Neutrophil Elastase Acts as a Biased Agonist for Proteinase-activated Receptor-2 (PAR2)*

Received for publication, November 19, 2010, and in revised form, May 13, 2011 Published, JBC Papers in Press, May 16, 2011, DOI 10.1074/jbc.M110.201988

Rithwik Ramachandran†§‡, Koichiro Miha†, Hyunjae Chung‡¶, Bernard Renaux†challenge, Chang S. Lau§, Daniel A. Muruve†, Kathryn A. DeFea§, Michel Bouvier‡, and Morley D. Hollenberg†

From the †Department of Physiology and Pharmacology and ‡Department of Medicine, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 4N1, Canada, the §Division of Biomedical Sciences and Biochemistry and Molecular Biology, University of California, Riverside, California 92521, and the ¶Department of Biochemistry, Institute for Research in Immunology and Cancer, Groupe de Recherche Universitaire sur le Médicament, Université de Montréal, CP 6128 Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada

Human neutrophil proteinases (elastase, proteinase-3, and cathepsin-G) are released at sites of acute inflammation. We hypothesized that these inflammation-associated proteinases can affect cell signaling by targeting proteinase-activated receptor-2 (PAR2). The PAR family of G protein-coupled receptors is triggered by a unique mechanism involving the proteolytic unmasking of an N-terminal self-activating tethered ligand (TL). Proteinases can either activate PAR signaling by unmasking the TL sequence or disarm the receptor for subsequent enzyme activation by cleaving downstream from the TL sequence. We found that none of neutrophil elastase, cathepsin-G, and proteinase-3 can activate Gq-coupled PAR2 calcium signaling; but all of these proteinases can disarm PAR2, releasing the N-terminal TL sequence, thereby preventing Gq-coupled PAR2 signaling by trypsin. Interestingly, elastase (but neither cathepsin-G nor proteinase-3) causes a TL-independent PAR2-mediated activation of MAPK that, unlike the canonical trypsin activation, does not involve either receptor internalization or recruitment of β-arrestin. Cleavage of synthetic peptides derived from the extracellular N terminus of PAR2, downstream of the TL sequence, demonstrated distinct proteolytic sites for all three neutrophil-derived enzymes. We conclude that in inflammation, neutrophil proteinases can modulate PAR2 signaling by preventing/disarming the Gq/calcium signal pathway and, via elastase, can selectively activate the p44/42 MAPK pathway. Our data illustrate a new mode of PAR regulation that involves biased PAR2 signaling by neutrophil elastase and a disarming/silencing effect of cathepsin-G and proteinase-3.

Although one main neutrophil function is to target invading microorganisms and to respond to damaged tissue, there are many host cell responses triggered by the released neutrophil serine proteinases. In inflammation, apart from their bactericidal actions, the serine proteinases trigger a variety of effects ranging from cytokine, kinin, and growth factor generation to the clustering of integrins (1). PARs belong to a novel four-member family of G-protein-coupled receptors that are proteolytically activated by the unmasking of an N-terminal tethered ligand (TL) sequence by serine proteinases (2–4). Of note, many proteinases can silence/disarm PARs by cleaving downstream of the TL sequence, so as to prevent further activation by proteinases, but not by synthetic PAR-activating peptides with sequences derived from the TL (2, 5). In previous work, we found that neutrophil elastase (NE) and cathepsin-G (CG) can block Gq-mediated calcium signaling by trypsin-activated proteinase-activated receptor-2 (PAR2), presumably by removing the enzyme-targeted tethered ligand sequence (6, 7). PAR2 activation by serine proteinases is widely considered to cause a proinflammatory response in various pathological conditions, in part via a neurogenic mechanism, and thus its disarming by neutrophil proteinases could be considered as an anti-inflammatory effect. There are also intriguing data that assign a protective function to PAR2 activation in certain inflammatory conditions (8–11). The mechanism(s) for this potentially biphasic action of PAR2 is not known, but it may relate to the ability of PAR2 to exhibit biased signaling either via a Gq-coupled calcium signal or via G12/13-coupled p44/42 MAPK signals (12). Our recent report showed that proteolytically revealed mutated rat TL sequences and one of the PAR2-activating peptides we tested were able to trigger such biased agonism, activating MAPK but not calcium signaling pathways, without either receptor internalization or the recruitment of arrestins (12). Evidence for biased signaling by PAR2 also comes from the observation that a novel peptidomimetic PAR2 antagonist could inhibit intracellular calcium release induced by a PAR2 agonist but failed to inhibit ERK signaling (13). It is, however, not known if endogenous agonists can trigger biased sig-

*This work was supported by grants from the Canadian Institutes of Health Research (to M. D. and M. B.). This work was also supported in part by National Institutes of Health Grant RO1GM066151 (to K. D.).

† An Alberta Innovates Health Solutions/Alberta Heritage Foundation for Medical Research (AI-HS/AFHMR) postdoctoral fellow. To whom correspondence should be addressed: Dept. of Physiology and Pharmacology, Faculty of Medicine, University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta T2N4N1, Canada. Tel.: 403-220-3663; Fax: 403-270-0979; E-mail: rramacha@ucalgary.ca.

‡ Supported by a CH/FOMD University of Alberta Emerging Teams Grant graduate scholarship.

The abbreviations used are: PAR, proteinase-activated receptor; TL, tethered ligand; NE, neutrophil elastase; CG, cathepsin-G; PR3, proteinase-3; HPT, human proximal tubular; MEF, mouse embryobiofibrostain; BRET, bioluminescence resonance energy transfer; BAB, biarsenical fluorochrome binding domain; AMC, 7-amido-4-methylcoumarin.

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VOLUME 286 • NUMBER 28 • JULY 15, 2011
 signaling through PAR2. In trying to uncover if such biased signaling might occur in vivo, we hypothesized that neutrophil proteinases that can cleave the PAR2 N terminus but do not trigger PAR2 calcium signaling might nonetheless activate MAPK to act as endogenous biased agonists for PAR2. We show here that neutrophil proteinases (NE, CG, and proteinase-3 (PR3)) can all disarm PAR2-dependent calcium responses to trypsin, whereas NE (but neither CG nor PR3) concomitantly activates MAPK signaling. We show further that all three neutrophil proteinases can target the PAR2 N terminus to remove the TL but via cleavage at distinct cleavage sites. We propose that under inflammatory conditions, this novel TL-independent signaling via PAR2, biased toward the MAPK pathway, may underlie differential PAR2-dependent responses in inflammation.

**EXPERIMENTAL PROCEDURES**

*Chemicals and Other Reagents—*Culture medium (DMEM and DMEM/F-12) and fetal bovine serum (FBS) were from Invitrogen. Fluo-4/AM no wash calcium indicator dye and the ReASH fluorescent probe were from Invitrogen. Fugene-6 transfection reagent was obtained from Roche Applied Science. Calcium ionophore A23187, porcine trypsin (catalogue no. T-7418; Calcium ionophore A23187, porcine trypsin (catalogue no. T-7418; Sigma. A maximum specific activity of 20,000 units/mg was used to calculate the approximate molar concentration of trypsin in the incubation medium (1 unit/ml, 1 units/ml for NE and PR3) can all disarm PAR2-dependent calcium responses to trypsin, whereas NE (but neither CG nor PR3) concomitantly activates MAPK signaling. We propose that under inflammatory conditions, this novel TL-independent signaling via PAR2, biased toward the MAPK pathway, may underlie differential PAR2-dependent responses in inflammation.

**Measurement of Trypsin and NE Activity**—NE activity was measured using the substrate methyl-O-succinyl-Ala-Ala-Pro-Val-AMC (elastase substrate V, EMD Chemicals), 50 μl of 200 milliliters/ml elastase in Hanks’ balanced salt solution containing 10 mM HEPES, pH 7.0, 1.5 mM MgCl2, 1.5 mM CaCl2, and 0.2% Nonidet P-40 was incubated with 50 μl of 200 μM elastase-specific substrate methyl-O-succinyl-Ala-Ala-Pro-Val-AMC (elastase substrate V, EMD Chemicals). The fluorescence from the free AMC as an index of proteolytic activity was measured using the substrate methyl-O-succinyl-Ala-Ala-Pro-Val-AMC (elastase substrate V, EMD Chemicals), 10 mM EDTA and subcultured as appropriate. Mouse embryonic fibroblasts from wild type (MEFwt) and β-arrestin-1/2 double knock-out animals (MEFβarrDKO) were a gift from Dr. Robert Lefkowitz and have been described previously (14, 15).

Primary human proximal tubular (HPT) cells were isolated as described before (16). Briefly, normal renal cortical tissue obtained from an adult kidney undergoing nephrectomy was dissected, minced, digested with collagenase IV, and passed through a 75-μm mesh. Filtered cells were washed in Hanks’ balanced salt solution and resuspended in hormonally defined medium and plated in a Petri dish. After 30 min, the non-adherent HPT cells were transferred to a collagen-coated plate and cultured in DMEM/F-12 (Invitrogen) supplemented with 1% FBS, 1% penicillin/streptomycin, 125 ng/ml prostaglandin E1, 25 ng/ml epidermal growth factor, 1.8 μg/ml l-thyroxine, 3.38 ng/ml hydrocortisone, and 2.5 mg/ml insulin/transferrin/sodium selenite supplement. Experiments involving the use of human tissue were approved by the Conjoint Health Research Ethics Committee at the University of Calgary.

**Calcium Signaling**—KNRK cells or HPT cells were incubated for 30 min with Fluo-4/AM no wash calcium indicator and assayed for calcium signaling responses as described previously (12) on a vior X4 fluorescent plate reader (PerkinElmer Life Sciences) or an Aminco Bowman fluorescence spectrophotometer (Thermo Life Science). Receptor disarming was monitored essentially as described previously (18), with minor modifications. Briefly, PAR2-expressing KNRK cells or HPT cells
Neutrophil Proteinase Cleavage of Synthetic PAR2-derived Peptides—Cleavage of the PAR2-derived synthetic peptides spanning the TL regions (human PAR sequences; NATLDPRSFLLRNPNNDKYE (PAR1), GTNRSSKGKSLIGKVDGTSHV (PAR2), and GDDSTPSILPAPRPGYQV (PAR4)) or encompassing the region downstream of the PAR2 TL region (rat PAR2 sequence ITGKGAPVEGFVSDFASVLTGKL, highly homologous with the comparable human sequence, VTGKYTVEFVSVDFASVLTGKL) was done as described previously for trypsin IV and tissue kallikreins (20, 21). Peptides (100 μM in a total volume of 150 μl) were incubated with neutrophil proteinases (0.7 unit/ml) for 15 min at 37 °C. Reactions were stopped by adding 150 μl of ice-cold 0.1% tri-fluoroacetic acid (TFA) in water. Samples were fractionated by reverse-phase high performance liquid chromatography (5–40% acetonitrile gradient in 0.1% TFA over 30 min at a flow rate of 1 ml/min), and eluted peptides were analyzed by MALDI mass spectrometry. Neutrophil proteinase cleavage of peptides spanning the TL regions of PAR1 (NATLDPRSFLLRNPNNDKYE) and PAR4 (GDDSTPSILPAPRPGYQV) were also assessed as described above for the PAR2 peptides.

**BRET-based Detection of β-Arrestin-1 Interaction with PAR2**—PAR2 was conjugated with YFP at the C terminus (PAR2-YFP), and BRET to Renilla luciferase-tagged β-arrestin-1 (Rluc-β-ar1) was determined essentially as described previously (22). HEK-293 cells were used instead of the KNRK cell line, because the HEK cells provide for a much greater transfection efficiency that is required for the BRET studies. In brief, HEK-293 cells were transiently transfected with 1 μg of PAR2-YFP construct along with 0.1 μg of the Rluc-β-ar1 construct. Cells were plated in white 96-well culture plates (PerkinElmer Life Sciences), and interactions between the receptors and β-arrestin-1 were detected by measuring BRET at timed intervals over 60 min following the addition of 5 μM coelenterazine (Nanolight Technology, Pinetop, AZ) on a Mithras fluorescence plate reader (Berthold, Mandel Scientific (Guelph, Canada)) in luminescence mode using the appropriate filters.

**Monitoring PAR2 Internalization**—Using the approach described in more detail elsewhere (12), HEK cells were plated on glass bottom Petri dishes (MatTek Corp., Ashland, MA) and transiently transfected with 1 μg of PAR2-YFP construct. After 48 h, the medium in the Petri dish was replaced with serum-free medium, and the cells were treated with trypsin (10 nm), SLIGRL-NH2 (10 μM), or NE (3 units/ml) for 30 min at 37 °C. After fixing cells with 4% formaldehyde, the cellular localization of the receptor tag YFP signal was detected using an Olympus Fluoview system software. Internalization of cell surface receptor was quantified morphometrically by counting the number of intracellular fluorescent speckles per cell in the images, indicative of receptor internalization to endocytic vesicles. Speckles in all cells were counted in a randomly selected representative ×40 image. The average number of speckles per cell was calculated, and the observation was repeated for comparable fields for cells observed in three independently conducted experiments.
substrate representing the rat PAR2 sequence downstream of neutrophil proteinases, we used a synthetic 27-mer peptide and as shown in Fig. 1 did not affect either its ability to cause calcium signaling or to preincubating trypsin with NE (as opposed to its target cells) trypsin to activate PAR2 was not due to neutrophil proteinase cleavage was verified by detection of the fluorescently labeled chromosome binding motif in the cell supernatant after enzyme unavailable for trypsin-mediated unmasking. This N-terminal domain of PAR2 from the cell surface (Fig. 2), making the TL unavailable for trypsin-mediated unmasking. This N-terminal cleavage was verified by detection of the fluorescently labeled receptor N-terminal fragment containing a biarsenical fluorochrome binding motif in the cell supernatant after enzyme treatment. To assess similarities or differences in the cleavage of the PAR2 N-terminal extracellular sequence by the three neutrophil proteinases, we used a synthetic 27-mer peptide substrate representing the rat PAR2 sequence downstream of the TL and just C-terminal to a series of three prolines that would segregate this sequence from the upstream TL moiety (Fig. 3). HPLC separation and mass spectral identification of the cleavage products showed that each of the enzymes cleaved at a distinct site in this peptide with predominant cleavage observed: at Ser68-Val69 for NE, at Phe65-Ser66 for CG, and at Val62-Asp63 for PR3 (Fig. 3).

**RESULTS**

Neutrophil Proteinases Target Distinct Cleavage Sites and Disarm PAR2 by Cleaving the Receptor N Terminus Downstream of the TL Sequence—As we anticipated from our previous work (6, 7) and as shown in Fig. 1A (representative data for NE) and Fig. 1B, pretreatment of PAR2-expressing cells with NE, CG, or PR3 abrogated subsequent trypsin-mediated calcium signaling, without affecting the response to the PAR-activating peptide, SLIGRL-NH2. The abrogation of the ability of trypsin to activate PAR2 was not due to neutrophil proteinase digestion of trypsin itself as confirmed by experiments where preincubating trypsin with NE (as opposed to its target cells) did not affect either its ability to cause calcium signaling or to cleave a fluorogenic substrate (supplemental Fig. S2). This loss of PAR2 sensitivity to trypsin activation upon pretreating cells with the neutrophil proteinases can be explained by the ability of all three enzymes to cleave and release the N-terminal domain of PAR2 from the cell surface (Fig. 2), making the TL unavailable for trypsin-mediated unmasking. This N-terminal cleavage was verified by detection of the fluorescently labeled receptor N-terminal fragment containing a biarsenical fluorochrome binding motif in the cell supernatant after enzyme treatment. To assess similarities or differences in the cleavage of the PAR2 N-terminal extracellular sequence by the three neutrophil proteinases, we used a synthetic 27-mer peptide substrate representing the rat PAR2 sequence downstream of the TL and just C-terminal to a series of three prolines that would segregate this sequence from the upstream TL moiety (Fig. 3). HPLC separation and mass spectral identification of the cleavage products showed that each of the enzymes cleaved at a distinct site in this peptide with predominant cleavage observed: at Ser68-Val69 for NE, at Phe65-Ser66 for CG, and at Val62-Asp63 for PR3 (Fig. 3).

In contrast with the cleavage of the PAR2 27-mer that represents a PAR2 sequence downstream from the TL, we failed to see any cleavage of the peptide corresponding to the human PAR2 N-terminal region spanning the TL cleavage activation site, GTNRSSKGK ↓ SLIGKVDGTSHV (where the downward arrow denotes the cleavage site that unmasks the TL) (data not shown). These studies indicated that the neutrophil-derived proteinases are unable to reveal the PAR2-activating TL and agree completely with previous proteomic work demonstrating the inability of neutrophil proteinases to cleave sequences representing the TL of PAR2 (23). Thus, neutrophil enzymes disarm PAR2 by removing the N-terminal TL receptor-activating sequence, whereas the retention of calcium signaling in response to SLIGRL-NH2 after treatment with neutrophil proteinases showed that the extracellular receptor loops involved in signaling remained functional.

**NE Activates PAR2-dependent p44/42 MAPK Signaling, whereas CG and PR3 Do Not**—Because we previously observed that PAR2 can selectively couple to the p44/42 MAPK pathway without triggering calcium release (12), we wondered if the neutrophil proteinases might act as endogenous biased agonists at PAR2, selectively activating MAPK but not calcium transients. Following activation of KNRK cells stably expressing rat-PAR2 (KNRK-PAR2) with NE for 10 min (but not with either CG or PR3) we indeed observed a robust p44/42 MAPK signaling with an EC50 for NE of about 0.7 unit/ml (Fig. 4). This response was not observed in empty vector-transfected KNRK cells (Fig. 4C).
Despite being able to cleave the PAR₂ N terminus to prevent trypsin signaling, neither CG nor PR3 was able to activate MAPK signaling in PAR₂-transfected KNRK cells (Fig. 4A).

Given that short synthetic peptides corresponding to the trypsin-revealed N terminus of PAR₂ can activate the receptor, we synthesized peptides derived from the N-terminal sequence...
of PAR$_2$ exposed by CG (SASVLTGKTLTTVFL-NH$_2$) and NE (VLTGKTLTTVFL-NH$_2$) (Fig. 3). In contrast with the trypsin-revealed TL-derived peptide (SLIGRL-NH$_2$), the NE/CG-revealed TL peptides (10–100 U/ml) were unable to trigger either a calcium signal or MAPK signal in PAR$_2$-transfected KNRK cells (supplemental Fig. S1).

**NE Disarms Calcium Signaling and Activated MAPK Signaling in HPT Cells That Endogenously Express PAR$_2$**—In order to confirm the existence of NE-dependent biased signaling in cells that endogenously express PAR$_2$, we have examined the effect of NE on calcium and MAPK signaling in HPT cells that naturally express high levels of PAR2 (24). Treatment of HPT cells with NE did not stimulate a calcium signal but significantly attenuated subsequent trypsin-stimulated calcium transients (Fig. 5, A and B). In contrast, treatment of HPT cells with NE for 5 min stimulated robust p44/42 MAPK signaling that declined but remained higher than base line at 15 and 30 min post-treatment (Fig. 5, C and D).

**Rho-kinase Inhibitors and Elafin Block NE-activated PAR$_2$-dependent p44/42 MAPK Signaling**—The NE-stimulated MAPK activation was attenuated by Rho-kinase inhibitors (Fig.

**FIGURE 4.** NE activates PAR$_2$-dependent p44/42 MAPK signaling, whereas CG and PR3 do not. Rat PAR$_2$-expressing KNRK cells or empty vector-expressing cells (pcDNA3-KNRK) were serum-starved and stimulated with either trypsin, SLIGRL-NH$_2$, NE, CG, or PR3 for 10 min. Samples were resolved on SDS-polyacrylamide gels, and activation of p44/42 MAPK was monitored by immunoblotting with phospho-p44/42-specific antibodies. A, representative image showing activation of p44/42 MAPK by NE but not by CG and PR3 in rPAR$_2$-KNRK cells. B, histogram showing densitometry analysis of multiple scanned Western blot images to quantify the percentage increase over base line of p44/42 activation by the different proteinases in rPAR$_2$-KNRK cells. C, representative image showing no activation of p44/42 MAPK by NE in pcDNA3-KNRK cells. D, histogram showing densitometry analysis of multiple scanned Western blot images to quantify the percentage increase over base line of p44/42 activation by the different proteinases in pcDNA3-KNRK cells. E, representative image showing activation of p44/42 MAPK in PAR$_2$-KNRK cells in response to different concentrations (0.75–3 units/ml) of NE. F, histogram showing densitometry analysis of multiple scanned Western blot images to quantify the percentage increase over base line of p44/42 activation by increasing concentrations of NE. Increases in p44/42 MAPK phosphorylation were quantified relative to the β-actin or total p44/42 signal detected in the same samples (mean ± S.E. (error bars), n = 3; *, p < 0.05). NT, no treatment.
In order to confirm the specificity of NE-stimulated MAPK activation, we monitored the ability of NE to activate MAPK following incubation with the specific NE inhibitor elafin (supplemental Fig. S3). We found that the NE-stimulated MAPK activation was completely inhibited by 10 μM elafin and that this inhibition was dependent on the elafin concentration, with less inhibition at lower concentrations (Fig. 7).

**NE-cleaved PAR₂ Does Not Recruit β-Arrestin and Is Retained on the Cell Surface**—PAR₂ activation by trypsin is followed by rapid receptor phosphorylation, recruitment of β-arrestin to the receptor, and subsequent internalization and targeting of the activated PAR₂ either to lysosomes for degradation or to an intracellular scaffold for further signaling via MAPK (12, 14, 25). A lack of β-arrestin recruitment would be expected to result in a different signaling response and differences in receptor trafficking compared with trypsin-activated PAR₂. In order to determine if NE-cleaved PAR₂ interacts with β-arrestin, we transfected cells expressing YFP-tagged PAR₂ along with the RLuc-β-ar11 and employed an energy transfer (BRET) assay to detect receptor β-arrestin-1 interactions after PAR₂ activation. Trypsin and PAR₂-activating peptide (2f-LIGRLO-NH₂) stimulation of PAR₂-expressing cells resulted in a substantial increase in the BRET ratio, indicating a recruitment of β-arrestin-1 to the activated receptor (Fig. 8A). However, following activation of PAR₂ with NE, we failed to observe any increase in the BRET ratio (Fig. 8A). Similar data were

**FIGURE 5. NE disarms calcium signaling and activated p44/42 MAPK in HPT cells.** HPT cells were incubated with the Fluo-4 no wash calcium indicator for 30 min, and trypsin-stimulated calcium signaling was monitored in cells that were preincubated or not with 3 units/ml NE for 20 min. A, top, representative trace showing trypsin signaling in HPT cells. Bottom, representative trace showing the lack of NE-stimulated calcium signaling in HPT cells and the subsequent attenuation of trypsin triggered calcium signaling in these cells (// indicates 20 min). The PAR₂-AP SLIGRL-NH₂ is still able to stimulate calcium signaling in cells that are disarmed by NE (mean ± S.E. (error bars), n = 3; *, significant decrease in trypsin-stimulated calcium signal in HPT cells that were exposed to NE compared with cells that were not preincubated with NE; p < 0.05). B, histogram showing trypsin-stimulated calcium signaling in HPT cells that were preincubated or not with 3 units/ml NE as a percentage of calcium stimulated by the calcium ionophore A23187 (mean ± S.E., n = 3). *, significant increase in p44/42 MAPK signal compared with no treatment (NT) (p < 0.05). C, representative image showing activation of p44/42 MAPK by SLIGRL-NH₂, trypsin, and NE in HPT cells. D, histogram showing densitometry analysis of multiple scanned Western blot images to quantify the percentage increase over base line of p44/42 activation by SLIGRL-NH₂, trypsin, and NE in HPT cells (mean ± S.E., n = 3). *, significant increase in P44/42 MAPK signal compared with the NT (p < 0.05).
obtained when monitoring recruitment of β-arrestin-2 (data not shown). The lack of β-arrestin recruitment to NE-activated PAR2 correlated with a lack of internalization of the receptor in cells that had been activated with NE (Fig. 9). As seen for NE, neither CG nor PR3 caused receptor internalization (Fig. 9, lower panels). In contrast, activation of cells with either trypsin or SLIGRL-NH₂ resulted in loss of cell surface receptor expression and increases in intracellular PAR2-YFP levels in endosome-like structures (Fig. 9, upper panels).

To determine if NE-stimulated p44/42 MAPK activation was independent of β-arrestins, we further examined p44/42 MAPK phosphorylation in embryonic fibroblasts from wild type (MEFwt) and PAR2-null (MEF/βarrDKO) mice. Trypsin, 2f-LIGRLO-NH₂, and NE all promoted increased phosphorylation of p44/42 MAPK (14-, 5-, and 6-fold over untreated, respectively) in MEFwt. However, in MEF/βarrDKO, whereas trypsin- and 2f-LIGRLO-NH₂-stimulated p44/42 MAPK phosphorylation was reduced by 90 and 80%, relative to MEFwt, NE-stimulated p44/42 MAPK activation in MEF/βarrDKO was not significantly different from that observed in MEFwt (Fig. 8, B and C). Thus, NE-stimulated PAR2 signaling to MAPK, in contrast with trypsin and 2f-LIGRLO-NH₂, appears to be independent of β-arrestins.

DISCUSSION

The discovery that serine proteinases can signal to cells by cleaving and activating PARs via a unique tethered ligand mechanism established a novel paradigm for inflammatory cell regulation (26, 27). Our new work described here demonstrates for the first time that neutrophil elastase, an endogenously expressed proteinase, can act as a biased agonist at PAR2. In an inflamed tissue, increased neutrophil infiltration and release of neutrophil serine proteinases will probably shift the balance of PAR2 signaling toward this newly identified signaling mechanism and could have important implications for our understanding of how these receptors regulate inflammation.

Our new data broaden considerably, well beyond the trypsin-related serine proteinase family, the spectrum of proteinases that can cause tissue responses via the PARs, including the possibility of PAR2 biased signaling (MAPK versus calcium), demonstrated here for elastase but not for the other two neutrophil proteinases. Previous work demonstrating the disarming of PAR2 by neutrophil and Pseudomonas elastases (6, 28) considered only the silencing of the calcium signal and did not recog-
nize the potential impact on MAPK activation that our new observations reveal. Interestingly, the mechanism of MAPK activation also appears to be distinct from the trypsin-triggered response. We observe that the elastase-activated receptor is impaired in its ability to internalize and fails to interact with \(\beta\)-arrestin.

\(\beta\)-Arrestin interaction is known to be important for PAR2 internalization (29). Further, \(\beta\)-arrestin scaffolds the internalized receptor to Raf-1 and activated ERK-MAPK and ensures cytosolic retention of the activated ERK (30), whereas ERK-MAPK activation by PAR2 receptors that are unable to interact with \(\beta\)-arrestin results in the nuclear translocation of activated ERK. These differences in localization of signaling complexes would suggest that the MAPK signaling pathway triggered by NE, which is independent of \(\beta\)-arrestin interaction, would result in ERK activation of nuclear transcription, whereas trypsin-activated PAR2 ERK-MAPK signaling would target non-nuclear substrates. This possible difference in MAPK signaling will probably impact on the inflammatory response that PAR2 directs. The inhibition of the NE-triggered MAPK signal by inhibitors of Rho-associated kinase indicate that this response is occurring through the coupling of the receptor to the G\(_{12/13}\) (31, 32), but the implications of selectively activating this arm of PAR2 signaling by NE remain to be fully understood.

Although a number of studies have described the ability of neutrophil-derived enzymes to inactivate PAR2-dependent cal-

![Image](imageLink)
cium signaling (6, 7), few studies have described the ability of these enzymes to modulate cellular responses through this receptor. A recent study examining the regulation of epithelial barrier permeability has shown that NE and PR3 activation of PAR$_1$ (and PAR$_3$) on the basolateral aspect of colonic epithelial cells increases barrier permeability (33). The authors proposed that this effect was a mechanism for allowing increased neutrophil transepithelial migration. More recently, in an in vivo mouse model of colitis, it was shown that inhibition of NE by elafin significantly suppresses inflammatory mediators and strengthens the intestinal epithelial barrier functions in colonic tissues from mice as well as in intestinal epithelial cells (34). Given that PAR$_2$ is believed to be a key player in the pathogenesis of colitis (35, 36), it will be interesting to evaluate further the signaling responses and trafficking of the receptor in the colonic epithelial cells to assess the possible relationship to data in this report. Similarly our data in kidney proximal tubular cells, which express an abundance of PAR$_2$, demonstrate that neutrophil infiltration, which is a hallmark of renal inflammation, will shift the balance of signaling via this receptor. This NE-mediated impact on tubular cell signaling in the kidney may trigger an epithelial to mesenchymal transition so as to play a role in renal fibrosis.

The cleavage of the PAR$_2$ N terminus downstream of the TL-revealing site by the neutrophil proteinases can also be compared with our preliminary evaluation of their cleavage of the human PAR$_1$$^*$, PAR$_2$$^*$, and PAR$_4$$^*$-derived sequences spanning the TL domains (supplemental Table 1). NE and PR3 were able to cleave the TL-spanning peptide of PAR$_1$ at sites that would prevent subsequent activation by thrombin, and thus this novel signaling mechanism that we have uncovered for PAR$_2$ may reflect a general mechanism for PAR regulation by neutrophil proteinases. There is some evidence to suggest that PAR$_2$ can exhibit biased agonism. For instance, elastase, which we show cannot unmask the PAR$_2$ TL sequence in vitro, is nonetheless able to induce apoptosis in human lung epithelial cells via a process that appears to be PAR$_2$-mediated (37, 38). Further, platelet PAR$_1$ was recently reported to be cleaved by the metalloproteinase MMP-1 to reveal a “non-canonical” receptor-activating tethered ligand that is distinct from that revealed by thrombin (39). The MMP-1-dependent activation of PAR$_2$ was shown to bias signaling toward the Rho-GTP and MAPK pathways. In addition, activated protein C has been shown to cause a PAR$_2$-mediated increase in endothelial barrier integrity, whereas thrombin activation of PAR$_2$ decreases endothelial barrier function (40, 41). Functional selectivity of the coupling of PAR$_2$ to different G-protein-mediated responses, with differences in coupling between the receptor activation by thrombin and agonist peptide activation of the receptor, has also been reported (42). Whether neutrophil proteinase-mediated disarming of either PAR$_1$ or PAR$_3$ triggers biased signaling by these receptors, as we describe here for PAR$_2$, is currently unknown. Nonetheless, the influx of inflammatory neutrophils in response to injury or infection, via the actions of elastase, proteinase-3, and cathepsin-G acting on the PAR$_2$s, could in principle stimulate a complex set of responses by activating PAR$_2$ (cathepsin-G) (7, 43), disarming PAR$_1$ and PAR$_2$ (cathepsin-G and proteinase-3) (7, 23), and selectively activating a unique arm of the PAR$_2$ MAPK signaling pathway (elastase). In summary, we identify in neutrophil elastase an endogenously expressed biased enzyme agonist for PAR$_2$. We also propose that proteinase-triggered biased signaling via PAR$_2$ shown here for the first time for neutrophil elastase, may underpin the distinct responses stimulated by PAR$_2$ in inflammatory settings.

Acknowledgments—We thank the live cell imaging core and the molecular instrumentation core laboratories of the Snyder Institute of Infection, Immunity, and Inflammation at the University of Calgary. We thank Dr. Gerald Zamponi for access to the Mithras LB940 plate reader for performing the BRET experiments and Dr. Ed Conway for critically reading the manuscript. We also thank Dr. Wenjie Wang for help with isolating human proximal tubular cells.

REFERENCES

1. Meyer-Hoffert, U. (2009) Front. Biosci. 14, 3409–3418
2. Ramachandran, R., and Hollenberg, M. D. (2008) Br. J. Pharmacol. 153, Suppl. 1, S263–S282
3. Coughlin, S. R. (2005) J. Thromb. Haemost. 3, 1800–1814
4. Adams, M. N., Ramachandran, R., Yau, M. K., Suen, J. Y., Fairlie, D. P., Hollenberg, M. D., and Hooper, J. D. (2011) Pharmacol. Ther. 130, 248–282
5. Hollenberg, M. D., and Compton, S. J. (2002) Pharmacol. Rev. 54, 203–217
6. Dulon, S., Candé, C., Bunnent, N. W., Hollenberg, M. D., Chignard, M., and Pidard, D. (2003) Am. J. Respir. Cell Mol. Biol. 28, 339–346
7. Ramachandran, R., Sadofsky, L. R., Xiao, Y., Botham, A., Cowen, M., Morice, A. H., and Compton, S. J. (2007) Am. J. Physiol. Lung Cell Mol. Physiol. 292, L788–L798
8. Laukkarinen, J. M., Weiss, E. R., van Acker, G. J., Steer, M. L., and Perides, G. (2008) J. Biol. Chem. 283, 20703–20712
9. Singh, V. P., Bhagat, L., Navina, S., Sharif, R., Dawra, R. K., and Saluja, A. K. (2007) Gut 56, 958–964
10. Fiorucci, S., Menecarelli, A., Palazetti, B., Distritti, E., Vergnolle, N., Hollenberg, M. D., Wallace, J. L., Morelli, A., and Cirino, G. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 13936–13941
11. Cocks, T. M., Fong, B., Chow, J. M., Anderson, G. P., Frauman, A. G., Goldie, R. G., Henry, P. J., Carr, M. J., Hamilton, J. R., and Moffatt, J. D. (1999) Nature 398, 156–160
12. Ramachandran, R., Mihara, K., Mathur, M., Rochdi, M. D., Bouvier, M., Defea, K., and Hollenberg, M. D. (2009) Mol. Pharmacol. 76, 791–801
13. Goh, F. G., Ng, P. Y., Nilsson, M., Kanke, T., and Plevin, R. (2009) Br. J. Pharmacol. 158, 1695–1704
14. Kumar, P., Lau, C. S., Mathur, M., Wang, P., and DeFea, K. A. (2007) Am. J. Physiol. Cell Physiol. 293, C346–C357
15. Stalheim, L., Ding, Y., Gullapalli, A., Paing, M. M., Wolfe, B. L., Morris, D. R., and Trejo, J. (2005) Mol. Pharmacol. 67, 78–87
16. White, L. R., Blanchette, J. B., Ren, L., Awn, A., Trpkov, K., and Muruve, D. A. (2007) Am. J. Physiol. Renal Physiol. 292, F567–F576
17. Al-Ani, B., Hansen, K. K., and Hollenberg, M. D. (2004) Mol. Pharmacol. 65, 149–156
18. Kawahata, A., Saiedfied, M., Al-Ani, B., Leblond, L., and Hollenberg, M. D. (1999) J. Pharmacol. Exp. Ther. 288, 358–370
19. Martin, B. R., Giepmans, B. N., Adams, S. R., and Tsien, R. Y. (2005) Science 310, 1308–1314
20. Nakanishi, H., Kato, K., and Hirose, Y. (2001) J. Biol. Chem. 276, 10759–10765
21. Oikonomopoulou, K., Hansen, K. K., Saiedfied, M., Tea, I., Blaber, M., Blaber, S. I., Scarisbrick, I., Andrade-Gordon, P., Cottrell, G. S., Bunnett, S. I., and Blaber, M. (2007) J. Biol. Chem. 282, 32095–32112
22. Hamdan, F. F., Rochdi, M. D., Breton, B., Fessart, D., Michaud, D. E., Charest, P. G., Laporte, S. A., and Bouvier, M. (2007) J. Biol. Chem. 282, 24647
Elastase-triggered Biased Signaling by PAR₂

29089–29100

23. Loew, D., Perrault, C., Morales, M., Moog, S., Ravanat, C., Schuhler, S., Arcone, R., Pietropaolo, C., Cazenave, J. P., van Dorsselaer, A., and Lanza, F. (2000) Biochemistry 39, 10812–10822

24. Vesey, D. A., Kruger, W. A., Poronnik, P., Gobé, G. C., and Johnson, D. W. (2007) Am. J. Physiol. Renal Physiol. 293, F1441–F1449

25. Defea, K. (2008) Br. J. Pharmacol. 153, Suppl. 1, S298–S309

26. Rasmussen, U. B., Vouret-Craviari, V., Jallat, S., Schlesinger, Y., Pagès, G., Pavirani, A., Lecocq, J. P., Pouysségur, J., and Van Obberghen-Schilling, E. (1991) FEBS Lett. 288, 123–128

27. Vu, T. K., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068

28. Dulon, S., Leduc, D., Cottrell, G. S., D’Alayer, J., Hansen, K. K., Bunnett, N. W., Hollenberg, M. D., Pidard, D., and Chignard, M. (2005) Am. J. Respir. Cell Mol. Biol. 32, 411–419

29. Déry, O., Thoma, M. S., Wong, H., Grady, E. F., and Bunnett, N. W. (1999) J. Biol. Chem. 274, 18524–18535

30. DeFea, K. A., Zalevsky, J., Thoma, M. S., Déry, O., Mullins, R. D., and Bunnett, N. W. (2000) J. Cell Biol. 148, 1267–1281

31. Scott, G., Leopardi, S., Parker, L., Babiarz, L., Seiberg, M., and Han, R. (2003) J. Invest. Dermatol. 121, 529–541

32. McCoy, K. L., Traynelis, S. F., and Hepler, J. R. (2010) Mol. Pharmacol. 77, 1005–1015

33. Chin, A. C., Lee, W. Y., Nusrat, A., Vergnolle, N., and Parkos, C. A. (2008) J. Immunol. 181, 5702–5710

34. Motta, J. P., Magne, L., Descamps, D., Rolland, C., Squarzoni-Dale, C., Rousset, P., Martin, L., Cenac, N., Balloy, V., Huerre, M., Jenne, D., Wartelle, J., Belaouaj, A., Mas, E., Vinel, J. P., Alric, L., Chignard, M., Vergnolle, N., and Sallenave, J. M. (2011) Gastroenterology 140, 1272–1282

35. Vergnolle, N. (2005) Gut 54, 867–874

36. Hansen, K. K., Sherman, P. M., Cells, L., Andrade-Gordon, P., Pan, Z., Baruch, A., Wallace, J. L., Hollenberg, M. D., and Vergnolle, N. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 8363–8368

37. Suzuki, T., Yamashita, C., Zemans, R. L., Briones, N., Van Linden, A., and Downey, G. P. (2009) Am. J. Respir. Cell Mol. Biol. 41, 742–755

38. Suzuki, T., Moraes, T. J., Vachon, E., Ginzberg, H. H., Huang, T. T., Matthey, M. A., Hollenberg, M. D., Marshall, J., McCulloch, C. A., Abreu, M. T., Chow, C. W., and Downey, G. P. (2005) Am. J. Respir. Cell Mol. Biol. 33, 231–247

39. Trivedi, V., Boire, A., Tchernychev, B., Kaneider, N. C., Leger, A. J., O’Callaghan, K., Covic, L., and Kuliopulos, A. (2009) Cell 137, 332–343

40. Riewald, M., and Ruf, W. (2005) J. Biol. Chem. 280, 19808–19814

41. Schuepbach, R. A., Feistritzer, C., Brass, L. F., and Riewald, M. (2008) Blood 111, 2667–2673

42. McLaughlin, J. N., Shen, L., Holinstat, M., Brooks, J. D., Dibenedetto, E., and Hamm, H. E. (2005) J. Biol. Chem. 280, 25048–25059

43. Sambrano, G. R., Huang, W., Faruqi, T., Mahrus, S., Craik, C., and Coughlin, S. R. (2000) J. Biol. Chem. 275, 6819–6823