Complement Activates the c-Jun N-Terminal Kinase/Stress-Activated Protein Kinase in Glomerular Epithelial Cells

Hongwei Peng, Tomoko Takano, Joan Papillon, Krikor Bijian, Abdelkrim Khadir and Andrey V. Cybulsky

*J Immunol* 2002; 169:2594-2601; doi: 10.4049/jimmunol.169.5.2594

http://www.jimmunol.org/content/169/5/2594

Why *The JI*?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References  This article cites 39 articles, 17 of which you can access for free at: http://www.jimmunol.org/content/169/5/2594.full#ref-list-1

Subscription  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2002 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Complement Activates the c-Jun N-Terminal Kinase/Stress-Activated Protein Kinase in Glomerular Epithelial Cells

Hongwei Peng, Tomoko Takano, Joan Papillon, Krikor Bijian, Abdelkrim Khadir, and Andrey V. Cybulsky

In the rat passive Heymann nephritis model of membranous nephropathy, complement C5b-9 induces sublethal glomerular epithelial cell (GEC) injury and proteinuria. C5b-9 activates cytosolic phospholipase A₂ (cPLA₂), and products of cPLA₂-mediated phospholipid hydrolysis modulate GEC injury and proteinuria. In the present study, we demonstrate that C5b-9 activates c-Jun N-terminal kinase (JNK) in cultured rat GECs and that JNK activity is increased in glomeruli isolated from proteinuric rats with passive Heymann nephritis, as compared with control rats. Stable overexpression of cPLA₂ in GECs amplified complement-induced release of arachidonic acid (AA) and JNK activity, as compared with neo (control) GECs. Activation of JNK was not affected by indomethacin. Incubation of GECs with complement stimulated production of superoxide, and pretreatment with the antioxidants, N-acetylcysteine, glutathione, and α-tocopherol as well as with diphenylene iodonium, an inhibitor of the NADPH oxidase, inhibited complement-induced JNK activation. Conversely, H₂O₂ activated JNK, whereas exogenously added AA stimulated both superoxide production and JNK activity. Overexpression of a dominant-inhibitory JNK mutant or treatment with diphenylene iodonium exacerbated complement-dependent GEC injury. Thus, activation of cPLA₂ and release of AA facilitate complement-induced JNK activation. AA may activate the NADPH oxidase, leading to production of reactive oxygen species, which in turn mediate the activation of JNK. The functional role of JNK activation is to limit or protect GECs from complement attack. The Journal of Immunology, 2002, 169: 2594–2601.

The complement system mediates inflammation, cytosis, and phagocytosis. The actions of complement are, in part, due to insertion of the C5b-9 membrane attack complex into plasma membranes of nucleated cells, which may lead to sublethal cell injury or lysis (1, 2). Injury in diverse renal glomerular diseases is mediated by complement (3). For example, in the rat passive Heymann nephritis (PHN) model of membranous nephropathy, impairment of glomerular capillary wall permeability (proteinuria) is associated with visceral glomerular epithelial cell (GEC) injury, which is mediated by Ab and C5b-9 (3–6). To better understand how C5b-9 injures GECs, we have used rat GECs in culture to characterize biochemical changes induced by sublytic complement attack (7–12). We have shown that C5b-9 activates protein kinases, such as protein kinase C, as well as mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK)/stress-activated protein kinase. C5b-9 also activates cytosolic phospholipase A₂-α (cPLA₂), which leads to release of arachidonic acid (AA). In GECs, metabolites of AA are metabolized to prostanoids via the constitutively expressed cyclooxygenase-1 and the C5b-9-inducible cyclooxygenase-2. Activation of cPLA₂ and production of prostanoids exacerbate GEC injury and proteinuria in PHN (13–16). Other studies have demonstrated that assembly of C5b-9 in GECs leads to production of reactive oxygen species (ROS), possibly via the NADPH oxidase (17, 18). ROS may contribute to glomerular capillary wall injury and, by analogy to other cells, ROS may play a potential role in signal transduction (19, 20).

The MAPKs are a family of serine/threonine protein kinases that transmit extracellular signals to nuclear or cytoplasmic targets. There are several MAPK pathways, including the JNK pathway, which consists of a cascade of kinases that are activated by diverse stimuli, e.g., proinflammatory cytokines (TNF and IL-1) and “stress” (heat-shock, irradiation, UV light exposure) (21, 22). JNK exists as a family of isoforms with several splice-variants, resulting in at least 12 different JNK polypeptides. Activation of JNK is by dual phosphorylation on threonine and tyrosine by upstream stimuli, e.g., proinflammatory cytokines (TNF and IL-1) and “stress” (heat-shock, irradiation, UV light exposure) (21, 22). JNK exists as a family of isoforms with several splice-variants, resulting in at least 12 different JNK polypeptides. Activation of JNK is by dual phosphorylation on threonine and tyrosine by upstream stimuli, e.g., proinflammatory cytokines (TNF and IL-1) and “stress” (heat-shock, irradiation, UV light exposure) (21, 22). JNK exists as a family of isoforms with several splice-variants, resulting in at least 12 different JNK polypeptides. Activation of JNK is by dual phosphorylation on threonine and tyrosine by upstream stimuli, e.g., proinflammatory cytokines (TNF and IL-1) and “stress” (heat-shock, irradiation, UV light exposure) (21, 22). JNK exists as a family of isoforms with several splice-variants, resulting in at least 12 different JNK polypeptides. Activation of JNK is by dual phosphorylation on threonine and tyrosine by upstream stimuli, e.g., proinflammatory cytokines (TNF and IL-1) and “stress” (heat-shock, irradiation, UV light exposure) (21, 22).
22). In a recent study, we demonstrated that in GECs, Csb-9-mediated induction of cyclooxygenase-2 is, in part, mediated via activation of JNK (12).

The aim of the present study was to investigate the pathways involved in complement-mediated GEC injury, specifically focusing on the activation of JNK and its potential interaction with cPLA2. We demonstrate that complement activates JNK in GECs in culture and in PHN. Assembly of Csb-9 results in activation of cPLA2 and release of AA. The latter stimulates production of ROS (i.e., superoxide), probably via activation of the NAPDH oxidase, and production of superoxide stimulates activation of JNK. Finally, activation of JNK reduces complement-mediated GEC injury, implying that JNK confers protection or limits complement attack.

Materials and Methods

Materials

Tissue culture reagents were obtained from Life Technologies (Burlington, Ontario, Canada). C8-deficient human serum, purified C8, AA, indomethacin, N-acetylcycteine (NAC), glutathione (GS), α-tocopherol (vitamin E), diphenylene iodonium (DPI), and luciginc (bis-N-thymylacridinium nitrate) were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-phospho-JNK Thr183/Tyr185 Ab was purchased from New England Biolabs (Mississauga, Ontario, Canada). This Ab is directed against a synthetic phospho-Thr183/Tyr185 peptide corresponding to the residues around Thr183/Tyr185 of JNK. Rabbit anti-JNK Ab was from New England Biolabs. Materials were used in this study are described in the Methods section.

Cell culture and transfection

Rat GEC characterization has been published previously (7, 8). GECs were cultured in K1 medium on plastic substratum. Strains were used with cells between passages 8 and 60. Subcones of GECs that stably overexpress cPLA2, or dominant-negative JNK, as well as neo GECs, were used in this study. Production and characterization of the GECs that stably overexpress cPLA2 or dominant-negative JNK were described previously (7, 8). GECs that express dominant-negative JNK were produced by stable transfection of kinase-inactive JNK1 by a method analogous to that for cPLA2.

Incubation of GECs with complement

The standard protocol involved incubation of GECs in monolayer culture with rabbit anti-GEC or sheep anti-Fx1A antiserum (5%, v/v) in modified Krebs-Henseleit buffer containing 145 mM NaCl, 5 mM KCl, 0.5 mM MgSO4, 1 mM NaHPO4, 0.5 mM CaCl2, 5 mM glucose, and 20 mM HEPES, pH 7.4, for 40 min at 22°C (7, 8). GECs were then incubated with normal human serum (NS; diluted in Krebs-Henseleit buffer) or heat-inactivated (decomplemented) human serum (HS; 56°C, 30 min) in controls, for the indicated times at 37°C. In some experiments, Ab-sensitized GECs were incubated with C8-deficient human serum or C8-deficient human serum reconstituted with purified C8 (80 μg/ml). As in previous studies, we have generally used heterologous complement to minimize possible signaling via complement-regulatory proteins (7, 8). Except for studies of cytolsis, experiments were conducted at concentrations of complement that induced minimal or no lysis (NS; 2.5%, v/v). Previous studies have shown that in GECs, complement is not activated in the absence of Ab (7).

Induction of PHN in rats

PHN was induced by a single i.v. injection of 0.4 ml of sheep anti-Fx1A antiserum, as described previously (25). Urine was collected on days 14–17, and rats were then sacrificed and glomeruli were isolated by differential sieving.

Preparation of cell and glomerular lysates

GECs were scraped from culture dishes into homogenization buffer containing 50 mM HEPES, 0.25 mM sucrose, 1 mM EDTA, 1 mM EGTA, 20 μM leupeptin, 20 μM pepstatin, and 0.1 mM PMSF, pH 7.4 (4°C), and were centrifuged at 1,500 × g for 3 min at 4°C. Cell pellets were solubilized in immunoprecipitation buffer containing 1.0% Triton X-100, 125 mM NaCl, 20 mM Tris, 20 μM leupeptin, 20 μM pepstatin, 0.2 mM PMSF, 25 mM NaF, 2 mM Na3VO4, 5 mM Na3P-O4, 1 mM EDTA, and 1 mM EGTA, pH 7.4 (4°C). The mixture was centrifuged at 14,000 × g for 10 min, and supernatant was then used for immunoblotting.

Glomeruli were isolated from rat kidney cortices by differential sieving (25). Glomeruli were centrifuged, resuspended in homogenization buffer, and homogenized with 25 strokes. The homogenate was centrifuged at 1,300 × g for 5 min to sediment nuclei. The supernatant was removed, and the pellet was homogenized for a second time, as above. After centrifugation, the supernant of the second homogenization was combined with that of the first and was centrifuged at 15,000 × g for 10 min. The supernatants were then used for immunoblotting.

Immunoblotting

Samples containing equal amounts of proteins were dissolved in Laemmli buffer and were separated by 10% SDS-PAGE under reducing conditions. Proteins were then electrophoretically transferred to a nitrocellulose membrane, blocked for 1 h in TBS with 0.01% (v/v) Tween 20 and 5% BSA, and incubated with rabbit anti-phospho-JNK or JNK Ab in TBS overnight at 4°C and then with HRP-conjugated secondary Ab. The blots were developed using the ECL technique (Amersham Pharmacia Biotech, Piscataway, NJ). Protein content was quantified by scanning densitometry, using NIH Image software. Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

Measurement of superoxide anion production

Superoxide anion production was determined according to published methods with some modifications (27). Glomeruli were prepared and supplement, cells were washed, scraped in homogenization buffer, and pelleted by centrifugation at 200 × g for 5 min at 4°C. The pellet was resuspended in assay buffer containing 130 mM NaCl, 5 mM KCl, 1 mM MgCl2, 35 mM sodium phosphate, and 20 mM HEPES (4°C). Cells were counted in a hemocytometer and were then centrifuged again at 200 × g for 5 min at 4°C and resuspended in assay buffer. The measurement of superoxide generation was conducted in a Berthold-Lumat LB 9507 luminometer (Bad Wildbad, Germany). To start the assay, luciginc (final concentration, 250 μM) was added to 1 × 106 cells in a total volume of 1 ml of assay buffer. Luciginc exhibits chemiluminescence that is reported to be sensitive to the superoxide anion. Photoemission in terms of relative light units (RLU) was measured every 5 s for 200 s. A buffer blank (<5% of the signal) was subtracted from each reading.

Measurement of complement-dependent cytotoxicity

Complement-mediated cytolysis was determined by measuring release of lactate dehydrogenase (LDH), similar to the method described previously (7). Specific release of LDH was calculated as (NS – HIS)/100 – HIS. Where NS represents the percent of total LDH released into cell supernatants in incubations with NS, and HIS is the percentage of total LDH released into cell supernatants in incubations with HIS.

Statistics

Data are presented as mean ± SEM. The t statistic was used to determine significant differences between two groups. One-way ANOVA was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the t statistic and adjusting the critical value according to the Bonferroni method. Two-way ANOVA was used to determine significant differences in multiple measurements among groups.

Results

Csb-9 induces phosphorylation of JNK in GECs and in PHN

Sublytic Csb-9 activates phospholipases and protein kinases in cultured GECs and in PHN (7–10). By monitoring phosphorylation of GST-c-Jun, we previously demonstrated that complement activates JNK in GECs and that JNK activation requires assembly of Csb-9 (12). JNK activation is dependent on dual phosphorylation of Thr183/Tyr185. Thus, in the present study, JNK activation was monitored by immunoblotting, using an Ab directed to phospho-JNK Thr183/Tyr185. GECs were incubated with anti-Fx1A and
complement (NS) at a sublytic concentration. Compared with control incubations (HIS), activation of complement produced an increase in JNK Thr\(^{183}\)/Tyr\(^{185}\) phosphorylation (Fig. 1A). However, complement did not affect JNK protein expression (Fig. 1B). It should be noted that JNK proteins of \(\sim 46\) and \(\sim 54\) kDa are expressed and phosphorylated in GECs. Complement increased the phosphorylation of both JNK proteins to a similar extent, although basal and stimulated phosphorylation was generally greater in the 46-kDa JNK. UV light (positive control) induced phosphorylation of the same 46- and 54-kDa JNK proteins (Fig. 1A). The JNK Ab also identified a protein between 46 and 54 kDa (Fig. 1B). This band may represent another species of JNK that does not undergo phosphorylation, or it may be a degradation product. In addition, the phospho-JNK Ab identified an \(\sim 42\)-kDa protein in complement-stimulated GECs (Fig. 1A). According to the manufacturer, the phospho-JNK Ab may cross-react with dually phosphorylated (activated) ERK. Therefore, this band probably represents phospho-ERK, in keeping with our earlier studies, which have demonstrated complement-induced ERK phosphorylation directly (9). To address the kinetics of JNK activation, Ab-sensitized GECs were incubated with complement for time periods ranging from 10 min to 24 h (Fig. 1, C–E). JNK activation was evident at 40 min and persisted at 60 min (data not shown). By 4 h, JNK activity declined toward basal levels.

To verify that complement-induced JNK phosphorylation was actually due to C5b-9 assembly, Ab-sensitized GECs were incubated with C8-deficient human serum reconstituted with purified C8 (which assembles C5b-9) or with C8-deficient human serum alone (which assembles C5b-7). JNK phosphorylation was increased markedly in incubations with C8-deficient serum reconstituted with C8, whereas phosphorylation was weak in incubations with C8-deficient serum, and was comparable to HIS incubations (Fig. 1F). Thus, C5b-9 is essential for JNK phosphorylation, whereas C5b-7 or other serum components are not active. In addition, incubation of Ab-sensitized GECs with normal rat serum stimulated JNK phosphorylation, as compared with heat-inactivated rat serum, indicating that homologous (rat) complement also activated JNK (Fig. 1G).

It is important to demonstrate that pathways analogous to those activated in cultured GECs by complement are also activated in vivo, specifically in the PHN model of membranous nephropathy, where C5b-9 assembles in GEC plasma membranes and induces sublytic injury and proteinuria. Studies were conducted in rats with autologous-phase PHN (day 14–17), which is known to be complement mediated (3). At this time point, rats demonstrated severe proteinuria (180 mg/24 h; normal, <25 mg/24 h). By analogy to cultured GECs, \(\sim 46\)- and \(\sim 54\)-kDa JNK proteins were expressed in rat glomeruli, and whereas both demonstrated increased phosphorylation in PHN (Fig. 2, A and C), there were no significant changes in protein expression (Fig. 2B). These data are consistent with earlier results, where it was shown that glomerular lysates of rats with PHN (but not normal rats) induced phosphorylation of GST-c-Jun (12). Similarly to cultured GECs, in glomeruli the anti-JNK Ab identified a band between 46 and 54 kDa, which may represent another species of JNK that does not undergo phosphorylation or may be a degradation product (Fig. 2B).

**Role of cPLA\(_2\) in C5b-9-stimulated JNK activation in GECs**

In the next series of experiments, we focused on the mechanisms of complement-induced JNK activation. Previously, we demonstrated that C5b-9 activates cPLA\(_2\) (but not secretory PLA\(_2\) isoforms) in GECs (10). In GECs that stably overexpress cPLA\(_2\), the C5b-9-induced release of AA is amplified, as compared with neo GECs, which express cPLA\(_2\) at a low level (7–10) (Fig. 3). By C5b-9-induced release of AA is amplified, as compared with neo GECs, which express cPLA\(_2\) at a low level (7–10) (Fig. 3). By...
compared with neo GECs (p, H11569). Ab-sensitized GECs that overexpress cPLA\(_2\) or neo GECs were incubated (8), precedes JNK activation (Fig. 1). Complement-induced AA release, which begins at 10 min and reaches a maximum at 30 min (8), is delayed, as compared with the time course of AA activation. It is important to note that the time course of JNK activation is, at least in part, dependent on the activation of cPLA\(_2\).

FIGURE 2. JNK phosphorylation in PHN. Glomeruli were isolated from rats with PHN (day 14–17) and from control rats, and glomerular homogenates were subjected to immunoblotting with anti-phospho-JNK (A and C) or anti-JNK Abs (B). A and B. Representative immunoblots, showing two PHN and two control rats. C. Densitometric quantification of pJNK bands. JNK phosphorylation is increased in PHN (*, p < 0.005, PHN vs control for 46- and 54-kDa bands; nine rats per group).

analogy to AA release, complement increased JNK phosphorylation ~4-fold in GECs that stably overexpress cPLA\(_2\), and ~2-fold in neo GECs (Fig. 3). Thus, cPLA\(_2\) overexpression amplified JNK activation. It is important to note that the time course of JNK activation was delayed, as compared with the time course of AA release reported earlier (8). Thus, complement-induced AA release, which begins at 10 min and reaches a maximum at 30 min (8), precedes JNK activation (Fig. 1E). Together, these data indicate that complement-induced JNK activation is, at least in part, dependent on the activation of cPLA\(_2\).

cPLA\(_2\) releases AA from membrane phospholipids, and in GECs the free AA is metabolized to prostanoids via cyclooxygenases-1 and -2 (11). To determine whether the free AA activated JNK or whether JNK activation was secondary to an AA metabolite, GECs were incubated with exogenous AA. Addition of AA (30 μM) increased JNK phosphorylation at 10 min (Fig. 4). Phosphorylation returned to near-basal levels by 30 min (Fig. 4). To evaluate the contribution of AA metabolites, complement-mediated JNK activation was measured in the presence of the cyclooxygenase inhibitor indomethacin. At concentrations known to inhibit prostaglandin generation in GECs (5–10 μM) (11), indomethacin did not affect JNK activation (Fig. 4). Furthermore, the increase in JNK phosphorylation induced by exogenous AA was also not inhibited in the presence of indomethacin (data not shown). Thus, JNK activation is most likely due to the effect of unmetabolized AA.

Role of ROS in the activation of JNK by complement

To determine whether the NADPH oxidase may be involved in the complement-induced activation of JNK, we first assessed whether complement stimulates superoxide production in cultured GECs (Fig. 5). Superoxide production was evident after 10 min of exposure to complement (Fig. 5B). A similar amount of superoxide was generated by GECs exposed to complement for 20 min, whereas 40-min exposure increased superoxide production markedly (Fig. 5, A and B). The flavoprotein inhibitor DPI can be used to block ROS production by the NADPH oxidase, which contains a flavocytochrome enzyme subunit (28). DPI inhibited complement-induced superoxide production by 100% (Fig. 5C). Superoxide production in GECs was also induced by the addition of exogenous AA, and this increase was also inhibited completely by DPI (Fig. 5D).

To determine whether ROS were involved in the complement-induced activation of JNK, we examined the effects of several antioxidants on JNK phosphorylation (Fig. 6). The antioxidants, NAC and GSH, both inhibited complement-mediated JNK activation in a dose-dependent fashion (Fig. 6, A and B). In addition, the addition of AA stimulated JNK phosphorylation (Fig. 4). Furthermore, the increase in JNK phosphorylation induced by exogenous AA was also not inhibited in the presence of indomethacin (data not shown). Thus, JNK activation is most likely due to the effect of unmetabolized AA.

Role of ROS in the activation of JNK by complement

To determine whether the NADPH oxidase may be involved in the complement-induced activation of JNK, we first assessed whether complement stimulates superoxide production in cultured GECs (Fig. 5). Superoxide production was evident after 10 min of exposure to complement (Fig. 5B). A similar amount of superoxide was generated by GECs exposed to complement for 20 min, whereas 40-min exposure increased superoxide production markedly (Fig. 5, A and B). The flavoprotein inhibitor DPI can be used to block ROS production by the NADPH oxidase, which contains a flavocytochrome enzyme subunit (28). DPI inhibited complement-induced superoxide production by 100% (Fig. 5C). Superoxide production in GECs was also induced by the addition of exogenous AA, and this increase was also inhibited completely by DPI (Fig. 5D).

To determine whether ROS were involved in the complement-induced activation of JNK, we examined the effects of several antioxidants on JNK phosphorylation (Fig. 6). The antioxidants, NAC and GSH, both inhibited complement-mediated JNK activation in a dose-dependent fashion (Fig. 6, A and B). In addition, the addition of AA stimulated JNK phosphorylation (Fig. 4). Furthermore, the increase in JNK phosphorylation induced by exogenous AA was also not inhibited in the presence of indomethacin (data not shown). Thus, JNK activation is most likely due to the effect of unmetabolized AA.

FIGURE 3. Role of cPLA\(_2\) in complement-mediated JNK activation. Ab-sensitized GECs that overexpress cPLA\(_2\), or neo GECs were incubated with NS or HIS for 40 min. Lysates were immunoblotted with anti-phospho-JNK Ab. A, Representative immunoblot. B, Densitometric quantification. JNK activation is greater in the GECs that overexpress cPLA\(_2\), as compared with neo GECs (⁎, p < 0.025, cPLA\(_2\) vs neo, four experiments; Untr, untreated). JNK activation by UV light (positive control) was equally potent in both cell lines. C, C5b-9-induced release of \(^{3}H\)AA is greater in GECs that overexpress cPLA\(_2\), as compared with neo (adapted from Ref. 10).

FIGURE 4. Effect of AA and its metabolites on JNK activation. Upper panel, GECs that overexpress cPLA\(_2\) were incubated with AA (10 or 30 μM) for 10 or 30 min. Lower panel, GECs were pretreated with indomethacin (Indo, 5 or 10 μM) for 30 min and were then incubated with Ab and complement (as in Fig. 1). Lysates were immunoblotted with anti-phospho-JNK Ab, and immunoblots were quantified using densitometry. Exogenous AA stimulated JNK phosphorylation (⁎, p < 0.03, AA vs control, five experiments). Inhibition of AA metabolism to prostanoids (by indomethacin) did not affect complement-induced JNK phosphorylation (†, p < 0.001, HIS vs NS and NS + Indo, four experiments).
stimulatory effect of complement on JNK was inhibited significantly by the antioxidant α-tocopherol (vitamin E) (Fig. 6C), as well as by the NADPH oxidase inhibitor DPI (Fig. 6A and D). Conversely, incubation of GECs with H$_2$O$_2$ (1 mM) stimulated JNK phosphorylation significantly (Fig. 6, A and E). Together, these results support a role for the NADPH oxidase and superoxide anion production in mediating the complement-induced activation of JNK.

Effect of JNK activation on complement cytotoxicity

The potential functional roles of JNK may include modulation of cell injury. There are presently no specific pharmacological inhibitors of JNK. To block endogenous JNK activity in GECs, we used stable transfection to express a dominant-negative (dominant-inhibitory) form of JNK, JNK1-APF (24). In this mutant, the phosphorylation site, TPY, is altered to APF, thereby inhibiting phosphorylation and activation of the kinase, and the mutant is predicted to compete for binding of upstream MAP kinase kinases (MKKs). Thirty-two clones of transfected, G418-resistant GECs were screened by immunoblotting with anti-JNK Ab for JNK protein expression. Fifteen of these clones demonstrated increased JNK total protein levels, in keeping with overexpression of dominant-negative JNK-expressing clones, as compared with neo GECs (Fig. 7A). An additional clone of GECs that expressed only the neo gene (neo35) was used as control, as well as clone neo16, which had been produced earlier (Fig. 7A). Basal and complement-mediated phosphorylation of endogenous JNK were attenuated in dominant-negative JNK-expressing clones, as compared with neo GECs (Fig. 7B). However, complement-induced phosphorylation of ERK was not affected by dominant-negative JNK expression (Fig. 7C), implying that complement activation, C5b-9 assembly, and activation...
of other pathways were not altered in dominant-negative JNK-expressing clones. The GECs that express dominant-negative JNK were indistinguishable morphologically from neo GECs and proliferated at comparable rates.

GEC injury was quantified by monitoring release of LDH, a sensitive measure of cell viability. GECs that express dominant-negative JNK and neo GECs were incubated with Ab and serially increasing concentrations of complement that induce minimal to moderate lysis. This protocol allows for complement to activate JNK, but with increasing incubation time and complement dose a portion of the cells will undergo lysis. After 18 h, lysis was significantly greater in the three GEC clones that express dominant-negative JNK, as compared with the neo clones (Fig. 8A). These results imply that complement-induced JNK activation limits or protects GECs from complement attack. When GECs were incubated with complement for only 40 min, lysis was significantly greater in two of the three GEC clones that express dominant-negative JNK, as compared with the two neo clones (Fig. 8B). In the third dominant-negative JNK clone, JNK inhibition was perhaps not as complete as in the two others. Thus, the effect of JNK activation on cell injury appears to be both acute and delayed, the latter occurring at a time point when JNK activation is no longer detectable (Fig. 1).

In GECs, DPI effectively inhibits complement-induced superoxide production (Fig. 5C) and JNK activity (Fig. 6D). To substantiate the above results, which show that overexpression of dominant-negative JNK exacerbates complement lysis, GECs that overexpress cPLA$_2$ were incubated with Ab and serially increasing concentrations of complement, in the presence or absence of DPI. After 18 h of incubation, complement lysis was enhanced in the presence of DPI (Fig. 8C), in keeping with the dominant-negative JNK expression studies. It should be noted that DPI was not independently cytotoxic. In a previous study (9), we showed that complement activates the ERK pathway and that ERK pathway activation is blocked by PD98059 (100 μM), an inhibitor of MKK1,2 (the kinase just upstream of ERK). To determine whether inhibition of the ERK pathway would also affect complement lysis,
GECs that overexpress cPLA₂ were incubated with Ab and serially increasing concentrations of complement, in the presence or absence of PD98059. After 18 h of incubation, there were no significant differences between PD98059-treated and untreated GECs (Fig. 5D). Similar results were evident after 40 min of incubation with complement (data not shown). Thus, the functional consequences of JNK and ERK pathway activation are distinct. Finally, GECs were incubated for 30 min with or without H₂O₂, an activator of JNK (Fig. 6E). After a period of 18 h, GECs were incubated with Ab and serially increasing concentrations of complement for 40 min. Complement lysis was reduced significantly by H₂O₂ pretreatment (Fig. 8E). Although it cannot be concluded that JNK was the sole target of H₂O₂, these data provide further support for a protective role of JNK and are in keeping with the results of the dominant-negative JNK or DPI experiments.

Discussion

We have demonstrated that C5b-9 stimulates JNK activity in cultured GECs and in experimental membranous nephropathy in vivo (12). The present study confirms these observations and characterizes the mechanisms of JNK activation, as well as the functional consequences. Two JNK polypeptides were activated in parallel in GECs. Complement-induced JNK activation was mediated via AA and involved production of superoxide anion, most likely via the NADPH oxidase. This mechanism is distinct from complement-induced activation of the ERK pathway in GECs, which involves protein kinase C and Ras (Ref. 9 and our unpublished observations). Interestingly, the functional role of JNK activation is to limit or protect GECs from complement-mediated injury.

In earlier studies, we used multiple approaches to demonstrate that in GECs, complement releases AA via activation of cPLA₂ (7, 8). Using analogous approaches, the present study establishes that complement-induced JNK activation is, at least in part, due to cPLA₂-induced release of AA (Figs. 1, 3, and 4). The effect of AA on JNK activation appeared to be mediated via the NADPH oxidase, a multicomponent plasma membrane enzyme consisting of flavocytochrome b₅₅₈ (a heterodimeric complex of gp91phox and p22phox protein subunits) and p47phox and p67phox subunits, plus Rac, a GTP-binding protein (19, 20). Activation of the NADPH oxidase leads to production of superoxide anion, which can be further metabolized to H₂O₂. Although the NADPH oxidase is best characterized in phagocytic cells (20), cultured GECs were shown to express mRNAs encoding for components of the NADPH oxidase (30). In the same study, incubation of GECs with ATP stimulated superoxide production via the NADPH oxidase (30), whereas xanthine oxidases did not contribute to superoxide production significantly. In the present study, complement or exogenously added AA stimulated production of superoxide in GECs (Fig. 5, B and D), and both the complement- and AA-induced superoxide production were blocked by DPI (Fig. 5, C and D). Moreover, preincubation of GECs with DPI or with antioxidants inhibited complement-induced JNK activation (Fig. 6), and exogenously added H₂O₂ stimulated JNK significantly. Together, these results imply that complement-induced AA release leads to production of superoxide via the NADPH oxidase and that ROS stimulate JNK activity. Generation of ROS by C5b-9 has also been reported in rat mesangial cells, although the mechanism was not addressed in detail (31).

The present study demonstrates a link among cPLA₂, AA, ROS, and JNK activation. Certain aspects of these interactions have been addressed previously. In rabbit proximal tubular epithelial cells, exogenous AA and linoleic acid (30–60 μM) were shown to activate JNK (27). The effect of AA was associated with superoxide production and was blocked by NAC. The authors concluded that the NADPH oxidase was involved. In a human myeloid cell line, incubation with phorbol myristate acetate or opsonized zymosan resulted in the activation of the NADPH oxidase and superoxide production only if the cells expressed cPLA₂, but not if cPLA₂ expression was abolished by antisense inhibition (32). In the presence of cPLA₂, phorbol myristate acetate also increased free AA, and exogenous AA (10–25 μM) stimulated superoxide production. Finally, in rat mesangial cells, activation of JNK by IL-1 was associated with AA release and was blocked by the nonspecific PLA₂ inhibitor aristiocholic acid (33). Exogenous AA also activated JNK independently of AA metabolism, but the potential role of ROS was not addressed.

Classically, production of ROS is believed to be associated with cell or tissue damage, but in recent years, ROS, particularly at low concentrations, have been shown to function in signal transduction (19, 20). For example, sublethal H₂O₂ was shown to activate ERK and JNK, whereas pretreatment of cells with NAC inhibited JNK activation. At low concentrations, ROS may activate some protein kinases and inhibit protein phosphatases. Activation of the JNK pathway is complex (21). JNKs are phosphorylated and activated by MKK4 and MKK7. These protein kinases are activated by another large group of kinases, including MAP kinase kinase kinase (ASKs), transforming growth factor-β-activated kinase 1, Tpl2, and others. Of potential relevance to this study is ASK1, a protein kinase that may be a target for ROS (34, 35). Under basal conditions, ASK1 is complexed and inhibited by thioredoxin. Activation of the TNF receptor stimulates ROS production, which leads to ROS-dependent thioredoxin dissociation from ASK1, recruitment of ASK1 to the membrane, dimerization, and activation. ASK1 can then potentially activate MKK4 or MKK7, which in turn can phosphorylate JNK. Other potential targets of ROS are JNK-directed phosphotyrosine phosphatases, dual-specificity phosphatases, or protein phosphatase 2A (19, 20, 22, 23). These molecules generally have a cysteine residue in the active site, which is essential for activity, and can be regulated in a redox-dependent manner. In resting cells, where levels of ROS are low, the activity of phosphatases in cells would predominate, because the specific activity of phosphatases tends to be higher than the activity of kinases. Stimulation of ROS production may transiently inactivate phosphatases and permit an increase in kinase activity. The precise mechanism by which complement-induced ROS activate JNK in GECs will require further study.

The biological roles of JNK activation appear to be diverse and may depend on cell type. For example, the JNK pathway may mediate withdrawal from the cell cycle, induce apoptosis, prevent apoptosis, or initiate cell repair (21, 22). JNK activation by C5b-9 has been described in oligodendrocytes, where activated JNK facilitated progression through the cell cycle (36). In GECs, induction of cyclooxygenase-2 by C5b-9 is, in part, dependent on JNK (12). In contrast to previous studies on cytoprotection that have focused on the role of JNK in mediating apoptosis, the present study implicates JNK in protecting GECs from cytolysis (Fig. 8). Measurement of cytolysis is a practical and accurate method to address complement-mediated injury in a cell culture system, although GEC injury in PHN is generally sublethal and manifests as proteinuria. JNK is activated in glomeruli of rats with PHN (12) (Fig. 2), and based on the mechanisms and functional role in cultured GECs, it is reasonable to propose that JNK activation may also restrict the amount of GEC injury in PHN. However, relatively large amounts of ROS may be generated in glomeruli of rats with PHN, and these ROS may independently damage glomerular structures and contribute to proteinuria (18). Thus, the role of ROS and JNK activation in PHN will require
further study. Nevertheless, our observations provide a rationale for developing nontoxic methods to induce activation of JNK in vivo, which may eventually have applications to therapy of glomerular disease. The potential importance of these results extends beyond complement-induced GEC injury, in that C5b-9 may be pathogenic in various diseases. Thus, JNK activation and potential role in cytoprotection could be tested in disease models of systemic lupus erythematosus, myositis, myocardial infarction, multiple sclerosis, and others (1, 2). Finally, activation of JNK before xenotransplantation might be a means of reducing hyperacute xenograft rejection, which is complement dependent (37).

“Ectocytosis,” or shedding of C5b-9 complexes from plasma membranes of cells (1, 2), or reassembly of cytoskeletal proteins (38) are two other mechanisms that may protect cells from complement attack. The present study shows that the protective effect of JNK in GEC injury may occur acutely (when JNK activation is evident) or may be delayed (when JNK activity has returned to basal levels). The former is in keeping with posttranslational effects, whereas the latter suggests induction of gene transcription. For example, JNK could potentially alter expression or phosphor-

References


29. Wojtaszek, P., A. L. E. Heeg, G. Siriwandana, and T. Berl. 1998. Dominant-negative c-Jun NH 2-terminal kinase 2 sensitizes renal inner medullary collecting duct cells to hypertonicity-induced injury (29), another possible role of JNK may be to correct the changes induced by ion and fluid fluxes that occur secondary to C5b-9 assembly in the plasma membrane. Further studies to define the subcellular target(s) of JNK in GECs are in progress.