Nitric oxide production in renal cells by immune complexes: Role of kinases and nuclear factor-κB

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Background. Interaction of deposited immune complexes (IC) with Fc receptors (FcR) on tissue cells elicits the release of inflammatory mediators leading to tissue damage. Nitric oxide (NO) radicals generated by inducible NO synthase (iNOS) are important mediators in inflammatory processes. To analyze the role of NO in IC-mediated glomerular inflammation, we studied the in vitro and in vivo expression of iNOS in renal cells [resident mesangial cells (MC), and infiltrating monocytes] induced by IC, and the possible intermediate steps between FcR occupancy and iNOS induction.

Methods. MC and monocytes were stimulated with IgG- and IgA-containing IC, and NO production (nitrite accumulation), iNOS transcription (luciferase assay) and their expression was measured by RT-PCR and Western blot. The involvement of FcR, transcription factor nuclear factor-κB (NF-κB), and protein kinases was assessed by using Fc fragments and specific inhibitors. Immune glomerulonephritis was induced in rats, and iNOS expression and NF-κB activation were analyzed.

Results. In MC and monocytes, IC enhanced iNOS transcription/expression and NO generation, which were attenuated by specific inhibitors of NF-κB. In addition, mitogen-activated protein kinase (MAPK) inhibitors decreased NO production, but did not interfere with NF-κB activity, suggesting that both pathways may converge downstream in the induction of iNOS. In experimental immune glomerulonephritis, increased iNOS expression correlated with proteinuria levels, and appeared colocalized with NF-κB in glomerular infiltrating cells. Treatment of animals and cells with Fc fragments prevented iNOS induction and NF-κB activation by IC.

Conclusions. These results indicate that IC, through activation of FcR, induce iNOS expression in renal resident and recruited cells by mechanisms involving MAPK and NF-κB, and support the idea of the important role of local NO generation in IC-mediated glomerular injury.

Key words: immune complexes, Fc receptors, nitric oxide, glomerulonephritis, NF-κB, tissue injury, inflammation.

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Nitric oxide (NO) is an important effector molecule both in physiological conditions and in disease states characterized by inflammation. NO radicals are synthesized from L-arginine by NO synthase, from which three isoforms can be distinguished: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) nitric oxide synthase [1]. In the kidney, a constitutive NO release within the glomerular vasculature may be protective by decreasing glomerular capillary pressure and autoregulating renal blood flow [2]. However, in pathological conditions, local generation of high output induced by various stimuli through the expression of iNOS may result in damaging effects. NO generated by activated cells may amplify the injury through the production of peroxynitrite and reactive oxygen species [3]. In vitro, iNOS expression can be induced in leukocytes and renal cells after exposure to cytokines and endotoxin [4–7]. In human and experimental nephritis, the glomerular induction of iNOS mediates renal injury [3, 8–10], suggesting that inhibition of high output NO release by blocking iNOS expression or activity may be a useful strategy for treatment of inflammatory disorders. However, conflicting results exist between the published studies of pharmacologic NO regulation by unselective or iNOS-specific inhibitors and the experimental glomerulonephritis in iNOS deficient mice [3, 11–14].

Inducible NOS gene expression is strictly controlled, mostly at the transcriptional level. The promoter region of iNOS gene has been cloned and sequenced in different species [4, 15], and contains at least one nuclear factor-κB (NF-κB) consensus site that has been shown to be important for iNOS transcription in macrophages and mesangial cells (MC) [7, 16]. NF-κB is a ubiquitous and rapid transcription factor in several cells in response to cytokines and other stimuli, and exerts its effect by regulating the expression of cytokines, chemokines, cell adhesion molecules, growth factors, and immunoreceptors [6, 7, 16–20]. In this manner, NF-κB contributes to immuno-
logically mediated diseases such as glomerulonephritis, allograft rejection, and rheumatoid arthritis [17, 21].

Deposition/formation of immune complexes (IC) in the glomeruli is the pathogenic factor that triggers the inflammatory cascade leading to tissue damage [22]. In addition to their capacity to activate the complement system, IC interact with Fc receptors (FcR) of resident and infiltrating cells and trigger several signaling pathways, thus resulting in the secretion of a wide array of chemical mediators [23]. In cultured MC, IgG- and IgA-containing IC elicit synthesis of proinflammatory and profibrogenic cytokines through a mechanism depending on the Fc region of the Ig [24–28]. These data are of particular importance because the MC, the major determinant in the regulation of glomerular filtration rate [29], is known to play a key role during immune glomerulonephritis, with an active participation in proliferation, synthesis of mediators and matrix deposition [30]. In our current study, we have focused on a highly versatile member of this orchestra of inflammatory mediators, NO, because its excessive formation may alter the glomerular filtration, but also may cause tissue injury and thus contribute to the pathogenesis of IC-mediated glomerulonephritis. We examined the in vitro and in vivo expression of iNOS in renal cells (resident MC and infiltrating monocytes) after stimulation with IC. Because the signaling pathway utilized by IC in iNOS induction is not well elucidated, we also studied the possible intermediate steps between FcR occupancy and iNOS expression in MC and monocytes.

METHODS
Reagents and chemicals

Monomeric human IgG and IgA were purified from patient serum by affinity and gel filtration chromatography as described [26], and were heat aggregated at 63°C for 30 and 150 minutes, respectively. After removing insoluble aggregates and monomeric immunoglobulins (Igs), soluble aggregates were used for cell stimulation because they present physicochemical properties similar to IC. IgG Fc fragments were obtained by digestion with activated papain (Sigma Chemicals, St. Louis, MO, USA), IgA fragments were obtained by digestion with pepsin [F(ab’)]2 or with protease from Haemophilus Influenzae (Fc and Fab) [31, 32]. The presence of endotoxin in all preparations was excluded with the Limulus amebocyte assay (Ingelheim Diagnostica, Barcelona, Spain). Pyrrolidine dithiocarbamate (PDTC), parthenolide, lipopolysaccharide (LPS), N-nitro-L-arginine methyl ester (L-NAME) and polymyxin B were provided by Sigma. Genstein, MG132, SB203580 and PD98059 were purchased from Calbiochem (La Jolla, CA, USA). Immunogenex Corporation (Los Angeles, CA, USA) provided us with recombinant human tumor necrosis factor-α (rhTNF-α), recombinant human interferon-γ (rHIFNγ), and recombinant human interleukin-1β (rhIL-1β). The specific antibodies (Abs) used were: rabbit anti-rat iNOS, goat anti-human iNOS, and rabbit anti-p50 (Chemicon International, Temecula, CA, USA); goat anti-IkBα, goat anti-p65, rabbit anti-p38, mouse anti-p-p38, goat anti-ERK1/2, and mouse anti-p-extracellular-signal-related kinase (ERK) (sc-371, sc-109, sc-535, sc-7973, sc154, and sc-7383; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell culture

Mesangial cells (MC) were obtained from human and rat kidneys as described [31] and cultured in RPMI 1640 medium with 25 mmol/L HEPES, pH 7.4, supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine (Life Technologies, Paisley, Scotland, UK). MC were characterized by phase contrast microscopy, positive staining for desmin and vimentin and negative staining for factor VIII and cytokeratin. The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI with 10% FCS. Cells were made quiescent by 24 hours of incubation in the medium containing 0.5% FCS. In some cases, cells were preincubated for 90 minutes with inhibitors or Ig fragments, washed and then stimulated with IC.

Experimental model of immune nephritis

Female Wistar rats (200 g) were immunized with ovalbumin according to a previously described protocol [33]. The study groups included healthy control rats (N = 8) and those with spontaneous development of nephritis (N = 12). Rats were maintained in metabolic cages and 24 hour proteinuria was measured by the sulfosalicylic acid and 100 mmol/L HCl). Medium was as-
pirated, centrifuged and incubated with 1 mmol/L 2,3-diaminophosphalene. The absorbance at 548 nm was measured and compared with a standard of NaNO₂. The production of NO was expressed as nmol of NO₃ per milligram of protein.

**Analysis of mRNA expression**

Total RNA from cells or renal cortex was obtained by the acid guanidine-thiocyanate-phenol-chloroform method and the iNOS mRNA expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using the following primers. Human iNOS (259bp): sense, 5'-CGGGTGCTGTATTTCTTACGAGCCAAGAG-3'; antisense, 5'-GTTCTAGTGGGTGAGGAGGT-3'. Rat iNOS (223 bp): sense, 5'-TGCTAGGGACCAGTGATAGAAC-3'; antisense, 5'-GTTTCTGGTCGATGTCATGAG-3'. The expression of GAPDH was used as internal control.

**Transient transfection and luciferase assay**

The expression of GPDH was used as internal control.

**Western blot analysis**

Mesangial cells were fixed with 3% paraformaldehyde, and permeabilized with 0.5% Triton X-100. Detection of NF-κB complex subunits was made by incubation with anti-p50 (2 μg/mL) and anti-p65 (5 μg/mL) Abs, followed by peroxidase-conjugated secondary Ab and a chemiluminescent detection system (ECL; Amersham, Buckinghamshire, UK). After probing phosphorylated proteins, the membranes were stripped and probed with anti-p38 or anti-ERK1/2 Abs for determining equal loading of proteins between samples.

**Kinase assay**

The p38 and ERK proteins were immunoprecipitated using specific Abs at 4°C for 16 hours (200 μg protein/1 μg Ab). Protein G agarose beads were added for four hours, and precipitates were washed and diluted in kinase buffer [20 mmol/L Hepes pH 7.6, 20 mmol/L MgCl₂, 20 mmol/L glycerophosphate, 0.2 mmol/L dithiothreitol (DTT), 10 mmol/L NaF, 0.2 mmol/L Na₃VO₄]. Kinase reactions were performed in the presence of 10 μg of the myelo basic protein (MBP) as a substrate and 1 μCi [γ-³²P]ATP at 30°C for 30 minutes, and then stopped by additions of 5× Laemmli sample buffer. Samples were resolved on 15% acrylamide gels, and phosphorylation of substrate was examined by autoradiography. As the control, immunoprecipitates were electrophoresed, transferred to membranes and probed with anti-p38 or anti-ERK Abs.

**Immunofluorescence analysis**

Mesangial cells were fixed with 3% paraformaldehyde, and permeabilized with 0.5% Triton X-100. Detection of NF-κB complex subunits was made by incubation with anti-p50 (2 μg/mL) and anti-p65 (5 μg/mL) Abs, followed by FITC-labeled anti-IgG. Controls were stained with nonimmune serum.

**Immunohistochemistry and Southwestern in situ**

Immunohistochemistry for iNOS was analyzed in paraffin-embedded renal tissues by incubation with 2.5 μg/mL anti-rat iNOS in PBS containing 4% bovine serum albumin (BSA) and 6% goat serum, treatment with peroxidase-conjugated secondary Ab, development with 3,3'-diaminobenzidine tetrahydrochloride and counterstaining with hematoxylin. Negative controls without primary Ab were run in parallel. Glomerular lesions were evaluated after hematoxylin and eosin (H&E) and Masson's trichrome staining. For histological analysis, approximately 20 fields from each animal were examined by an
investigator who had no prior knowledge of the experimental design. The glomerular staining was semiquantitatively graded on the following scale: 0, negative, 1, weak, 2, moderate, and 3, strong staining.

In situ distribution of activated NF-κB was analyzed in paraffin-embedded tissue sections fixed in 0.5% paraformaldehyde by incubation with 50 pmol of digoxigenin-labeled DNA probe in buffer containing 0.25% BSA and 1 μg/mL poly(dI-dC), followed by alkaline phosphatase-conjugated anti-digoxigenin IgG and colorimetric detection [34]. In some cases double immunohistochemistry for iNOS was carried out on slides directly from the final wash of the Southwestern histochemistry protocol (counterstain with hematoxylin was omitted).

Statistical analysis

Data are expressed as mean ± SD or representative result, when indicated. Statistics was performed by using analysis of variance (ANOVA) or Tukey-Kramer tests. Differences were considered to be significant at \( P < 0.05 \).

RESULTS

NO generation in human MC and monocytes induced by IC

Exposure of human MC and monocytes to IC (200 μg/mL) induced a time-response production of NO, measured as accumulation of nitrites (a stable oxidative end product of NO) by fluorimetric assay (Fig. 1). Detectable amounts of nitrite were evident at six hours, with a peak of production after 24 hours of incubation. Monocytic cells (THP-1 line) produced greater amounts of NO than MC. The kinetics of IgA-IC (Fig. 1A) and IgG-IC (Fig. 1B) were very similar, however, IgG-IC was a stronger stimulus. The response to the same concentration of monomeric Igs was undetectable in both cell types (human MC, IgA 7 ± 3; IgG 9 ± 4; monocytes, IgA 12 ± 3; IgG 9 ± 1 nmol/mg protein at 24 h).

The possibility of endotoxin contamination in the Ig preparations was discarded, since preincubation with polymyxin B (10 μg/mL) did not significantly decrease mesangial NO generation by IgG-IC and IgA-IC (8 ± 2 and 10 ± 3% inhibition, respectively). Additionally, endotoxin concentration in the samples was less than 0.25 U/mL, as determined by Limulus amebocyte assay.

In these experiments the combination of LPS (10 μg/mL) and cytokines (TNF-α 100 ng/mL; IFN-γ 100 U/mL; IL-1β 100 U/mL), used as a positive control, induced a high NO production in MC and monocytes (183 ± 11 and 237 ± 12 nmol/mg protein at 24 h). NO formation was abolished upon treatment with 50 μmol/L L-NAME, a competitive NOS inhibitor (MC 89 ± 7%, monocytes 93 ± 3% inhibition).

IC induce iNOS expression in MC and monocytes

To assess whether the modulation of NO levels by IC is associated with changes in the corresponding mRNA steady state levels for iNOS, RT-PCR analysis using specific primers for human iNOS were performed. In human MC, the time course of iNOS induction was parallel to the NO production, increasing up to seven (IgA-IC) and 14 (IgG-IC) hours, and decreasing to near control levels after 48 hours (Fig. 2A). In monocytes, IgA-IC dose-dependently induced iNOS mRNA expression (1.9-, 3.2- and 3.8-fold increases with 100, 200 and 300 μg/mL at 7 h), and the temporal course was very different from that of MC, observing a continuous increase (Fig. 2B). Similar results were obtained with IgG-IC (not shown).

Monomeric Igs or F(ab’)2 fragments did not elicit iNOS expression in both cell types (less than 1.5-fold vs. basal). The iNOS protein induction by IC was analyzed by Western blot using cytosolic fractions from MC and monocytes. As shown in Figure 2C, IgA-IC drastically up-regulated the iNOS protein production after 24 hours of incubation, whereas monomeric IgA had no effect. The protein detected presented a similar size (130 kD) in both cell types, although it was more abundant in monocytes. Similar results were observed after incubation with IgG monomeric and IC (not shown).

IC increase the transcriptional activity of the iNOS gene

Growth-arrested human MC were transfected with the plasmids containing the 0.7 kb and 0.3 kb fragments of
the 5′ regulatory region of iNOS (0.7 kb and 0.3 kb piNOS-Luc), and luciferase activity was measured after 24 hours of stimulation with IC. Increasing concentrations of IgA-IC and IgG-IC activated the expression of the reporter iNOS plasmids, observing a maximal response with 300 μg/mL of IgA-IC and IgG-IC (5- and 6.5-fold increase of the 5′ flanking region of the iNOS gene), pGL-3 (empty plasmid), vs. control cells; N = 3). In monocytes, increased luciferase activity was observed after incubation with 150 μg/mL of IgA-IC and IgG-IC (5- and 6.5-fold increase vs. control cells; N = 4, P < 0.05), while monomeric Igs had no effect (1.2- and 1.4-fold vs. control, N = 3).

Modulation of IC-induced NO generation by inhibitors of kinases and NF-κB

The signal transduction pathways involved in the IC-induced NO generation were analyzed by using inhibitors of tyrosine kinases, mitogen-activated protein kinases (MAPK) and NF-κB pathway. As indicated in Figure 4A, NO released by human MC stimulated with IgA-IC and IgG-IC was significantly decreased in the presence of genistein (tyrosine kinase inhibitor), PD98059 (MEK inhibitor) and SB203580 (p38 MAPK inhibitor). However, the major inhibition percentages (around 60%) were observed after treatment with several agents known to inhibit the NF-κB activation at different levels: oxidative pathway (PDTC), IκB tyrosine phosphorylation (parthenolide), and IκB degradation (MG132) [35–37]. These compounds were unable to block iNOS enzyme activity (by indirect measurement of NO at 7 h), as no inhibition was observed when the agents were added at the last hour of incubation with IC (not shown).
Fig. 4. Mesangial NO production and iNOS expression induced by IC require activation of protein kinases and NF-κB. (A) Human MC were incubated with 150 μg/mL of IC alone or after pretreatment with genistein (150 μmol/L), PD98059 (50 μmol/L), SB203580 (30 μmol/L), PDTC (150 μmol/L), MG132 (10 μmol/L), or parthenolide (10 μmol/L). Symbols are: (●) IgA-IC; (●) IgG-IC. The NO production after 24 hours was analyzed by fluorimetric assay. Data of nitrite production (nmol/mg protein) are mean ± SD of 4 experiments performed in triplicate (*P < 0.05 vs. IC alone). (B) Human MC were preincubated with the indicated inhibitors before stimulation with IgA-IC for 6 hours, and iNOS mRNA expression was analyzed by RT-PCR. A representative experiment is shown in the upper part of the panel. Densitometry of the iNOS band was corrected by the GAPDH expression, and data are expressed as fold increases respect to control conditions. Data are means ± SD of three experiments (*P < 0.01 vs. IC alone).

The mesangial iNOS mRNA expression induced by IC also was significantly reduced when MAPK or NF-κB activity was blocked (Fig. 4B). To extend the above observations to rodent cells, the modulation of rat iNOS expression was assessed. Rat MC stimulation with IgG-IC (150 μg/mL) for six hours increased the iNOS mRNA expression (5-fold vs. control cells, N = 3), which was reduced by preincubation with PD98059, parthenolide and MG132 (52 ± 6, 62 ± 6, and 59 ± 8% inhibition; N = 3, P < 0.05). Similarly, preincubation of monocytes with the MEK inhibitor or proteasome inhibitor decreased the iNOS expression induced by IgA-IC and IgG-IC (PD98059 48 ± 9 and 43 ± 5% inhibition; MG132 63 ± 10 and 57 ± 8% inhibition; N = 4, P < 0.05). Cell viability assessed by trypan blue exclusion was consistently greater than 90% under all experimental conditions studied, indicating that these inhibitory effects were not the result of cytotoxicity.

NF-κB and MAPK are involved in the transcriptional induction of iNOS gene by IC

To examine in a more direct manner the upstream signaling cascades involved in the transcriptional induction of iNOS, transfected human MC and monocytes were pretreated with several inhibitors before IC stimulation. In monocytes, inhibition of NF-κB or MAPK pathways partially decreased iNOS transcription induced by IgG-IC and IgA-IC (Fig. 5A). Similarly, the luciferase activity in IC-stimulated human MC was dramatically attenuated by parthenolide or MG132 and, to a lesser extent, by the p38 inhibitor SB203580 (Fig. 5B). Interestingly, the MEK inhibitor PD98059 was effective in cells transfected with the 0.7 kb plasmid, but not with 0.3 kb plasmid, which contains NF-κB, but not AP-1 sites. This suggests that MEK activates iNOS transcription through the regulation of AP-1.

The pharmacological modulation of NF-κB activity in IC-stimulated cells was assessed by gel shift assay and Western blot for IkBα. Nuclear extracts from IgG-IC and IgA-IC stimulated cells showed a strong DNA binding activity compared with the control, as previously described [18, 20, 28], and cytosolic proteins revealed a decrease in the intensity of IkBα band (Fig. 5 C, D). NF-κB activity was attenuated by pretreatment with antioxidants, IkB phosphorylation inhibitors and proteasome inhibitors (Table 1), which also prevented IkBα degradation (Fig. 5C). Interestingly, MAPK inhibitors (SB203580 and PD98059) did not significantly affect both IkBα degradation and NF-κB nuclear translocation (Fig. 5 C, D and Table 1), indicating that NF-κB activation of IC-stimulated cells occurs independently of MAPK pathway. No differences in the subunits of NF-κB complex were detected in cells treated with the different inhibitors (PD98059, SB203580, PDTC, parthenolide) compared with non-treated cells after IC stimulation, as assessed by supershift assay (Fig. 5D and data not shown).
Fig. 5. NF-κB and MAPK pathways independently regulate the IC-induced iNOS transcription. Monocytes transfected with 0.7 kb piNOS-Luc (A) and human MC transfected with 0.7 kb and 0.3 kb piNOS-Luc (B) were pretreated with different inhibitors of NF-κB and MAPK, and then stimulated for 24 hours with 200 μg/mL of IgG-IC (A) and IgA-IC (A, B). Data of luciferase activity are expressed as fold increases vs. control cells; data are means ± SD of 4 experiments in duplicate (*P < 0.05 vs. IC alone). Symbols in panel A arc: (□) IgA-IC; (■) IgG-IC. (C) Degradation of the inhibitory subunit IκBα in the cytosol of human MC stimulated with IgA-IC (200 μg/mL, 60 min) was prevented when NF-κB inhibitors, but not MAPK inhibitors, were used. Western blots are representative of three experiments. (D) DNA-binding properties of nuclear proteins from MC activated with IgA-IC were analyzed by gel shift assays using consensus NF-κB oligonucleotide and p65 and p50 Abs. Pretreatment with the MEK inhibitor PD98059 did not modify the intensity of the bands and the composition of activated complexes. Arrows indicate the position of specific (NF-κB) and supershifted (ss) bands. Gel is representative of two experiments.

Further experiments were performed to examine the effects of IC on the activation of MAPK members ERK and p38. Since activation of MAPK is dependent on the phosphorylation by their respective upstream MAPK kinases, mesangial lysates were immunoblotted using phospho-specific Abs to p38 and ERK. As shown in Figure 6A, phosphorylation of p38 occurred after five minutes of IgA-IC stimulation, peaking at 15 minutes, and decreased near to control values at three hours. The pattern of ERK phosphorylation was more prolonged, gradually increasing up to one hour, and remaining sustained above the basal level after three hours of stimulation (Fig. 6B). A similar pattern of MAPK activation was observed after IgG-IC incubation (not shown). To determine whether this augmentation in phosphorylation of ERK and p38 was accompanied also by an increase in kinase activity, another portion of the whole cell lysate was subjected to in vitro kinase assay with
Table 1. Inhibition of IC-induced NF-κB activity in mesangial cells and monocytes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% Inhibition</th>
<th>Mesangial cells</th>
<th>Monocytes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IgG-IC</td>
<td>IgA-IC</td>
<td>IgG-IC</td>
</tr>
<tr>
<td>MG132 10 μmol/L</td>
<td>64 ± 2%</td>
<td>59 ± 17a</td>
<td>ND</td>
</tr>
<tr>
<td>TPCK 25 μmol/L</td>
<td>ND</td>
<td>69 ± 12b</td>
<td>73 ± 14b</td>
</tr>
<tr>
<td>PDTC 150 μmol/L</td>
<td>ND</td>
<td>75 ± 15c</td>
<td>ND</td>
</tr>
<tr>
<td>Genistein 150 μmol/L</td>
<td>43 ± 13e</td>
<td>41 ± 9f</td>
<td>50 ± 8g</td>
</tr>
<tr>
<td>Parthenolide 10 μmol/L</td>
<td>58 ± 12i</td>
<td>56 ± 10j</td>
<td>65 ± 10k</td>
</tr>
<tr>
<td>PD98059 50 μmol/L</td>
<td>17 ± 10m</td>
<td>22 ± 9n</td>
<td>ND</td>
</tr>
<tr>
<td>SB203580 30 μmol/L</td>
<td>20 ± 10p</td>
<td>18 ± 8q</td>
<td>ND</td>
</tr>
<tr>
<td>Fc fragments 100 μg/mL</td>
<td>75 ± 9s</td>
<td>71 ± 10t</td>
<td>73 ± 16u</td>
</tr>
<tr>
<td>F(ab')2, fragments 100 μg/mL</td>
<td>ND</td>
<td>13 ± 5w</td>
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Abbreviations are in the Appendix. Growth-arrested cells were pretreated during 90 minutes with the indicated inhibitors and then incubated for 60 minutes with IC (100 μg/mL). Cells were lysed and the NF-κB binding activity in nuclear extracts was analyzed by gel shift assay as described. Data of densitometry are expressed as percentage of inhibition vs. IC-treated cells and are mean ± SD of 3 to 5 experiments.

MBP as the substrate. The kinetics of p38 and ERK activation in human MC induced by IgA-IC was consistent with the phosphorylation assay (Fig. 6). Incubation of cells with the MEK inhibitor PD98059 avoided the phosphorylation of ERK, but had no effect on activation of p38. Similarly, blocking p38 had no effect on either phosphorylation levels or kinase activity of ERK (data not shown).

iNOS expression and NF-κB activation in experimental glomerulonephritis

Our previous studies described the evolution of the IC proliferative glomerulonephritis, which is characterized by marked glomerular immune deposits, cell proliferation, matrix accumulation, inflammatory cell infiltration, and intense proteinuria [32, 33]. Then, we evaluated whether overexpression of iNOS could be implicated in the pathogenesis of IC-mediated glomerulonephritis, analyzing iNOS expression levels in two different phases of the experimental model. Moderate proteinuria and mesangial immune deposits appear in the mild phase, while the severe phase is characterized by intense proteinuria, morphological lesions and immune deposits in the mesangium and glomerular capillary wall [32, 33]. The renal mRNA expression of iNOS was measured by RT-PCR on cortex samples, and the results were expressed as ratio of iNOS/GAPDH expression. Control animals presented very low expression levels of iNOS mRNA, which were increased after induction of the disease, being markedly higher (5-fold vs. control) in the severe phase of nephritis (Fig. 7A). By immunohistochemistry, few glomerular and tubular cells positive for iNOS protein were observed in the kidney of healthy control rats (not shown). In diseased kidney, iNOS protein was increased in glomeruli, with positivity found mainly in the mesangial area, some epithelial and endothelial cells, and in several tubular epithelial cells and interstitial infiltrating cells (Fig. 7B). A semiquantitative scoring system was applied for glomerular intensity of iNOS staining, which correlated with the severity of the disease, as determined by proteinuria levels (Fig. 7C).

Since previous papers described the activation of NF-κB during experimental glomerulonephritis [32, 38], in the next set of experiments we studied the simultaneous localization of the iNOS protein and the transcription factor. Therefore, we subsequently performed Southwestern histochemistry with consensus oligonucleotide for NF-κB and standard immunohistochemistry with anti-iNOS Ab. As shown in Figure 7D, colocalization of NF-κB (nuclear staining in dark) and iNOS (cytoplasmic staining in brown) was observed in several glomerular and tubular cells, suggesting that in vivo activation of this factor may be involved in the transcriptional regulation of iNOS.

Implication of FcR in renal cell activation by IC

To analyze whether IC activate MC and monocytes by a FcR-dependent mechanism, cells were incubated with different fragments obtained by enzymatic digestion of IgG. In human MC, the presence of IgA or IgG Fc fragments in the incubation medium reduced NO production induced by IgA-IC or IgG-IC (71 ± 2 and 74 ± 3% inhibition, N = 4), whereas no inhibition was observed in the presence of F(ab)2, or Fab fragments. In IC-stimulated monocytes, pretreatment with Fc fragments attenuated the luciferase activity of 0.7kb-piNOS-Luc plasmid (Fig. 8A) and the iNOS mRNA expression (IgA Fc fragments, 66 ± 6% inhibition, N = 4). By gel shift assay, a marked decrease in NF-κB activity was detected in the presence of Fc, but not F(ab')2, fragments, in both cell types (Table 1). Additionally, the nuclear translocation of p65/p50 heterodimer induced by IC in human MC (Fig. 8B, C) was prevented by Fc fragments (Fig. 8D, E).

To evaluate in vivo whether the interaction of IC with FcR is responsible for the increased iNOS expression in renal tissues, animals with ongoing nephritis were treated with IgG Fc fragments and followed for one and two weeks. We have previously demonstrated that Fc fragment administration prevents the development of renal injury in experimental immune glomerulonephritis [32]. Consistent with these data, the renal iNOS expression (mRNA and protein) was decreased in animals injected with Fc fragments as compared with untreated nephritic animals, observing significant differences after one and two weeks of study (Table 2). The decrease in iNOS expression was temporally associated with a reduction in proteinuria levels and glomerular lesions. No changes in proteinuria, renal morphology and iNOS expression were
observed in healthy control rats receiving daily injection of Fc fragments for two weeks (Table 2).

**DISCUSSION**

This study demonstrates that IC alone modulate NO generation by inducing iNOS in infiltrating and renal resident cells through a mechanism involving FcR activation. Moreover, in these cells, activation of the MAPK pathway and translocation of NF-κB into the nucleus are important steps in overexpression of iNOS upon IC activation.

Exposure of cultured cells to IC was accompanied by a marked time-dependent increase in steady-state iNOS mRNA levels, which was initially detected after few hours of incubation, peaked at 7 to 14 hours and decreased after 48 hours. The kinetics of iNOS expression in monocytes was different, and had a linear pattern. The iNOS is a ubiquitous enzyme, but the degree of NO production in response to the same stimuli is closely associated with the cell type-specific function [1, 3]. This regulatory complexity reflects the inducibility of the iNOS gene, and may explain why monocytes produce larger amounts of nitrites than MC in response to the same concentration of IC.

Renal iNOS activity is significantly increased in various pathophysiological conditions, including glomerulonephritis, tubulointerstitial nephritis and sepsis [2, 3, 8–11], suggesting a role for NO in the pathogenesis of immune-mediated inflammation. Recent reports using non-selective or iNOS-specific inhibitors in experimental models of acute immune glomerulonephritis and lupus nephritis have described the protective effects of blocking NO production in the development of proteinuria and renal lesions [12, 13]. In contrast, NOS inhibition in other models of immune renal injury presented deleterious effects, such as increased proteinuria and intraglomerular coagulation [3, 14]. This controversy suggests that immune-mediated renal disease probably will not be effectively treated in a very distal point to the IC deposition.

It has previously been described that the major source for NO production in the nephritic glomerulus seems to be the monocytes/macrophages [9]. NO also can be produced by neutrophils in the acute phase of immune glomerulonephritis, although glomerular iNOS inhibition does not affect the primary mechanism of injury (leukocyte infiltration) [12]. However, the concomitant participation of intrinsic glomerular cells in the synthesis/release of NO during glomerular inflammation cannot be excluded, since iNOS induction in different cells may peak at different time points. In our experimental model of immune glomerulonephritis in rats (mild and severe phase) there was an up-regulation of the renal iNOS expression in temporal association with the appearance of proteinuria and glomerular lesions (proliferation and inflammatory infiltration). Moreover, increased iNOS expression was detected even in the mild phase of the disease, which is characterized by moderate proteinuria and renal lesions, and mesangial IC deposits [32, 33]. This is consistent with previous data showing increased glomerular iNOS expression in renal biopsies from patients with proliferative forms of lupus nephritis and IgA

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**Fig. 6. Activation of MAPK by IC in human MC.** Cells were exposed to IgA-IC for different periods of time and the activation of p38 (A) and ERK (B) in the cell lysates was analyzed. Phosphorylation of p38 and ERK was detected by immunoblotting with phospho-specific Abs to each of the MAPK. For MAPK activity, proteins were immunoprecipitated with anti-p38 or anti-ERK Abs, and kinase assay was made using MBP as substrate. As control, total cell lysates and immunoprecipitates were immunoblotted with anti-p38 or anti-ERK Abs. Densitometric relative units are expressed as fold increase vs. control conditions. Results are representative of three independent experiments.
Fig. 7. Expression of iNOS in experimental immune complex glomerulonephritis. (A) The RNA from renal cortex of healthy control animals and rats with nephritis (mild and severe phases) was reverse transcribed and a PCR reaction (33 cycles) was performed with specific primers for rat iNOS, and GAPDH as control. Blot is representative of 4 different experiments. (B, C) Immunohistochemical detection of iNOS protein in renal tissues was analyzed by immunoperoxidase and semiquantitatively graded in a scale 0 to 3. (B) In animals with severe nephritis the strong iNOS staining was localized in the cytoplasm of resident and infiltrating cells. (C) The iNOS score was represented against the proteinuria levels of each individual (correlation coefficient $r = 0.973$). (D) Colocalization of activated NF-κB and iNOS in nephritic rats was analyzed by Southwestern histochemistry with digoxigenin-labeled NF-κB oligonucleotides followed by immunoperoxidase with anti-iNOS. The arrows indicate the double staining, positive nucleus (NF-κB) surrounded by a brown cytoplasm (iNOS) in glomerulus and tubule. Magnification $\times 200$.

Fig. 8. FcR are involved in the iNOS expression and NF-κB activation induced by IC. (A) Monocytic cells THP-1 cotransfected with 0.7 kb pNOS-Luc and pRL-TK were stimulated for 24 hours with 200 μg/mL of monomeric Igs or IC (in the presence or absence of Fc fragments). Data are the mean ± SD of 4 experiments in duplicate ($^*P < 0.05$ vs. control unstimulated cells). Symbols arc: (□), IC; (□), IC + Fc fragments; (□), monomeric. (B–E) Human MC were stimulated with IgA-IC (100 μg/mL, 60 min) in the absence (B, C) or presence (D, E) of 100 μg/mL IgA Fc fragments. Nuclear translocation of p65 (B, D) and p50 (C, E) subunits of activated NF-κB complex was detected by immunofluorescence analysis. Micrographs are representative of three independent experiments.

Table 2. In vivo blockade of FcR in rats with immune glomerulonephritis reduces iNOS renal expression

<table>
<thead>
<tr>
<th></th>
<th>UN (N = 3)</th>
<th>Fc (N = 4)</th>
<th>UN (N = 4)</th>
<th>Fc (N = 4)</th>
<th>UN (N = 4)</th>
<th>Fc (N = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS mRNA</td>
<td>2.4 ± 0.3a</td>
<td>1.3 ± 0.2b</td>
<td>5.2 ± 0.9a</td>
<td>1.5 ± 0.4c</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>iNOS protein</td>
<td>1.9 ± 0.2a</td>
<td>1.0 ± 0.1a</td>
<td>2.7 ± 0.2a</td>
<td>1.1 ± 0.2b</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Glomerular lesions</td>
<td>2.1 ± 0.1a</td>
<td>0.9 ± 0.1b</td>
<td>2.8 ± 0.2a</td>
<td>1.3 ± 0.2c</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>229 ± 60a</td>
<td>77 ± 12b</td>
<td>386 ± 31a</td>
<td>85 ± 16c</td>
<td>8 ± 1</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

The RNA from renal cortex of control animals, rats with untreated (UN) nephritis or treated with 5 mg/kg of Fc fragments (Fc) was isolated and analyzed in triplicate by RT-PCR for rat iNOS. After densitometry of the iNOS band and correction by GAPDH, data were expressed as fold increases with respect to untreated control. Immunohistochemical detection of iNOS protein and glomerular lesions in renal tissues was analyzed by immunoperoxidase and Masson’s staining, respectively, and semiquantitatively graded in a scale of 0 to 3. Urinary protein excretion was expressed as mg/24 h. Results are mean ± SD of the total number of animals from each group analyzed in duplicate.

$^aP < 0.01$ vs. untreated control rats

$^bP < 0.01$ vs. untreated nephritic rats after one or two weeks of study
nephropathy, but not in non-proliferative diseases [3, 10]. We showed that iNOS protein could be synthesized not only by infiltrating cells but also by resident glomerular cells (principally MC) and tubular cells. Similarly, in human glomerulonephritis, double immunohistochemical labeling for iNOS and macrophages (anti-CD 68) showed that most of the iNOS staining was present in CD68 negative cells, which morphologically could have been mesangial or visceral epithelial cells [10].

In cultured MC and monocytes, whereas IC induced high levels of iNOS and NO, monomeric Igs caused only a small increase, and none was detected when the cells were treated with F(ab')2 fragments. Additionally, incubation with Fc fragments prior to IC stimulation prevented iNOS induction and NO release. These data confirm that specific FcR for IgA or IgG are involved in IgA-IC or IgG-IC cell activation, as previously reported [18, 24–28]. To confirm these data in vivo, we evaluated the effects of FcR blockade on the protection from high output NO release and glomerulonephritis. Treatment with Fc fragments (inhibitors of IC binding to FcR) in cultured cells) largely prevented iNOS mRNA and protein expression in infiltrating and resident cells, together with an improvement in the renal lesions. These data are consistent with our previous article showing the prevention of proteinuria, cytokine production and matrix accumulation in the immune nephritis by Fc fragment administration [32], and expands the concept that FcR may have a key role in the initiation/progression of inflammatory responses during immune glomerular injury.

The iNOS gene has multiple transcription factor binding sequences, including NF-κB sites [4, 15]. The transcriptional regulation of iNOS gene was studied using two fragments from the 5′ regulatory region of the iNOS. The 0.7 kb fragment contains consensus sequences of the TATA box, Oct-1, NF-IL-6, NF-κB and TNF-RE, all included in the 0.3 kb fragment, and also contains additional γ-IRE and AP-1 sites [4, 7]. The data obtained with MC and monocytes transfectected with these two plasmids indicate that, although NF-κB activation represents a crucial step in the transcription of iNOS, it is possible that other promoter elements, such as AP-1, may be necessary for maximal iNOS gene induction. This is compatible with previous studies suggesting that other transcription factors can physically associate with and/or synergize with NF-κB for full induction of the iNOS gene in other systems [39, 40].

Studies in experimental and human glomerulonephritis have provided some evidence to support the involvement of the transcription factor NF-κB in the pathogenesis of glomerulonephritis [32, 38]. Additionally, increased expression of NF-κB–dependent genes, such as chemokines and adhesion molecules has been demonstrated in glomerular cells [19, 28, 41]. Various reagents have already proven effective in inhibiting NF-κB-inducible gene expression, and they may be classified into antioxidants, protease inhibitors, and IkB phosphorylation blockers [35–37]. In our work, pharmacological modulation of NF-κB activation with agents inhibiting NF-κB pathway at different stages produced parallel effects on iNOS expression and nitrite production, suggesting that NF-κB sites may be essential for iNOS induction by IC in cultured MC and monocytes. Moreover, NF-κB activation and iNOS expression were simultaneously detected in glomerular and tubular cells of nephritic rats, since both mediators appeared colocalized in the same cells (nuclear staining for NF-κB and cytoplasmic staining for iNOS). These data indicate that NF-κB has an important role in iNOS expression, although we do not discard the contribution of other signaling cascades in concert with NF-κB as crucial for iNOS induction by IC in renal cells.

The intracellular mechanisms controlling NO synthesis by different cell types are the subject of current interest. Thus, the expression of iNOS appears to be regulated in a cell-specific manner by several protein kinases [42]. Interestingly, the involvement of MAPK in iNOS induction is diverse, since the principal family members (ERK, p38, JNK) may play a positive, negative or neutral regulatory role—depending on the stimulus and cell type—in iNOS expression [43, 44]. We observed that NO generation and iNOS expression in MC and monocytes were attenuated by a general tyrosine kinase inhibitor (genistein) and specific inhibitors of MEK (PD98059) and p38 MAPK (SB203580) (a pharmacological inhibitor of JNK is not commercially available). This indicates that tyrosine kinases and MAPK play a positive regulatory role in the induction of iNOS by IC. Other authors described that tyrosine phosphorylation mediates iNOS up-regulation by insoluble IgG-IC in rat peritoneal macrophages [20]. The activation of tyrosine kinases by IC also was reported previously in MC [28, 31], but this is the first study describing the involvement of the MAPK family in the signaling pathways activated by FcR in these cells.

The involvement of MAPK in the activation of NF-κB in different systems has been illustrated by numerous reports. Thus, p38/ERK and ERK/JNK cascades are required for activation of NF-κB regulated genes by cytokines and antioxidants [45, 46]. Our data demonstrate activation of p38 and ERK by IC, temporally consistent with the kinetics of NF-κB activation and iNOS expression. However, studies employing specific inhibitors of MAPK members revealed that either IkBα phosphorylation, composition of the activated NF-κB complex or DNA-binding ability were independent of the activation of MEK and p38 cascades. Indeed, although NF-κB and MAPK activation is mediated by different pathways, both may converge downstream, as previously reported in other systems [47]. Therefore, we hypothesize that activation of downstream members in the MAPK path-
Fig. 9. Schematic summary of IC-activated signaling pathways controlling iNOS expression in MC and monocytes. Multiple cascades are activated by FcR that culminate in activation of NF-κB pathway, in which MAPK members appear necessary for inducing full expression of iNOS. Dotted lines show the paths of action of the different MAPK and NF-κB inhibitors used in this study.

APPENDIX

Abbreviations used in this article are: AP-1, activator protein-1; FcR, Fc receptors; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IC, immune complexes; IFN-γ, interferon-γ; Ig, immunoglobulin; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; L-NAME, N-nitro-l-arginine methyl ester; LPS, lipopolysaccharide; MC, mesangial cells; mAb, monoclonal antibody; MEK, ERK kinase; MAPK, mitogen-activated protein kinase; MBP, myelob basic protein; NF-κB, nuclear factor-κB; NO, nitric oxide; PDTC, pyrrolidine dithiocarbamate; rh, recombinant human; TNF-α, tumor necrosis factor-α.

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