated cells in SNAP- and PAO-treated glomeruli were colocalized with the rat nephrin (yellow, arrows).

**Discussion**

In this study, we provide evidence that NO has the potential to disrupt the glomerular barrier function, as reflected by increased albumin permeability in an *in vitro* assay system.
Furthermore, we demonstrated that this action of NO is related to augmented phosphotyrosine levels of some glomerular proteins, which suggests the involvement of functional protein(s) in NO-induced glomerular hyperpermeability.

The assay for albumin permeability in isolated glomeruli used in this paper was established by Savin et al. (10–13). A modified method, based on the same principle but using a multisizer instead of a video recording system for the measurement of glomerular volume, has also been reported (22). We have employed protocols similar to those of Savin et al. (10–13) with some modifications regarding image treatment. For analyzing the microscope images of isolated glomeruli, pictures of the glomeruli were directly digitized into a computer via a CCD camera. The area of each glomerulus was automatically measured with the help of the National Institutes of Health image analysis software program. This approach avoids the tedious work of measuring glomerular diameters manually and is simpler and more objective.

Exposure of isolated glomeruli to three structurally unrelated NO donors, SNAP, DETA-NONOate, and SNP, all resulted in an enhancement of Pa. A cytotoxic effect of the NO donors and NO itself could be excluded as a major cause of the increased Pa, for a number of reasons. First, treatment of glomeruli with NO donors did not influence the enzymatic activity of mitochondria, as judged in the MTT assay. Second, the effect of SNAP and DETA-NONOate on Pa could be largely reversed by pretreatment of glomeruli with a tyrosine kinase inhibitor. Third, the concentrations of SNAP employed (80 to 500 μM) were consistent with those used by others (up to 1 mM) when testing for anti-mitogenic and permeability-enhancing effects of NO donors on cultured cells (3,23–26). Therefore, these results can be taken to indicate that NO has the potential to regulate glomerular albumin permeability.

Addition of 8-Br-cGMP, a stable analog of cGMP, to the assay system mimicked the action of NO and suggests that cGMP signaling may be associated with the enhancing effect of NO on glomerular permeability. The role of cGMP signaling in NO-induced microvascular and epithelial hyperpermeability has been documented elsewhere (8,27). However, the participation of cGMP-independent mechanisms cannot be excluded. The impairment of the permeability barrier by cAMP has been reported elsewhere (28). The products resulting from the interaction of NO with other oxygen radicals have also been demonstrated to be able to affect the permeability of epithelial cells (23,26). The exact role of these factors in the NO-induced enhancement of glomerular Pa remains to be addressed.

In this study, Western blot analysis of the proteins from three NO donors treated glomeruli revealed an enhancement of phosphotyrosine proteins of molecular weights around 120 and 70 kD. The time course of tyrosine phosphorylation induced by SNAP was closely correlated with the degree of Pa enhancement seen. Furthermore, we confirmed, using two kinds of NO donor, SNAP and DETA-NONOate, that the action of NO on Pa could be largely prevented by the tyrosine kinase inhibitor, genistein. In addition, incubation of isolated glomeruli with the tyrosine phosphatase inhibitor, PAO, mimicked the action of SNAP on albumin permeability. On the basis of these findings, it seems likely that tyrosine phosphorylation of certain glomerular proteins is a key event in the initiation of glomerular hyperpermeability. This notion is consistent with the results of recent study that used cultured endothelial cells, which showed that disturbance of the permeability barrier was related to the

**Figure 8.** Enhancement of tyrosine phosphorylation in isolated rat glomeruli by PAO. (A) Isolated rat glomeruli were incubated with 1 μM PAO for the indicated time periods and lysed with RIPA. Lysate was blotted with anti-phosphotyrosine antibody (PY20). The blot shown is a representative of three independent experiments. Right arrows indicate two major bands of increased tyrosine phosphorylation. (B) Isolated rat glomeruli were stimulated with the indicated concentrations of PAO for 15 min and lysed with RIPA. Lysate was blotted with anti-phosphotyrosine antibody (PY20). The blot shown is a representative of three independent experiments. Left arrows indicate two major bands of increased tyrosine phosphorylation.

Furthermore, we demonstrated that this action of NO is related to augmented phosphotyrosine levels of some glomerular proteins, which suggests the involvement of functional protein(s) in NO-induced glomerular hyperpermeability.
increased phosphorylation of focal adhesion proteins (29). Tyrosine phosphorylation is dynamically regulated by the competing activities of protein tyrosine kinases and protein tyrosine phosphatases. It has been demonstrated that NO is able to inhibit the activity of protein tyrosine phosphatases in one of the glomerular cell types, mesangial cells (30), under the assumption that NO may cause an increased phosphorylation of certain proteins on tyrosine residues.

The question as to why increased tyrosine phosphorylation of certain glomerular proteins could lead to the disruption of the glomerular barrier remains to be addressed. The glomerular permeability barrier is composed of the glomerular basement membrane, as well as glomerular epithelial and endothelial cells. It is considered that any disruption of the normal architecture of the glomerular barrier components, such as degradation of glomerular basement membrane by proteinases and/or disturbances of the cell-to-cell and cell-to-matrix interactions, could lead to enhanced albumin permeability (6,31–34). The phosphotyrosine-containing proteins have been found to be concentrated mainly along the regions of cell-to-cell and cell-to-matrix interaction, especially the proteins found in focal adhesion, adherent junctions, and tight junctions (35–37). Accumulating evidence demonstrates a critical role of tyrosine phosphorylation in the regulation of cell-to-cell and cell-to-matrix interactions and in the disintegration of the permeability barrier (18,20,21,29,32,38,39). In the cultured epithelial cell line, MDCK, PAO-induced hyperpermeability was associated with increased phosphorylation of the tight junction protein, ZO-1 (21). Enhanced protein phosphorylation at the location of tight junctions and at the basal membrane foot processes of isolated glomeruli was also reported in nephrotic rats (32). In this study, the exact properties of the enhanced phosphotyrosine proteins having molecular weights around 120 and 70 kD are unclear. In addition, treatment of glomeruli with PAO

Figure 9. Immunocytochemistry showing the effect of SNAP and PAO on tyrosine phosphorylation in isolated rat glomeruli. Isolated rat glomeruli were preincubated with either 400 μM SNAP for 1 h or 1 μM PAO for 15 min or left as untreated controls. Isolated rat glomeruli were then stained with anti-PY20 antibody as described in the Materials and Methods section.

Figure 10. Distribution of tyrosine phosphorylation after treatment of SNAP and PAO in isolated rat glomeruli. Isolated rat glomeruli were preincubated with either 400 μM SNAP for 1 h or 1 μM PAO for 15 min or left as untreated controls. Isolated rat glomeruli were then double stained with a monoclonal anti-PY20 antibody (FITC labeled, green) and a polyclonal rabbit anti-rat nephrin antibody (tetramethyl rhodamine B isothiocyanate–labeled, red), as described in the Materials and Methods section. Colocalization of anti-PY20 and anti-rat nephrin antibodies generates a yellow fluorescence (arrows), which indicates that most of positively phosphorylated cells in SNAP- or PAO-treated glomeruli (green, in Figure 9) were podocytes.
or NO donors could also result in an obvious dephosphorylation of protein at the molecular mass \( \sim 40 \) kD (Figure 5 and 8). The property of this protein also remains uncertain. Because the hyperpermeability elicited by NO could be completely prevented by preincubation of glomeruli with kinase inhibitor genistein (Figure 6), it is less likely that this dephosphorylation contributes to the increased glomerular albumin permeability. Immunohistochemical staining of phosphotyrosine proteins revealed that most of the positive cells in SNAP-treated glomeruli were situated in the surface region of the glomeruli. Using dual-label staining in combination with podocyte specific marker, nephrin, we demonstrated that most of the phosphotyrosine-positive cells corresponded to podocytes. A recent study on cultured podocytes has revealed that dephosphorylation of tyrosine proteins was necessary for maintaining the normal architecture of the foot processes and attachment of podocytes onto the underlying extracellular matrix (33). Taken together, it is highly likely that glomerular visceral epithelial cells are involved in NO-induced tyrosine phosphorylation, although participation of endothelial and mesangial cells cannot be excluded. Molecular and cellular identification of enhanced phosphotyrosine protein(s) will be a focus of our future research.

In summary, we have demonstrated that NO has the ability to impair the glomerular permeability barrier via a mechanism related to tyrosine phosphorylation of glomerular proteins. If tyrosine phosphorylation of specific functional proteins is a common signal pathway, leading to impairment of the glomerular barrier function and pharmacologic and selective modulations at the glomerular phosphotyrosine level might represent a new strategy in the treatment of certain glomerular diseases.

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