Neutrophil Elastase Promotes Interleukin-1β Secretion from Human Coronary Endothelium

Mabruka Alfaidi, Heather Wilson, Marc Daigneault, Amanda Burnett, Victoria Ridger, Janet Chamberlain, and Sheila Francis

From the Department of Cardiovascular Science, Medical School, University of Sheffield, Sheffield S10 2RX, United Kingdom

Background: The mechanism of IL-1 release from endothelial cells is not fully known.

Results: Neutrophil elastase causes secretion of bioactive IL-1 from endothelial cells via microvesicles.

Conclusion: A mechanistic link between IL-1 secretion from endothelial cells and neutrophil elastase in atherosclerotic plaques is revealed.

Significance: Neutrophil elastase could be a potential target for preventing atherosclerosis.

The endothelium is critically involved in the pathogenesis of atherosclerosis by producing pro-inflammatory mediators, including IL-1β. Coronary arteries from patients with ischemic heart disease express large amounts of IL-1β in the endothelium. However, the mechanism by which endothelial cells (ECs) release IL-1β remains to be elucidated. We investigated neutrophil elastase (NE), a potent serine protease detected in vulnerable areas of human carotid plaques, as a potential “trigger” for IL-1β processing and release. This study tested the hypothesis that NE potentiates the processing and release of IL-1β from human coronary endothelium. We found that NE cleaves the pro-form of IL-1β in ECs and causes significant secretion of bioactive IL-1β via extracellular vesicles. This release was attenuated significantly by inhibition of neutrophil elastase but not caspase-1. Transient increases in intracellular Ca2+ levels were observed prior to secretion. Inside ECs, and after NE treatment only, IL-1β was detected within LAMP-1-positive multivesicular bodies. The released vesicles contained bioactive IL-1β. In vivo, in experimental atherosclerosis, NE was detected in mature atherosclerotic plaques, predominantly in the endothelium, alongside IL-1β. This study reveals a novel mechanistic link between NE expression in atherosclerotic plaques and concomitant pro-inflammatory bioactive IL-1β secretion from ECs. This could reveal additional potential anti-IL-1β therapeutic targets and provide further insights into the inflammatory process by which vascular disease develops.

Atherosclerosis is a complex chronic inflammatory disease that involves inflammatory cell recruitment and release of pro-inflammatory cytokines (1). IL-1β has been implicated in several aspects of vascular inflammation and neointima formation (2). Endothelial cell dysfunction, promoted by IL-1, also plays a central role in atherogenesis by expression of adhesion molecules and cytokine secretion (3), facilitating leukocyte recruitment and plaque development. The culmination of two decades of preclinical experimental studies in the IL-1 field has led to the ongoing phase 3 clinical trial Cankinumab Anti-inflammatory Thrombosis Outcomes Study, which will test whether blocking IL-1β only will reduce the incidence of thrombotic events in patients after myocardial infarction who remain at high risk because of ongoing inflammation (4).

It has generally been assumed that IL-1β is produced predominantly by immune-derived cells (5). However, we have shown, in ischemic heart disease patients, that atherosclerotic coronary arteries synthesize and express significant IL-1β within the endothelium (6) compared with controls. Experimental studies have also indicated that cultured endothelial cells (ECs)2 synthesize IL-1β in response to different cytokine stimulations but that the released IL-1β is low and relatively inefficient (7). It is crucial, therefore, to understand the mechanism(s) of release of IL-1β from ECs, especially because IL-1β acts at a distance rather than just locally in the vessel wall.

IL-1β production is a two-step controlled process requiring an “initial” stimulus for transcription/translation of proIL-1β (31 kDa), which, in turn, is cleaved by an inflammasome-activated caspase-1 (8) (“a second hit”) into a biologically active isoform (17 kDa) before secretion. The cleavage of proIL-1β is a crucial step, and studies in monocytes show that caspase-1 (a cysteine protease) is a cardinal enzyme in this process (9). There is a spectrum of proposed cellular mechanisms responsible for IL-1 secretion in monocytes, including rescue from autophagy and subsequent release, release via microvesicles or multivesicular bodies, and terminal release (via pores), dependent on cell type and stimulus intensity (10).

In vivo studies have postulated that IL-1β can also be released in the absence of caspase-1 (11), suggesting an alternative and still unknown mechanism by which “leaderless” IL-1β is secreted. There are other potential enzymes that cleave proIL-1β into its mature form, including the serine proteases (neutrophil elastase, cathepsin G, and proteinase 3 (12, 13)). It is known...
Neutrophil Elastase Promotes Endothelial IL-1 Secretion

that, in cell-free systems, these serine proteases cleave purified proIL-1β into a biologically active IL-1β in vitro at distinct sites to caspase-1 with production of 18- and 20-kDa isofoms of IL-1β. However, whether and to what extent these proteases can contribute to IL-1β release in cells such as ECs is relatively unknown.

Neutrophil elastase (NE) is a potent serine protease that has wide substrate specificity (14, 15). Experimental studies have potentially focused on the destructive nature of NE, but interesting recent data show that NE can provoke a variety of pro-inflammatory responses, such as IL-8 release from bronchial epithelium and TGF-β production in bronchial smooth muscle cells (14). Moreover, deletion of NE in mice leads to a reduction of serum inflammatory biomarkers such as TNF-α, MCP-1, and IL-1 (16). One study has also demonstrated NE in macrophage-rich human atherosclerotic plaque shoulders (17), and it also appears to be critical in caspase-1-independent IL-1β generation in NE-induced lung (18) and renal injury (19). In this study, we sought to determine whether NE promotes biologically active IL-1β secretion from vascular endothelium. We show that NE stimulation leads to proIL-1β cleavage and increases IL-1β release from coronary artery ECs via a caspase-1-independent, vesicular release-mediated process. Furthermore, we demonstrate that IL-1β is colocalized with NE predominantly in the endothelium in experimental atherosclerosis. This first demonstration and explanation of active IL-1β release from the endothelium potentially provides additional novel strategies for inhibition of IL-1β activity in inflammatory cardiovascular disease.

Experimental Procedures

Human coronary artery endothelial cells (HCAECs) were purchased from PromoCell (Heidelberg, Germany) and cultured in supplemented media according to the recommendations of the manufacturer. The cells, at passages 2–5, were seeded into 6-well plates (2 × 10⁴ cells/well) and grown at 37 °C/5% CO₂ (v/v) until 70% confluent. The first step of stimulation was to up-regulate proIL-1β production by adding cytokines (TNF-α/IL-1α, 10 ng/ml each) for 48 h as described previously (7). Cells were then washed to remove all traces of stimulating cytokines before the media was replaced with serum-free media containing NE (1 μg/ml, equating to 60 IU). To ensure that the stimulating cytokines were removed completely, the final cell wash was tested for the presence of IL-1 via ELISA. No cytokines were detected in these washes (data not shown). In some experiments, cells were preincubated with NEIII (500 μM) (20), caspase-1 inhibitor I (YVAD, 50 μM) (8, 21), or BAF1 (50 nM) (22) for at least 30 min before the addition of NE. At the end of the incubations, supernatants were collected, and the cells were lysed in ice-cold 1% (v/v) Triton X-100 lysis buffer. Both supernatant and cell lysates were stored at −80 °C until the analysis was conducted.

Determination of Cell Viability—Cell viability was evaluated by trypan blue dye exclusion and measurement of lactate dehydrogenase levels in conditioned media. Lactate dehydrogenase detected in cell lysates was used as a positive control for total lactate dehydrogenase. Levels of lactate dehydrogenase were analyzed using a CytoTox 96 non-radioactive cytotoxicity kit (Promega) according to the instructions of the manufacturer.

NE Activity—NE activity was measured spectrophotometrically using a highly specific synthetic substrate (elastase substrate I, MeOSuc-APV-pNA, 100 μM) as described in detail previously (14, 23). Briefly, samples (supernatant and lysate) were added to assay buffer (0.45 Tris base and 2 mM NaCl (pH 8.0)) containing elastase substrate I for 6 h. The rate of substrate cleavage was measured using a plate reader (Thermo Scientific) at 410 nm.

ELISA for IL-1β—The concentrations of IL-1β (picograms per milliliter) in the supernatants and lysates were quantified by ELISA Quantikine kits (R&D Systems) according to the recommendations of the manufacturer.

Apoptosis—Apoptosis, detected via caspase-3/7 activity, was analyzed by Caspase-Glo® 3/7 assay (Promega) according to the recommendations of the manufacturer.

Western Blot Analysis for IL-1β Processing and Release—Samples (lysates and concentrated supernatants using 10k Amicon filter devices (Thermo Scientific)) were subjected to Western blotting.

Microvesicle Isolation—MV isolation was conducted as described previously (24). Freshly prepared MVs were used for analysis to avoid false positive effects caused by leakage of contents from MVs damaged by freezing and thawing.

Flow Cytometry Measurement of Extracellular Vesicles—A pellet of extracellular vesicles was resuspended in annexin V-binding buffer and labeled with annexin V PE-Cy7 fluorescence according to the instructions of the manufacturer (eBioscience). Events were acquired using an LSR II flow cytometer (BD Biosciences), and annexin V-positive extracellular vesicles were enumerated using Accu Count beads (Spherotech, 0.2–0.9 μm) and analyzed using FlowJo software (TreeStar).

Measurements of Intracellular Calcium Concentration—HCAECs in 96-well plates at a seeding density of 5 × 10⁴ were treated with cytokines or left untreated for 48 h. Fura-4 was then added to the cells according to the instructions of the manufacturer (Invitrogen). After washing off the dye, the cells were incubated with or without NE, and changes in cytosolic Ca²⁺ were recorded using a plate reader (Thermo Scientific) according to the recommendations of the manufacturer. EGTA (6 mM) and ionophore A23187 (10 μM) were used as controls as described previously (25).

Direct Effects of NE on Recombinant IL-1β/ProIL-1β—NE is known to undergo spontaneous autolysis (26) and has a proteolytic activity against many cytokines, such as TNF-α (15). For this reason, we tested the effect of NE with the studied concentration on IL-1β/proIL-1β itself. IL-1β standard (R&D Systems) at a concentration of 125 pg/ml and proIL-1β standard (R&D) at a concentration of 10,000 pg/ml were mixed separately with NE (1 μg/ml) and kept in the incubator for 30 min, 2 h, and 6 h. The samples were stored at −80 °C and tested by ELISA and Western blot analysis.

Detection of Extracellular Vesicular Shedding—HCAECs (2 × 10⁴) were plated in LabTek (Fisher) 8-well chamber slides and subjected to the abovementioned stimulation conditions. Annexin V-Alexa Fluor 488 (Invitrogen) was then added to the cells at 5 μl/well. MV shedding was visualized using image
acquisition software (inverted wide-field fluorescence microscope, Leica AF6000 time-lapse) after the addition of NE in a 5% CO₂/37 °C (v/v) heated chamber. The images were captured after 15 min, 30 min, 2 h, and 6 h and analyzed using Image J software (National Institutes of Health). MVs (0.1–1 μm) were quantified in blinded samples in a random field of cells.

**Immunofluorescence**—Cells were fixed with 4% w/v paraformaldehyde and permeabilized in 0.3% v/v Triton X-100. Non-specific binding was blocked for 30 min with 5% v/v goat or rabbit serum in 1% w/v BSA prior to sequential incubation of the cells with the following primary antibodies: anti-goat IL-1β and anti-rabbit LAMP-1 (1:100 dilutions). Alexa Fluor 488- and 647-conjugated secondary antibodies were used in 1% w/v BSA (1:200 dilution). The coverslips were washed with PBS and mounted onto glass slides using media containing DAPI.

The labeling of NE was conducted as described previously (27) using a Microscale protein labeling kit (Molecular Probes) according to the instructions of the manufacturer. 50 μg of NE was used for the reaction. The final concentration of Alexa Fluor 647-NE was 0.1 mg/ml in a volume of 100 μl.

**Determination of IL-1β Biological Activity**—The bioactivity of the secreted IL-1β by HCAECs following NE stimulation was assessed using an IL-8 luciferase reporter assay sensitive to picomolar concentrations of IL-1β, as described previously (9, 28, 29). Briefly, HeLa cells (5 × 10⁵) were grown to 70% confluence in 96-well plates and transfected with a total of 100 ng of DNA/well, including 60 ng of pIL-8-luc (reporter) and 40 ng of pRL-TK (internal control). After 24 h, the transfection efficiency was assessed, and cells were then stimulated for 6 h with 0.1 nm IL-1β as a control or with harvested supernatants from HCAEC stimulated with NE. IL-1β-neutralizing antibody (1 μg/ml, catalog no. MAB201, R&D Systems) was used in some wells to prove specificity. Cells were then lysed with passive lysis buffer (Promega) and transferred to white well plates to assay for luminescence intensity using LARII and Stop and Glo reagents (Promega). Luciferase activity was calculated by normalizing to the Renilla luminescence measured in each well according to the recommendations of the manufacturer (Promega).

**Conventional and Immunogold Labeling Electron Microscopy**—EC pellets (NE or untreated controls) were processed as described previously (30). Thin sections were immunogold-labeled with anti-goat IL-1β and anti-rabbit LAMP-1 (1:50 dilutions each) primary antibodies for 2 h at room temperature. After washing the grids, the sections were incubated with immunogold–conjugated secondary antibodies (20- and 10-nm gold particles, Agar Scientific) for 2 h. Control experiments were performed using PBS instead of the primary antibodies, and all sections were then post-stained with uranyl acetate and lead citrate.

**Mice and Diets**—ApoE⁻/⁻ male mice were bred in-house at the University of Sheffield. Food and water were given ad libitum in a controlled environment (temperature, 22–25 °C; humidity, 55% ± 5%; 12-h light cycle). At 8–10 weeks of age, the mice were housed individually and fed a high-fat, Western-type diet containing 21% fat, 0.15% cholesterol, and 0.296% sodium over a 12-week duration. Special Diet Services (Witham, UK) supplied the food. This high-fat diet was used specifically to study the effects of the diet on atherosclerosis, as described previously (31). All animal care and procedures were conducted under Animals (Scientific Procedures) Act 1986 (UK) and approved by The University of Sheffield Ethics Committee. At the end of the study, the mice were euthanized, and proximal aortae were harvested.

**Immunohistochemistry**—Sections were used for immunohistochemistry as described previously (32).

**Statistical Analyses**—Results are shown as mean ± S.E. Analyses were performed using GraphPad Prism version 6.04. For multiple-comparison tests, one way analysis of variance (ANOVA) followed by Tukey’s test was performed. Statistical significance was achieved when the p value was less than 0.05.

**Results**

**NE Promotes IL-1 Release in HCAECs**—To assess the contribution of NE to IL-1β secretion, cytokine-primed ECs were treated with varying concentrations of NE in serum-free media at different time points. As shown in Fig. 1A, after 2 h of stimulation, NE at 1 μg/ml caused significant (10×) release of IL-1β from cytokine-primed cells (198 ± 24.85 pg/ml, p < 0.0001) compared with primed cells without NE (12.1 ± 4.81 pg/ml). This release decreased significantly with higher concentrations of NE (2 μg/ml) because of a decrease in cell viability (50% ± 10%). Therefore, subsequent experiments used NE at 1 μg/ml to give the highest amount of IL-1β release without a significant increase in cell death (Fig. 1B).

After 6 h of stimulation, NE caused a significant increase in IL-1β secretion (Fig. 1C) compared with cytokine stimulation alone (272.8 ± 50 pg/ml versus 55.5 ± 17.3 pg/ml, p < 0.001). No IL-1β was detected in the media of unstimulated cells (Fig. 1C).

To specifically confirm that the release was due to NE, the cells were pretreated with the specific neutrophil elastase inhibitor NEII, resulting in a significant attenuation of IL-1β secretion. To determine the involvement of caspase-1, cytokine-primed ECs were pretreated with YVAD-CHO and then with NE. No significant changes in the secreted levels of IL-1β were seen compared with NE alone (Fig. 1C). Therefore, NE-mediated IL-1β release in endothelial cells was independent of caspase-1.

In unstimulated EC lysates, we did not detect any IL-1β. However, there was significant IL-1β production inside cells following treatment with pro-inflammatory cytokines (Fig. 1D). The IL-1β levels in the lysates were not significantly different among the groups (Fig. 1D). In addition, NE treatment alone did not provoke IL-1β production in the cells (Fig. 1D), suggesting no direct effects of NE on IL-1β generation inside the cells. There was also no effect seen on IL-1β production in cell lysates by NEIII or caspase-1 inhibitor (Fig. 1D).

To confirm that NE was active over the duration of the study, we measured NE activity using quantitative cleavage of a chromogenic specific substrate and, interestingly, in the lysates harvested from NE-treated cells, this was increased significantly (Fig. 1E).

Cell lysis as a mechanism of IL-1β secretion was ruled out by the absence of cytosolic enzyme lactate dehydrogenase levels in...
Neutrophil Elastase Promotes Endothelial IL-1 Secretion

There were no significant changes in lactate dehydrogenase levels following NE stimulation at 2 and 6 h compared with the control (Fig. 1F). Because IL-1 release could be a feature of cell apoptosis, we investigated caspase-3/7 activation in ECs under our stimulation conditions, and there was no significant increase in NE-treated cells compared with unstimulated ECs, cytokine-stimulated cells, or cells in which NE effects were attenuated using NEIII (Fig. 1G). In addition, we used propidium iodide in conjunction with annexin V to determine whether ECs were viable, apoptotic, or necrotic at any time point in this study. We did not detect any significant increase in propidium iodide/annexin V staining in NE-treated cells compared with untreated controls (data not shown).

In vitro, some proteases, including NE, have been shown to process proIL-1β into mature IL-1β (13). Therefore, because the ELISA does not distinguish between pro- and mature IL-1β,
we determined which IL-1β isoforms were present, and their relative respective levels, in cell lysates by immunoblotting. Unstimulated HCAEC lysates did not contain any detectable IL-1β, but full-length proIL-1β (31 kDa) was seen in cytokine-primed cell lysates (Fig. 2A, i). However, after the addition of NE, there was cleavage of the 31-kDa pro-form associated with a decrease in the 31-kDa proIL-1β in cell lysates (Fig. 2A, ii). Nor did we detect p20 (the product of active caspase-1) in cell media, indicating that cleavage and secretion had not occurred. NE is known for its proteolytic properties. Therefore, to confirm that NE cleaved the pro-form but not the mature form of IL-1, recombinant proIL-1β and recombinant mature IL-1β were incubated with NE in a cell-free system. NE cleaved the pro-form of IL-1 in vitro (Fig. 2C) but not the mature form (Fig. 2, D and E), supporting the hypothesis that NE specifically cleaves the pro-form of IL-1β only. Taken together, these data clearly suggest that NE increases cytokine-induced IL-1β secretion in ECs via an inflammasome/caspase-1-independent pathway.

**NE Induced Secretion of Extracellular Vesicles Containing Bioactive IL-1 from HCAECs**—In immune-derived cells, protected release of IL-1β (microvesicles, multivesicular bodies (MVBs), and exosomes) has been proposed as a mechanism for IL-1β trafficking to the extracellular environment (9, 33, 34). Therefore, we sought to determine whether extravesicular shedding occurred in response to NE and whether this was associated with IL-1β processing in HCAECs. Because phosphatidylserine exposure has been associated with MV shedding in monocytes (9) and ECs (24), we used annexin V binding (annexin V-Alexa Fluor 488) as a tool to visualize events in live HCAECs. Small particles (0.1–1 μm in diameter) were observed separating from the cells in real time...
using time-lapse imaging over a duration of 6 h (supplemental Movie S1). The first MV generation was at 10 min, with membrane alterations 30 min after NE stimulation, with large numbers of MVs observed at later time points (Fig. 3). The number of MVs was quantified using flow cytometry and gating for annexin V (Fig. 4A). Interestingly, there was a significant increase in the number of MVs isolated from ECs following NE treatment (2- to 3-fold) compared with controls. Importantly, NE inhibition effectively attenuated MV formation, and shedding induced by NE and caspase-1 inhibition had no significant effect (Fig. 4B).

We next investigated which IL-1β isoforms were inside MVs using immunoblotting. MVs from unstimulated cells contained no IL-1β, and, in MVs from cytokine-primed cells (6 h), prominent proIL-1β (31-kDa) forms were detected (Fig. 4C). In MVs isolated from the supernatants of NE-treated cells, cleavage of the 31-kDa IL-1β isoform to ~19 – 20 kDa was observed from as early as 30 min (Fig. 4D), with further cleavage to the 18- and 15-kDa isoforms after 6 h (Fig. 4C). Treatment of cells with NEIII abolished any cleavage of proIL-1β in these MVs, confirming that these bands are the result of direct NE activity. Cleavage of proIL-1β continued even in the presence of YVAD, with more prominent isoforms detected (Fig. 4C). MVs were assessed for caspase-1 and NLRP3 content, and, interestingly, active caspase-1 p20 and NLRP3 were not detected in MVs isolated from cells treated with NE or NE and YVAD together (data not shown), indicating that intravesicle cleavage of proIL-1β is independent of caspase-1 activation. These findings suggest that either NE cleaves the released proIL-1β inside MVs or that NE-treated cells continually generate more MVs containing IL-1β as a route of secretion.

We next asked whether the processed IL-1β released into cell supernatants in vesicles as a result of NE activation was bioactive. We collected total supernatants (containing MVs) from NE-treated or untreated cells for 6 h, applied them to HeLa cells expressing an IL-1RI-responsive reporter, and measured IL-8 activity. We compared the reporter assay output (IL-8) with media obtained from unstimulated or cytokine-primed ECs with a positive control (0.1 nM recombinant IL-1β) or with an IL-1β-neutralizing antibody. As shown in Fig. 4E, supernatants isolated from NE-activated ECs increased significantly IL-8 activity compared with unstimulated and cytokine-primed cells, and this was abrogated completely by the neutralizing antibody. To confirm that the bioactivity was due to released IL-1β and not a result of direct NE effects on HeLa cells, NE (1 μg/ml) was added to HeLa cells (as a spike), and this showed no significant IL-8 activation. These data indicate that the IL-1β in the MVs is bioactive. MVs containing IL-1β were confirmed using immunogold transmission electron microscopy, and we only detected 0.2-μm-diameter MVs containing immunogold-labeled IL-1β in NE-treated cells (Fig. 4F).

**Mechanisms of IL-1β Release in Endothelial HCAECs**—To study early MV shedding by NE, intracellular calcium changes were assessed. MV release has been linked with transient changes in intracellular calcium ([Ca^{2+}]) in specialized secretory cells (35) and IL-1β secretion in macrophages (33). Using a
Ca\(^{2+}\)-sensitive fluorometric dye, we assessed the role of [Ca\(^{2+}\)]\(_i\) in MV formation and release in response to NE performed experiments in the presence or absence of exogenous Ca\(^{2+}\). In this experiment, [Ca\(^{2+}\)]\(_i\) is released from intracellular stores during an initial stimulation/treatment in Ca\(^{2+}\)-free media, and application of CaCl\(_2\) during the second phase of the protocol allows Ca\(^{2+}\) influx inside ECs. In Ca\(^{2+}\)-free media, there was a small non-significant increase in cytosolic Ca\(^{2+}\) levels in NE-treated cells compared with untreated cells after NE stimulation (Fig. 5A). However, [Ca\(^{2+}\)]\(_i\) was increased significantly in NE-stimulated cells after the addition of CaCl\(_2\) compared with unstimulated and cytokine-primed cells (Fig. 5B). This finding suggests that NE treatment of ECs increases free [Ca\(^{2+}\)]\(_i\), by promoting Ca\(^{2+}\) influx into ECs and that this calcium influx is associated with MV release.

We detected small MVs (~200 nm) in response to NE (assessed by flow and electron microscopy), characteristic of exosomes secreted from MVBs, and this led us to study the compartmentalization of IL-1\(\beta\) in ECs (30). HCAECs were stained using an immunofluorescence technique for LAMP-1 (a late endolysosomal marker) and IL-1\(\beta\). Confocal images of cytokine-primed ECs stained for IL-1\(\beta\) and costained for LAMP-1 suggested a wide distribution of IL-1\(\beta\) throughout the cytoplasm and no colocalization with LAMP-1 (Fig. 5C). Not surprisingly, no signals were detected for IL-1\(\beta\) in unstimulated ECs (data not shown). However, in ECs incubated with NE
for increasing times (30 min, 1 h, and 2 h), IL-1β was colocalized with LAMP-1 (Fig. 5D). Indeed, following a 2-h stimulation of EC with NE, the majority of IL-1β was detected in MVBs (Fig. 5D).

To confirm that the site of IL-1β processing and secretion was indeed mediated by an endolysosomal mechanism, we evaluated the effect of bafilomycin A1 (BAF1), a lysosomal V/ATPase inhibitor that has been shown to prevent endolysosomal formation (36). Treatment of ECs with BAF1 (50 nM) before the addition of NE largely decreased IL-1 levels in the supernatants after 6 h, and that was associated with a reduction in pro-IL-1 cleavage in the lysates (Fig. 6, A and B). We subsequently performed transmission electron microscopy on cytokine-primed HCAECs ± NE and observed >200 nm structures in the cytosol with classical morphological features of MVBs (37). These were detected in close proximity to the plasma membrane in NE-activated ECs but not in unstimulated ECs (Fig. 6C, i). Interestingly, inside the cells, the majority of IL-1 was detected within the MVBs (Fig. 6C, ii).

**NE Is Detected in ECs and Is Colocalized with IL-1β in the Endothelium of Mature Atherosclerotic Plaques**—To follow the fate of NE in activated ECs, we used Alexa-Fluor 647-labeled NE and performed immunofluorescence staining. After permeabilization, we also labeled the internal endolysosomes with LAMP-1. Surprisingly, NE was detected inside cells. The enzyme colocalized with LAMP-1 (Fig. 7A).

Finally, we asked whether NE could be detected in atherosclerotic plaques in mice to ascertain whether NE could contribute to local IL-1β generation. Only in well developed atheromatous lesions of ApoE−/− mice fed a high-fat diet for 12 weeks was IL-1β detected, predominantly in endothelial cells. Interestingly, in these lesions, NE also appeared to be expressed in the luminal endothelium (Fig. 7B) and colocalized with IL-1β-positive stained ECs (Fig. 7C).

**Discussion**

Here we describe, for the first time, how coronary artery ECs release IL-1β, which has been a “holy grail” of endothelial biology for many years. We report that a considerable amount of IL-1β is released from ECs in response to NE via a caspase-1-independent vesicular pathway. This is supported by the following lines of evidence: bioactive IL-1β is secreted only in MVs following NE treatment, IL-1β secretion is unaffected by YVAD and occurs without caspase-1/NLRP3 activation, and MVBs are prevalent in primed NE-treated ECs and contain IL-1β protein. We propose that “protected release” (10) (i.e. contained within
Neutrophil Elastase Promotes Endothelial IL-1 Secretion

agents, perpetuating the cycle of inflammation. Several lines of evidence suggest that IL-1 is an apical cytokine in this process (39), but its mechanism of release from ECs is largely unknown. Furthermore, the biological pattern of the cross-talk has not been completely defined. We hypothesized that NE induces IL-1β secretion from the vascular endothelium.

Because caspase-1 has been identified as the main proteolytic enzyme to play a role in proIL-1β cleavage and secretion in monocytes and macrophages (40), we used a specific caspase-1 inhibitor (YVAD-CHO) as a potential means of attenuating IL-1β release. Our data show that caspase-1 appears to be non-essential in EC in this setting for IL-1β cleavage and release by NE. This is at odds with other in vitro studies in monocytes (21) but in agreement with more recent data from other cell types (41) and in vivo models (42, 43). Our findings are also supported by the findings of Guma et al. (11), who describe the presence of IL-1β in the synovial fluid of caspase-1−/− mice. Moreover, our data may explain why caspase-1 suppression did not show promise in vascular healing or atherosclerosis progression (32).

It has been shown previously that IL-1β lacks the signal peptide for directing it to the classical endoplasmic reticulum-Golgi secretory pathway (30). Therefore, IL-1β release has been proposed to occur by distinct mechanisms, including MV shedding and endolysosomal regulation (10). Strikingly, in HCAECs, NE-induced MV shedding occurred independently of caspase-1 activity, which is in contrast to previous investigations using immune cells which reported caspase-1 dependent MV shedding. We suggest that, at least in HCAECs, NE is able to directly cleave the IL-1β precursor, which is associated with protected release in membrane-bound vesicles. In MVs, the prominent forms of IL-1β released (also present inside cells) were 20, 18, and 15 kDa. These isoforms have been detected previously in vitro (13), and, although proposed as 5-10-fold less bioactive than the 17-kDa isoform, they are active enough for IL-1 to bind to its receptors and initiate signaling. Our study is the first to show, in intact cells, that NE is capable of cleaving proIL-1β at multiple sites and that these products are bioactive.

A common biogenesis has linked Ca2+-regulated MV shedding and IL-1β secretion with an increase in intracellular calcium levels (33). We are in agreement with this and demonstrated that NE transiently increased [Ca2+]i, suggesting that NE mobilized [Ca2+]i to a maximum after addition of exogenous Ca2+, suggesting that NE mobilized [Ca2+]i, mainly by influx of extracellular Ca2+. The secretory pathway identified here has been used to describe the secretion of other non-classical proteins, such as EGF (44). Although a few studies have begun to investigate NE-mediated IL-1β secretion in renal and lung disease, ours is the first to propose a direct effect of NE on IL-1β release in cardiovascular inflammation and to link this to a MV release mechanism.

Recent immunofluorescence staining data in murine macrophages have shown that IL-1β does not colocalize with LAMP-1 in macrophages (45). This finding is consistent with our data in ECs prior to NE treatment (cytokine-primed cells) where IL-1β appears to be distributed diffusely within the cytoplasm. Significantly, however, 2 h after NE treatment, colocalization increased, strongly suggesting that IL-1β compartmentalization may be induced acutely by NE and predestined for maturation by regulated transport. In ECs primed with cyto-

FIGURE 7. NE is detected in ECs and is colocalized with IL-1β in the endothelium of mature atherosclerotic plaques. A, confocal images showing LAMP-1 and NE in primed ECs after NE treatment. HCAECs were incubated with or without Alexa Fluor 647-labeled (1 μg/ml) NE for 2 h in serum-free media before washing in PBS, and colocalization was performed using an antibody against LAMP-1. Confocal images were analyzed using Zeiss image and Imaged software. Scale bars = 10 μm. B, immunohistochemical detection of NE and IL-1β in the luminal endothelium of mouse atherosclerotic plaques. Paraffin-embedded aortic sinuses from ApoE mice fed a high-fat diet for 12 weeks were stained with primary antibodies as indicated. The specificity of staining was confirmed by no primary negative control. Scale bars = 200 μm. C, colocalization of IL-1-β, NE, and von Willebrand Factor (vWF) in aortic atherosclerosis. NE positivity was detected predominantly in the endothelium (top right panel, arrows). IL-1β positive endothelium (top left panel) was also detected. The bottom panels show vWF-stained endothelium, and DAPI was used for the nuclei. The specificity of staining was confirmed by no primary negative control. Images are representative of histology data obtained from a total of six animals.

membrane-bound vesicles) of IL-1β is a prevalent mechanism in HCAECs.

The endothelium is fundamental in atherosclerotic plaque development not only during early lesion development but also later by controlling plaque instability (38). In atherosclerosis, cross-talk between circulating cells such as monocytes and neutrophils and the endothelium can cause ECs to liberate soluble
kines and treated with NE, we observed that MVBs with cytosolic IL-1β accumulated within cell invaginations, similar to that shown in Ref. 46. This is consistent with work from Rubartelli et al. (47) in monocytes. Because MVBs were not detected in cells that did not produce IL-1β (33), this strongly suggests that MVBs are a part of the secretory pathway of IL-1β.

The colocalization of IL-1β and LAMP-1 suggests that NE may trigger a signaling pathway that allows the IL-1β processing to occur in secretory endolysosomes (MVBs). However, the possibility of NE internalization within ECs, via endocytosis, cannot be ruled out, particularly because we detect enhanced NE activity in NE-treated EC lysates compared with untreated and cytokine-primed cells. In agreement with others who have shown that NE is internalized by macrophages (48) and tumor cells (27) by active transport, we also detected NE inside ECs using confocal imaging. Therefore, it is possible that, after internalization of NE by ECs, NE enters MVBs, where it cleaves proIL-1β.

It is possible that NE also causes the release of IL-1β because of a toxicity effect, with NE causing cell damage and leakage of cellular contents. However, at the NE concentration used, this is unlikely to be the case because cell viability was not affected significantly, nor was apoptosis proven. Indeed, IL-1β has been shown to increase caspase-3/7 in cells without inducing apoptosis (49), which is in agreement with our data. NE increases IL-1β levels, which, in turn, affects caspase-3/7 but does not affect apoptosis. We use recombinant NE throughout this study, raising the question of what the natural source of NE would be and whether levels of NE would be enough to activate ECs. Previous work by our group has shown that IL-1β is released from activated cocultures of ECs and monocytes at greater levels than from monocytes or ECs alone and that an unidentified monocyte-derived mediator contributed significantly to this response (50). Taken together with the findings reported here, we postulate that NE is the mediator released from monocytes and that it would be the natural source of NE in vivo.

Given the continued prominence and topicality of IL-1 in the generation and progression of atherosclerosis (51), we studied the expression of NE in vivo in a recognized atherosclerosis preparation: aortic root plaques taken from ApoE−/− mice fed a high-fat diet for 12 weeks. Although previous work has detected NE in coronary arteries (6), aortic aneurysms (52), and carotid plaques (17), our study is the first to investigate NE distribution in experimental atherosclerosis. Significantly, NE was mainly detected in the endothelium of plaques and was detected alongside IL-1β. The antibody used for these studies recognizes both the proIL-1β and mature forms, and we show a clear cellular colocalization with NE in support of our data showing that NE activates and promotes the secretion of IL-1β.

This colocalization of NE and IL-1β suggests that the IL-1β observed is likely to be active. We propose that circulating NE is assimilated into developing plaques from degranulating immune cells during their passage through the main vasculature or via the vasa vasorum, where these exist. This suggestion remains to be clarified, and a causal connection between IL-1β and NE needs to be confirmed in a future study utilizing NE−/− mice.

In conclusion, we significantly add to and cement the emerging role of NE in IL-1-induced inflammation. Furthermore, we suggest a novel mechanism for NE-mediated IL-1 secretion by ECs; namely, proIL-1 processing in the secretory endolysos-
Neutrophil Elastase Promotes Endothelial IL-1 Secretion

Generation of biologically active interleukin-1 beta by proteolytic cleavage of the inactive precursor. J. Biol. Chem. 263, 9437–9442

14. Lee, K. Y., Ho, S. C., Lin, H. C., Lin, S. M., Liu, C. Y., Huang, C. D., Wang, C. H., Chung, K. F., and Kuo, H. P. (2006) Neutrophil-derived elastase induces TGF-β1 secretion in human airway smooth muscle via NF-κB pathway. Am. J. Respir. Cell Mol. Biol. 35, 407–414

15. Henriksen, P. A., and Sallenne, J. M. (2008) Human neutrophil elastase: mediator and therapeutic target in atherosclerosis. Int. J. Biochem. Cell Biol. 40, 1095–1100

16. Talukdar, S., Oh da, Y., Bandypadhyay, G., Li, D., Xu, J., McNelis, J., Lu, M., Li, P., Yan, Q., Zhu, Y., Ofrecio, J., Lin, M., Brenner, M. B., and Olefsky, J. M. (2012) Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. Nat. Med. 18, 1407–1412

17. Dollery, C. M., Owen, C. A., Sukhova, G. K., Krettek, A., Shapiro, S. D., and Libby, P. (2003) Neutrophil elastase in human atherosclerotic plaques: production by macrophages. Circulation 107, 2829–2836

18. Couillin, I., Vasseur, S., Charron, S., Gasse, P., Tavernier, M., Guillet, J., Lagente, V., Fick, L., Jacobs, M., Coelho, F. R., Moser, R., and Ryffel, B. (2009) IL-1R1/MyD88 signaling is critical for elastase-induced lung inflammation and emphysema. J. Immunol. 183, 8195–8202

19. Schreiber, A., Pham, C. T., Hu, Y., Schneider, W., Luft, F. C., and Kettritz, R. (2012) Neutrophil serine proteases promote IL-1β generation and injury in necrotizing crescentic glomerulonephritis. J. Am. Soc. Nephrol. 23, 470–482

20. Karmarkar, M., Sun, Y., Hise, A. G., Rietsch, A., and Pearlman, E. (2012) Cutting edge: IL-1β processing during Pseudomonas aeruginosa infection is mediated by neutrophil serine proteases and is independent of NLRC4 and caspase-1. J. Immunol. 189, 4231–4235

21. Ward, J. R., West, P. W., Ariaans, M. P., Parker, L. C., Francis, S. E., Crossman, D. C., Sabroe, I., and Wilson, H. L. (2010) Temporal interleukin-1β secretion from primary human peripheral blood monocytes by P2X7-independent and P2X7-dependent mechanisms. J. Biol. Chem. 285, 23147–23158

22. Gupta, P., Goldenberg, D. M., Rossi, E. A., Cardillo, T. M., Byrd, J. C., Muthusamy, N., Furman, R. R., and Chang, C. H. (2012) Dual-targeting immunotherapy of lymphoma: potent cytotoxicity of anti-CD20/CD74 bispecific antibodies in mantle cell and other lymphomas. Blood 119, 3767–3778

23. Coffman, L. G., Brown, J. C., Johnson, D. A., Parthasarathy, N., D’Agostino, R. B., Jr., Lively, M. O., Hua, X., Tilley, S. L., Muller-Esterl, W., Willingham, M. C., Torti, F. M., and Torti, S. V. (2008) Cleavage of high-molecular-weight kininogen by elastase and trypstatin is inhibited by ferri tin. Am. J. Physiol. Lung Cell Mol. Physiol. 294, L505–515

24. Ettelaie, C., Collier, M. E., Maraveyas, A., and Ettelaie, R. (2014) Characterization of physical properties of tissue factor-containing microvesicles and a comparison of ultracentrifuge-based recovery procedures. J. Extracell. Vesicles 3, 23592–23605

25. Storey, R. F., Sanders, H. M., White, A. E., May, J. A., Cameron, K. E., and Heptinstall, S. (2000) The central role of the P2X7 receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. Br. J. Haematol. 110, 925–934

26. Liu, H., Lazarus, C. S., Caughey, G. H., and Fahy, J. V. (1999) Neutrophil elastase and elastase-rich cystic fibrosis sputum degranulate human eosinophils in vitro. Am. J. Physiol. 276, L28–34

27. Houghton, A. M., Rzymkiewicz, D. M., Ji, H., Gregory, A. D., Egea, E. E., Metz, H. E., Stolz, D. B., Land, S. R., Marconcini, L. A., Klimenti, C. R., Jenkins, K. M., Beaudieu, K. A., Mouled, M., Frank, S. J., Wong, K. K., and Shapiro, S. D. (2010) Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. Nat. Med. 16, 219–223

28. Evans, I., Dower, S. K., Francis, S. E., Crossman, D. C., and Wilson, H. L. (2006) Action of intracellular IL-1α (type 1) is independent of the IL-1 intracellular signalling pathway. Cytokine 33, 274–280

29. Kiss-Toth, E., Guesdon, F. M., Wyllie, D. H., Qwarnstrom, E. E., and Chang, C. H. (1996) Processing of precursor interleukin 1 in aortic endothelial cells. Blood 88, 3642–3650

30. Andrei, C., Dazzi, C., Lotti, L., Torrisi, M. R., Chimini, G., and Rubartelli, A. (1999) The secretory route of the leaderless protein interleukin 1β

Author Contributions—M. A. performed all experiments, analyzed the results, and contributed to writing the paper. H. W., M. D., A. B., and V. R. helped design, conduct, or analyze experiments involving microvesicles. J. C. and S. F. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

References
Neutrophil Elastase Promotes Endothelial IL-1 Secretion

involves exocytosis of endolysosome-related vesicles. Mol. Biol. Cell 10, 1463–1475
31. Chamberlain, J., Francis, S., Brookes, Z., Shaw, G., Graham, D., Alp, N. I., Dower, S., and Crossman, D. C. (2009) Interleukin-1 regulates multiple atherogenic mechanisms in response to fat feeding. PloS ONE 4, e5073
32. Chamberlain, J., Gunn, J., Francis, S., Holt, C., and Crossman, D. (1999) Temporal and spatial distribution of interleukin-1 β in balloon injured porcine coronary arteries. Cardiovasc. Res. 44, 156–165
33. Qu, Y., Franchi, L., Nunez, G., and Dubyak, G. R. (2007) Nonclassical IL-1 activity is not essential for apoptosis during thymocyte development. J. Immunol. 179, 1913–1925
34. Galliher-Beckley, A. J., Lan, L. Q., Aono, S., Wang, L., and Shi, J. (2013) Caspase-1-independent interleukin-1β is required for clearance of Borde-tella pertussis infections and whole-cell vaccine-mediated immunity. PloS ONE 9, e107188
35. Rodríguez, A., Webster, P., Ortego, J., and Andrews, N. W. (1997) Lyso-somes behave as Ca²⁺–regulated exocytic vesicles in fibroblasts and epithelial cells. J. Cell Biol. 137, 93–104
36. Sahoo, S., Klychko, E., Thorne, T., Misener, S., Schultz, K. M., Millay, M., Ito, A., Liu, T., Kamide, C., Agrawal, H., Perlman, H., Qin, G., Kishore, R., and Losordo, D. W. (2011) Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity. Circ. Res. 109, 724–728
37. Aird, W. C. (2008) Endothelium in health and disease. Pharmacol. Rep. 60, 139–143
38. Van Tassell, B. W., Toldo, S., Mezzaroma, E., and Abbate, A. (2013) Targeting interleukin-1 in heart disease. Circulation 128, 1910–1923
39. Dinarello, C. A. (2007) Mutations in cryopyrin: bypassing roadblocks in the caspase 1 inflammasome for interleukin-1β secretion and disease activity. Arthritis Rheum. 56, 2817–2822
40. Doerfler, P., Forbush, K. A., and Perlmutter, R. M. (2000) Caspase enzyme activity is not essential for apoptosis during thymocyte development. J. Immunol. 164, 4071–4079
41. Place, D. E., Muse, S. J., Kirimanjeswara, G. S., and Harvill, E. T. (2014) Caspase-1-independent interleukin-1β is required for clearance of Borde-