Mechanism of eosinophil induced signaling in cholinergic IMR-32 cells

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Curran, David R., Ross K. Morgan, Paul J. Kingham, Niamh Durcan, W. Graham McLean, Marie Therese Walsh, and Richard W. Costello. Mechanism of eosinophil induced signaling in cholinergic IMR-32 cells. Am J Physiol Lung Cell Mol Physiol 288:L326–L332, 2005. First published October 1, 2004; doi:10.1152/ajplung.00254.2004.—Eosinophils interact with nerve cells, leading to changes in neurotransmitter release, altered nerve growth, and protection from cytokine-induced apoptosis. In part, these interactions occur as a result of activation of neural nuclear factor (NF)-κB, which is activated by adhesion of eosinophils to neural intercellular adhesion molecule-1 (ICAM-1). The mechanism and consequence of signaling after eosinophil adhesion to nerve cells were investigated. Eosinophil membranes, which contain eosinophil adhesion molecules but not other eosinophil products, were coincubated with IMR-32 cholinergic nerve cells. The studies showed that there were two mechanisms of activation of NF-κB, one of which was dependent on reactive oxygen species, since it was inhibited with diphenyleneiodonium. This occurred at least 30 min after coculture of eosinophils and nerves. An earlier phase of NF-κB activation occurred within 2 min of eosinophil adhesion and was mediated by tyrosine kinase-dependent phosphorylation of interleukin-1 receptor-associated kinase-1 (IRAK-1). Coimmunoprecipitation experiments showed that both extracellular signal-regulated kinase 1/2 and IRAK-1 were recruited to ICAM-1 rapidly after coculture with eosinophil membranes. This was accompanied by an induction of ICAM-1, which was mediated by an IRAK-1-dependent pathway. These data indicate that adhesion of eosinophils to IMR-32 nerves via ICAM-1 leads to important signaling events, mediated via IRAK-1, and these in turn lead to expression of adhesion molecules.
phosphorylation of actin-associated proteins (5) and cytoskeletal rearrangement (6). Other molecules that have previously been shown to have a functional relationship with the cytoskeleton are involved in cell signaling (18, 28) and in particular NF-κB activation, in a variety of cells. ERK1/2 is one such molecule and exists in actin-enriched structures known as focal adhesion complexes (14). Thus ICAM-1 ligation could result in activation of NF-κB through mechanisms involving the phosphorylation of ERK1/2 or IRAK-1. In this study, we tested the hypothesis that, after adhesion, the cytoplasmic tail of ICAM-1 recruits and activates several molecules, including IRAK-1 and ERK1/2 and that these are responsible for NF-κB activation. We examined the effect of adhesion on nerve cell gene transcription of the adhesion molecule ICAM-1, since this NF-κB-regulated adhesion molecule is important in eosinophil recruitment to nerves.

MATERIALS AND METHODS

Materials. DMEM + glutamax, FCS, and penicillin-streptomycin solution were purchased from Gibco-BRL Life Technologies (Paisley, UK). The IMR-32 cell line was obtained from European Collection of Cell Cultures (Salisbury, UK) and depleted of fibroblasts using immunomagnetic anti-fibroblast microbeads and LD MACS separation columns purchased from Miltenyi Biotech (Bisley, UK). Gentamicin, Trypan blue, diphenylelenidionium (DPI), poly(dI-dC), CDP-Star chemiluminescent substrate solution, Igepal CA-630, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), genistein, Tri Reagent, Tween 20, and all common buffer constituents were obtained from Sigma (Poole, UK). Dulbecco’s PBS was obtained from Invitrogen (Paisley, UK), I-Block for Western blot blocking and Nitro-Block II, chemiluminescent substrate component for alkaline phosphatase (AP), were purchased from Tropix (Bedford, MA). Monoclonal mouse anti-human anti-CD1/18 antibody (clone 68-5A5, isotype IgG2a) was from Cymbus Biotechnology (Chandlers Ford, UK). NF-κB binding site consensus oligonucleotide (5’-AGT TGA GGG GAC TTT CCC AGG C-3’), anti-mouse IgG AP conjugate, anti-rabbit IgG AP conjugate, PD-98059, T4 polynucleotide kinase, Transfast transfection reagent, pSVβ-galactosidase control vector, β-galactosidase enzyme assay system with reporter lysis buffer, Taq polymerase, Thermophilic DNA 10× buffer, and MgCl2 (25 mM) were obtained from Promega (Madison, WI). Monoclonal mouse anti-human phosho-ERK antibody (isotype IgG2a), rabbit anti-human IκBα antibody (isotype IgG1), monoclonal mouse anti-human anti-IRAK-1 antibody (isotype IgG1), monoclonal mouse anti-human anti-ICAM-1 (clone P2A4, isotype IgG1), normal mouse control IgG, normal rabbit control IgG, and protein A/G PLUSagarose beads immunoprecipitation reagent were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ficoll-Paque PLUS was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). CD16 immunomagnetic beads and VS+ VarioMacs columns were purchased from Miltenyi Biotech. Diffy-Diff was obtained from Cln-Tech (Clacton-on-Sea, UK). [γ-32P]ATP was from NEN (Zaventem, Belgium). First-strand cDNA synthesis kits for RT-PCR (AMV) were bought from Roche (Leuven, UK). Forward and reverse primers for ICAM-1 (5’-GGCTGGAGCTGTTTGAGAAC-3’; 5’-ACTGTGGGGTTCAACCTCTG-3’) and β-actin (5’-TCTTGGT-GCATCCACGAAAAACT-3’; 5’-GAAGCATTCTTTGCGGTGAC-GAT-3’) were purchased from MWG-Biotech (Milton Keynes, UK). The Multiplex PCR kit for human chemokines was obtained from AMS Biotechnology (Oxon, UK). Monoclonal mouse anti-human anti-ICAM-1 and mouse IgG, isotype control fluorescein-conjugated antibodies were bought from R & D Systems (Minneapolis, MN). The dominant-negative construct for MyD88 was a gift from Dr. T. Carroll (Dept of Medicine, Royal College of Surgeons in Ireland, Ireland). This dominant-negative construct has been previously shown to attenuate IRAK-1 signaling in a number of cell lines (16, 26).

IMR-32 nerve cell culture. The human cholinergic neuroblastoma cell line IMR-32 was depleted of fibroblasts by incubating with immunomagnetic anti-fibroblast microbeads and applying to LD MACS separation columns, as recommended by the manufacturer. Fibroblast-depleted IMR-32 cells were used for all experiments. The cells were maintained in culture in proliferation medium (DMEM + glutamax, 5% FCS, 100 U/ml penicillin-streptomycin, and 10 μg/ml gentamicin) at 37°C in an atmosphere of 5% CO2. Upon achieving confluence, cells were plated at a density of 5 × 105/well in six-well cell culture dishes and grown in proliferation medium for 48 h. Proliferation medium was then replaced by differentiation medium [DMEM + glutamax, 2% FCS (vol/vol), 2 mM sodium butyrate, 100 U/ml penicillin-streptomycin, and 10 μg/ml gentamicin], and cells were used for experimentation after a further 6–7 days of differentiation in culture.

Eosinophil isolation. Eosinophils were prepared from 45 ml of peripheral blood from healthy human volunteers by a negative immunomagnetic selection technique, as described previously. Briefly, after phlebotomy, 15 ml of blood were added to 25 ml of PBS containing 100 units of heparin, and 30 ml of blood/PBS were layered on 23 ml of Ficoll (1.077 ± 0.001 g/ml). Centrifugation at 500 g for 20 min at room temperature was carried out, the upper serum and monocyte layers were discarded, and the resulting granulocyte- and red blood cell-containing pellet was subjected to hypotonic water lysis, for removal of red blood cells. This was performed with 18 ml of ice-cold dH2O for 30 s followed by the addition of 2 ml of PIPES-buffered salt solution (250 mM PIPES, 1.1 M NaCl, 50 mM KCl, and 420 mM NaOH; pH 7.47). After hypotonic lysis, the granulocyte pellet was washed in PAG buffer [PIPES-buffered salt solution + 55.5 mM glucose and 0.0003% (wt/vol) human serum albumin]. Granulocytes were then resuspended in MACS buffer [PBS + 2 mM EDTA and 0.5% (wt/vol) BSA] with an equal volume of human CD16 immunomagnetic microbeads (1 μl beads/106 cells) at 4°C for 30 min. After being washed and resuspended in MACS buffer, granulocytes were applied to a VS+ VarioMacs column. Immunomagnetically labeled neutrophils were retained on the column, and eluted eosinophils were collected and resuspended in differentiation medium. Cell viability was assessed by Trypan blue exclusion, and eosinophil purity was determined by Speedy-Diff staining. Only populations of eosinophils that were >98% pure and >95% viable were used in experiments.

Eosinophil membrane preparation. Immediately upon isolation, eosinophils were resuspended in cold, sterile dH2O, incubated on ice for 15 min, and then centrifuged at 1,500 g for 10 min at 4°C. This process was repeated two times, and the resulting lysed cell membranes were resuspended in differentiation medium and added to differentiated IMR-32 cells, plated as described above in six-well cell culture plates, in a concentration equivalent to 2 × 106 whole eosinophils/well.

Nuclear and cytoplasmic protein preparation. IMR-32 cells (5 × 105) were differentiated for 6–7 days with sodium butyrate, as described above, and then incubated with eosinophil membranes equivalent to 2 × 106 whole eosinophils for varying time periods from 2 min to 2 h. In some experiments, IMR-32 cells were pretreated with inhibitors of eosinophil adhesion for 1 h, and coculture experiments with eosinophil membranes were carried out in the presence or absence of these inhibitors. In other experiments, IMR-32 cells were pretreated with the tyrosine kinase inhibitor genistein (50 μM), the ERK1/2 inhibitor PD-98059 (50 μM) for 2 h, or the NADPH oxidase inhibitor diphenyleneiodonium chloride (1 μM) for 1 h, before coculture (9, 10). Nuclear and cytoplasmic extracts were isolated from IMR-32 cells after coculture. Briefly, cells were harvested in 1 ml of ice-cold PBS and pelleted by centrifugation at 3,800 g for 5 min at 4°C. Cells were resuspended in 1 ml of hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM PMSF, and 0.5 mM DTT) and pelleted by centrifugation at 13,000 g for 10 min.
PCR products on a 2% Tris borate-EDTA agarose gel containing 0.5 μg/ml ethidium bromide. The PCR products were imaged and documented using the Gene Genius Gel Documentation System (Syngene, Cambridge, UK).

Flow cytometry. After coculture experiments in six-well plates, the differentiation medium containing the eosinophil membranes was removed, and the IMR-32 cells were washed with ice-cold PBS. The nerve cells were then scraped off the wells and washed three times in an isotonic PBS buffer (supplemented with 0.5% BSA) by centrifugation at 500 g for 5 min. The IMR-32 cells were then resuspended in isotonic PBS buffer to a final concentration of 4 × 10^5 cells/ml, and 25 μl of this suspension were used for antibody staining. Before being stained, cells were first Fc-blocked by treatment with 1 μg of human IgG for 15 min at room temperature. Cells were then incubated with 10 μl of fluorescein-conjugated monoclonal anti-human ICAM-1 antibody or mouse IgG control antibody for 45 min at 4°C. Unreacted antibody was then removed by washing cells two times in isotonic PBS buffer before final resuspension in 400 μl of buffer for analysis by flow cytometry (EPICS XL-MCL; Coulter, Fullerton, CA).

Statistical analysis. Values are expressed as means ± SD. Comparisons between groups were made using an unpaired Student’s t-test. P < 0.05 was considered to be statistically significantly different.

RESULTS

Eosinophil coculture with IMR-32 cholinergic nerve cells results in nerve cell NF-κB activation that is only partly mediated by oxidative stress. Peripheral blood eosinophils were coincubated with differentiated IMR-32 cells for varying periods of time from 2 min to 2 h, as previously described (27). Nuclear extracts from the nerve cells were examined for NF-κB activation using electrophoretic mobility shift assay (EMSA), which confirmed our previous finding of a rapid and pronounced activation of NF-κB occurring within 2 min and sustained for a period of 2 h (Fig. 1A; see Ref. 27). Western blotting of cytoplasmic extracts also demonstrated degradation of IκBβ accompanying NF-κB activation (data not shown). However, when the nerve cells were pretreated with the NADPH oxidase inhibitor DPI before eosinophil coculture, only the later stages of NF-κB activation and IκBβ degradation were inhibited.

Thermocycling conditions for PCR were 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 59°C for 1 min, and 72°C for 30 s. A final extension step of 72°C for 10 min was followed by resolution of PCR products on a 2% Tris borate-EDTA agarose gel containing 0.5 μg/ml ethidium bromide. The PCR products were imaged and documented using the Gene Genius Gel Documentation System (Syngene, Cambridge, UK).

Electrophoretic mobility shift assay. Nuclear extracts (10 μg) were incubated with 1.6 kbq [γ-32P]ATP (3,000 Ci/mmol) and T4 polynucleotide kinase end-labeled oligonucleotide containing NF-κB consensus sequence. Incubations were performed for 30 min at room temperature in binding buffer [4% (vol/vol) glycerol, 1 mM EDTA, 10 nM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM DTT, 0.1 mg/ml nuclease-free BSA, and 2 μg poly(dI-dC)]. Reaction mixture was electrophoresed on native 5% polyacrylamide gel and viewed by phosphorimaging (Storm 820 Scanner; Molecular Dynamics).

Western blotting. Cytoplasmic protein extracts (10 μg) were heated to 95°C in sample buffer [100 mM Tris (pH 6.8), 2% (wt/vol) SDS, 0.002% (wt/vol) bromophenol blue, 20% (vol/vol) glycerol, and 10% (vol/vol) β-mercaptoethanol] and separated by SDS-PAGE on 10% polyacrylamide separating gel overlaid with 4% stacking gel at 500 volts for 2 h. The separated proteins were transferred to a nitrocellulose membrane in transfer buffer [20 mM Tris, 150 mM glycine, 0.01% (wt/vol) SDS, and 20% (vol/vol) methanol] at 500 volts overnight. Membranes were incubated in blocking buffer [1× Dulbecco’s PBS containing 0.2% (wt/vol) I-block and 0.1% (vol/vol) Tween 20] for 1 h at room temperature and then incubated for 2 h in blocking buffer containing the anti-IκBβ antibody (1:1,000) or the anti-IκBα antibody (1:500). After six 5-min washes in washing buffer [1× PBS, pH 7.4, and 0.1% (vol/vol) Tween 20], membranes were incubated for 1 h in blocking buffer containing the appropriate goat anti-mouse (IκB) or goat anti-rabbit (IκBβ) IgG AP conjugate (1:7,500). Membranes were then washed six times for 5 min each and exposed to CDP Star light-sensitive film.

Transfection of IMR-32 neuronal cells with a dominant-negative MyD88 construct (MyD88Δ). Transfection of IMR-32 cells with MyD88Δ (500 ng) was performed with Transfast Transfection Reagent in a 1:3 ratio according to the manufacturer’s instructions. The pSV-β-galactosidase control vector was used as a control for monitoring the transfection efficiency. The expression of β-galactosidase was then assessed using the β-galactosidase enzyme assay with reporter lysis buffer, as described in the manufacturer’s protocol. IMR-32 cells were incubated in the transfection mixture for 1 h at 37°C. The IMR-32 cells were then supplemented with additional differentiation medium (2 ml/well) for 48 h at 37°C before use in coculture experiments with eosinophil membranes.

RT-PCR. After coculture with eosinophil membranes, for varying time periods, total RNA was extracted from the IMR-32 cells using TRI Reagent according to the manufacturer’s instructions. RNA concentrations for each sample were estimated using spectrophotometry. Extracted RNA (1 μg) from each sample was reverse transcribed into first-strand cDNA using the First Strand cDNA Synthesis Kit for RT-PCR, as described in the manufacturer’s protocol. Optimum concentrations of MgCl2 for the ICAM-1 and β-actin primers were determined by varying the concentration of this reagent. Subsequent PCRs were performed in a reaction mixture containing MgCl2, 200 μM dNTPs, 1 unit of Taq DNA polymerase, and the relevant forward and reverse primers in PCR reaction buffer. The amplification reaction was performed using the PTC-200 DNA Engine (MJ Research), and
tion, i.e., those occurring at or after 1 h, were inhibited (Fig. 1B). These data suggest that the early phase of NF-κB activation is not mediated by oxidative stress.

We have previously shown, using eosinophil membranes, that adhesion alone leads to induction of NF-κB (27). Membrane preparations allow for analysis of adhesion-induced changes in neuronal gene and protein expression without contribution from eosinophil products in these coculture experiments. Therefore, in all further experiments, membrane preparations were used.

Eosinophil coculture with IMR-32 cholinergic nerve cells results in activation of the IRAK-1 pathway in the nerves. Because ligation of leukocyte CD11b/18 results in activation of IRAK-1 and subsequently leads to NF-κB activation (25), we investigated if a similar pathway occurs in nerve cells after ICAM-1 ligation. Western blots of cytoplasmic protein from IMR-32 cells cocultured with eosinophil membranes were probed with an antibody to IRAK-1. Activation and degradation of IRAK-1 occurred within 5 min of coculture (Fig. 2A). IRAK-1 activation was not inhibited by pretreatment of the nerve cells with DPI (Fig. 2B).

To determine whether the adapter protein, MyD88, facilitated eosinophil-induced IRAK-1 activation, the nerve cells were transfected with a dominant-negative form of the adaptor protein MyD88 (MyD88ΔC), or pretreated with anti-CD11/18 antibody (0.1 μg/ml for 30 min; D) before coculture with eosinophil membranes. Shown are representative results from 3 independent experiments. *P = 0.05; **P = 0.001.

Adhesion-induced IRAK-1 activation in IMR-32 cholinergic nerve cells is tyrosine kinase and ERK1/2 dependent. Eosinophil adhesion to cholinergic nerve cells, via ICAM-1, results in the rapid activation of tyrosine kinases (12) and the MAP kinase, ERK1/2 (27). Pretreatment of the IMR-32 cells with the nonspecific tyrosine kinase inhibitor, genistein, prevented IRAK-1 activation throughout the time course (Fig. 3A). Similarly, the mitogen/extracellular adhesion molecule 1 inhibitor PD-98059, which blocks phosphorylation-induced ERK1/2 activation, prevented IRAK-1 activation throughout the time course (Fig. 3B). These data suggest that tyrosine kinase and ERK1/2 activation are critical upstream steps in the activation of the IRAK-1 pathway in nerve cells.

ERK1/2 and IRAK-1 coimmunoprecipitate with ligated ICAM-1. After coculture of IMR-32 cells with eosinophil membranes, the nerve cell lysates were immunoprecipitated with an anti-ICAM-1 antibody. Immunoblots were probed with an anti-IRAK-1 antibody. These experiments demonstrated that ICAM-1-mediated eosinophil adhesion to IMR-32 cells recruited IRAK-1 to ICAM-1, with maximal association at 2

Fig. 2. Eosinophil (eos) membranes induce interleukin-1 receptor-associated kinase-1 (IRAK-1) activation in IMR-32 cholinergic nerve cells, facilitated by the adaptor protein myeloid differentiation protein (MyD88), independently of the production of intraneuronal reactive oxygen species and via intercellular adhesion molecule (ICAM)-1. Western blots (WB) of IRAK-1 in cytoplasmic extract from differentiated IMR-32 cells (0.5 × 10⁶) were grown in differentiation medium for 7 days and then coincubated with eosinophil membranes (2 × 10⁵) for the indicated time periods. Differentiated IMR-32 cells were not pretreated (A), pretreated with DPI (1 μM for 1 h; B), transfected with a dominant-negative form of the adaptor protein MyD88 (MyD88ΔC), or pretreated with anti-CD11/18 antibody (0.1 μg/ml for 30 min; D) before coculture with eosinophil membranes. Shown are representative results from 3 independent experiments. *P = 0.05; **P = 0.001.

Fig. 3. Tyrosine kinase or extracellular signal-regulated kinase (ERK) 1/2 inhibition abrogates eosinophil membrane-induced activation of IRAK-1 in IMR-32 cholinergic nerve cells. Shown are Western blots of IRAK-1 in cytoplasmic extract from differentiated IMR-32 cells pretreated with the tyrosine kinase inhibitor genistein (50 μM; A) or mitogen/extracellular adhesion molecule (MEK) 1 inhibitor PD-98059 (50 μM for 120 min; B) and then coincubated with eosinophil membranes (2 × 10⁵) for the indicated time periods. Shown are representative results from 3 independent experiments.
min (Fig. 4A). Furthermore, stripping and reprobing with anti-phospho ERK antibody demonstrated that phosphorylated ERK1/2 also coprecipitated with ICAM-1 after adhesion (Fig. 4B). Finally, stripping and reprobing with anti-ICAM-1 antibody demonstrated equal loading and specificity for ICAM-1 (Fig. 4C).

Eosinophil adhesion to cholinergic nerves results in upregulation of nerve cell ICAM-1 surface expression that is dependent on IRAK-1-mediated NF-κB activation. We next assessed the effects of eosinophil adhesion on the expression of the NF-κB-regulated gene ICAM-1 (17). Coculture experiments were performed, and the expression of nerve cell ICAM-1 was examined using RT-PCR. Nerve cell ICAM-1 mRNA was detected as early as 4 h after coculture, with a more pronounced increase in expression seen at 8 h (Fig. 5).

To determine the contribution of the IRAK-1 pathway to eosinophil-induced NF-κB activation, we transfected IMR-32 cells with MyD88Δ and performed coculture experiments using eosinophil membranes. Western blots were prepared using nerve cell cytoplasmic protein and probed with an anti-IκBβ antibody. Degradation of IκBβ occurred in wild-type nerve cells cocultured with eosinophil membranes but was inhibited at the 2-min time point in nerve cells transfected with MyD88Δ (Fig. 5A).

Because ICAM-1 gene expression is mediated by NF-κB and was upregulated in this system (Fig. 5A), the role of the IRAK-1 pathway on surface expression of ICAM-1 on the nerve cell was also examined. After coculture experiments using eosinophil membranes and either wild-type or MyD88Δ-transfected nerve cells, ICAM-1 surface expression was measured by flow cytometry. These experiments revealed an ~1.8-fold increase in ICAM-1 expression at 8 h of coculture in wild-type nerve cells (Fig. 5C). In contrast, transfection of IMR-32 cells with MyD88Δ significantly reduced the eosinophil membrane-induced ICAM-1 surface expression (Fig 5C).

Fig. 5. Eosinophil membrane adhesion to IMR-32 cholinergic nerve cells induces upregulation of ICAM-1 gene and protein expression via IRAK-1. A: total RNA was extracted from differentiated IMR-32 nerve cell lysates after coculture with eosinophil membranes (2 × 10⁶) over a 0- to 8-h time course. RNA (1 μg) was converted to cDNA and RT-PCR carried out to determine levels of ICAM-1 mRNA expression at the indicated time points compared with the levels of the “housekeeping” gene β-actin. Shown are representative results from 4 independent experiments. B: eosinophil membrane-induced upregulation of neural ICAM-1 surface expression. WB of IκBβ in cytoplasmic extracts from differentiated IMR-32 cells that were transfected with MyD88Δ or left untreated before coculture with eosinophil membranes (2 × 10⁶) for the indicated time periods. Wild-type and MyD88Δ-transfected nerve cells were treated with fluorescein-labeled anti-ICAM-1 antibody, following eosinophil membrane (2 × 10⁶) coculture for 8 h, and surface expression of the adhesion molecule was assessed using flow cytometry. Data indicating percentage increase in ICAM-1 surface expression in wild-type and transfected cells, compared with that in untreated cells, represent means ± SD from 3 independent experiments (C).
DISCUSSION

The results of this study indicate that adhesion of eosinophils to nerve cells leads to the recruitment and phosphorylation of ERK1/2 and IRAK-1 via nerve cell ICAM-1 and to the induction of the NF-κB-dependent gene ICAM-1. Our previous work indicated that there was a significant interaction between eosinophils and nerve cells (11, 21, 27). In particular, we have shown that adhesion of eosinophils to nerve cells occurs through the adhesion molecules ICAM-1 and vascular cell adhesion molecule-1 and that this influences nerve growth (12), survival (Morgan RK, Kingham PJ, Walsh MT, Curran DC, Durcan N, McLean WG, and Costello RW, unpublished observation), and neurotransmitter release (22). The signaling events that occur within the nerves after contact with eosinophils are critical to understanding how these effects occur. We have previously shown that adhesion of eosinophils via ICAM-1 leads to both the activation of ERK1/2 and the generation of neuronal ROS and that these events are associated with activation of NF-κB in the nerves (11, 27). In this study, we have investigated the mechanisms of activation of NF-κB and some of the consequences of activation of this transcription factor. We focused specifically on the effects of eosinophil adhesion by using membrane preparations. Membrane preparations allowed for analysis of adhesion-induced changes in neuronal gene and protein expression without contribution from eosinophil products in these coculture experiments.

We have previously reported that ROS are generated via ICAM-1 in IMR-32 cells after coculture with eosinophils. Because in other cells ROS can activate both ERK1/2 (4) and NF-κB (20), the relative contribution of ROS to eosinophil-induced signaling in IMR-32 cells was studied. We showed that there were two temporally separate mechanisms for adhesion-induced NF-κB activation, an early ROS-independent and a later ROS-dependent pathway. The mechanism of this early ROS-independent NF-κB activation was then investigated. It has recently been described that, in monocytes, NF-κB activation after CD11b/18 binding occurs via IRAK-1 activation (25). We hypothesized that a similar process may also occur after binding to ICAM-1, whereby the MyD88 adapter protein is recruited, leading to activation of IRAK-1 and subsequently NF-κB. We demonstrated that IRAK-1 activation occurred after 2–10 min of coculture with eosinophil membranes. IRAK-1 activation was dependent on the adapter protein MyD88 and inhibited by preventing eosinophil adhesion via ICAM-1. The intracellular domain of ICAM-1 does not possess a TIR domain, which in Toll receptors is critical for IRAK-1 activation. However, ICAM-1 ligation results both in a tyrosine kinase-dependent phosphorylation of a number of proteins and in MAP kinase activation (29), which we have also shown in IMR-32 nerve cells (12, 27). Thus we investigated if IRAK-1 activation resulted from a tyrosine kinase- or MAP kinase-dependent pathway. After pretreatment of IMR-32 cells with the tyrosine kinase inhibitor genistein or the ERK1/2 inhibitor PD-98509, there was no adhesion-induced IRAK-1 activation. In addition, immunoprecipitation experiments demonstrated that, after eosinophil adhesion, ICAM-1 and both IRAK-1 and the MAP kinase ERK coimmunoprecipitated.

We next sought to investigate whether IRAK-1 activation was associated with NF-κB activation. Our studies showed that transfection of IMR-32 cells with a dominant-negative mutant of the adapter protein MyD88 prevented degradation of the NF-κB inhibitory protein, IkBβ. We then investigated the functional consequences of these events by studying the expression of the NF-κB-dependent gene ICAM-1 (17). These studies showed that eosinophil adhesion resulted in the upregulation of the ICAM-1 gene. Furthermore, ICAM-1 protein expression was dependent on the IRAK-1 pathway, since it was inhibited by transfection of IMR-32 cells with the dominant-negative mutant of MyD88 before coculture with eosinophil membranes.

Thus the consequence of eosinophil binding via ICAM-1 in the nerve cells is an ERK1/2- and tyrosine kinase-dependent activation of IRAK-1, which in turn leads to NF-κB activation. This is associated with a subsequent increase in nerve cell ICAM-1 expression. Thus eosinophil adhesion to nerve cell ICAM-1 may lead to a self-perpetuating cycle that augments eosinophilic recruitment to nerves.

GRANTS

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REFERENCES


