Epidermal Growth Factor-Like Domain 7 Suppresses Intercellular Adhesion Molecule 1 Expression in Response to Hypoxia/Reoxygenation Injury in Human Coronary Artery Endothelial Cells

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Background—Epidermal growth factor-like domain 7 (Egfl7) is a chemoattractant for endothelial cells, and its expression is restricted to endothelial cells. Hypoxia/reoxygenation (H/R) induced endothelial injury that occurs during transplantation contributes to the subsequent development of allograft vasculopathy. We investigated the effect of Egfl7 on endothelial cell intercellular adhesion molecule 1 expression in response to H/R injury.

Methods and Results—Human coronary artery endothelial cells were submitted to hypoxia (0.1% O2) followed by normoxia (21% O2) in the presence or absence of Egfl7 (100 ng/mL). Hypoxia alone increased the expression of Egfl7×140±8% of control at 3 hours (n=6; *P*<0.05) and 385±50% of control at 6 hours (n=6; *P*<0.001). Incubation with Egfl7 during the reoxygenation period prevented intercellular adhesion molecule 1 upregulation (mean fluorescence intensity: 5.37±0.92 versus 3.81±0.21; *P*<0.05; n=4 per group). Nuclear factor-κB nuclear localization on H/R injury was blocked by Egfl7 administration (cytosolic/nuclear ratio of 0.93±0.01 versus 1.44±0.24; *P*<0.05; n=4 per group). Inhibitor of nuclear factor-κB protein level was significantly reduced on H/R injury (26±6.6% of control expression; *P*<0.05; n=4 per group); however, concurrent incubation with Egfl7 attenuated this reduction (46±6.2% of control expression; *P*<0.05 when compared with H/R injury alone; n=4 per group).

Conclusions—Our study reveals the novel observation that hypoxia upregulates human coronary artery endothelial cells expression of Egfl7 and that Egfl7 inhibits expression of intercellular adhesion molecule 1 subsequent to H/R injury. Mechanistically, Egfl7 prevented nuclear factor-κB nuclear localization and augmented inhibitor of nuclear factor-κB protein levels, suggesting that it inhibits nuclear factor-κB activation, a key step in the inflammatory activation of endothelial cells. Egfl7 may be protective against H/R injury incurred during transplantation and may modulate the events that lead to the development of graft vasculopathy. (Circulation. 2010;122[suppl 1]:S156–S161.)

Key Words: ischemia ■ reperfusion ■ endothelium ■ cell adhesion molecules

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ypoxia/reoxygenation (H/R), which simulates ischemia/reperfusion (I/R) in a cell culture model, is known to injure endothelial cells through oxidant production, which leads to the production of inflammatory cytokines and activates inflammatory cell signaling pathways. This endothelial inflammatory response includes the expression of cell adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), that allow circulating neutrophils to adhere to sites of endothelial injury. Activation of coronary endothelial cells after I/R injury can occur in a number of clinical settings, such as reperfusion, after cardioplogic arrest during cardiac surgery. In particular, I/R induced endothelial injury that occurs with cardiac allograft storage, and subsequent reperfusion during heart transplantation is believed to be a key event that incites subsequent development of allograft vasculopathy.

Epidermal growth factor-like domain 7 (Egfl7) is a novel protein that has been found to be expressed exclusively by endothelial and early endothelial progenitor cells. Studies in animal models of arterial injury have demonstrated Egfl7 to be upregulated in the regenerating endothelium after injury and have demonstrated that Egfl7 acts as a chemoattractant for embryonic endothelial cells. Upregulation of Egfl7 gene expression has also been demonstrated in the brains of neonatal rats subjected to global hypoxic preconditioning. Furthermore, overexpression of Egfl7 in pulmonary artery endothelial cells has been demonstrated to be protective against hyperoxic injury in both animal and cell culture models.
Whether hypoxic injury directly influences Egfl7 expression in endothelial cells has not been reported previously. Furthermore, the role of Egfl7 in the context of endothelial activation in response to injury has not been studied. We hypothesized that exposure to hypoxia would upregulate Egfl7 expression in cultured human coronary artery endothelial cells (HCAECs). We also hypothesized that Egfl7 would have a protective effect against H/R-induced ICAM-1 expression, via inhibiting activation of the nuclear factor-kB (NF-κB) signaling pathway.

Methods

Endothelial Cell Cultures

HCAECs (Lonza) were cultured in endothelial growth medium (EGM-2 MV) containing endothelial growth factors, 5% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in humidified air (5% CO₂). Cells passaged 3 to 5 times and at 80% to 90% confluence were used for this study. For hypoxia studies, confluent HCAECs were exposed to hypoxia (0.1% O₂) by incubation in a ProOx hypoxia chamber system (BioSpherix) at 37°C that subjected the cells to a controlled anoxic gas mixture consisting of N₂ and 5% CO₂. For reoxygenation and normoxic controls, cells were returned to incubation in the standard CO₂ incubator under humidified air (5% CO₂). Cells passaged 3 to 5 times and at 80% to 90% confluence were returned. For NF-κB and IκB signaling pathway during H/R injury, nuclear and cytoplasmic NF-κB fraction was determined by the cytoplasmic/nuclear NF-κB translocation of the nucleus was determined by the cytoplasmic/nuclear NF-κB protein ratio.

Western Immunoblotting

Western immunoblotting was performed using chemiluminescence detection. For Egfl7 and ICAM-1 protein detection, whole cell lysates were used. For NF-κB and IκB-α protein detection, separate nuclear and cytoplasmic protein extracts were obtained from cell pellets using an NE-PER extraction kit (Thermo Scientific) as per the manufacturer’s directions. Protein samples were separated using 4% stacking and 10% running Tris-glycine SDS-PAGE gels. Proteins were then transferred to polyvinylidene fluoride membranes. Blocking was performed in 5% milk solution for 1 hour at room temperature. The monoclonal primary antibodies were used at the following dilutions in 1% milk: Egfl7 1:2000 (Abnova), ICAM-1 1:5000 (R&D Systems), and NF-κB p65 and IκB-α 1:200 (Santa Cruz Biotechnology). Secondary horseradish peroxidase–conjugated antibodies were used at a concentration between 1:5000 and 1:15 000 (Santa Cruz Biotechnology) with ECL Plus used as a substrate (GE Healthcare). β-Actin was detected as a loading control for all of the blots. X-ray films were analyzed using a Bio-Rad GS-800 calibrated densitometer and Bio-Rad Quantity One software (Bio-Rad Laboratories).

Flow Cytometry

Flow cytometry was performed on cells grown on 60-mm plates detached and stained as follows. Cells were washed twice with PBS then incubated with 1 mL of Accutase cell detachment medium (eBioscience) at 37°C for 2 to 10 minutes to detach the cells. Once detached, 2 mL of staining buffer (1% BSA and 20 mmol/L of glucose in PBS) was added to the cell suspension, which was then divided into samples for ICAM-1 and isotype IgG staining. The samples were centrifuged at 400g for 5 minutes at 4°C, and resultant cell pellets were then resuspended in 100 μL of staining buffer containing a 1:100 dilution of either ICAM-1 monoclonal antibody or IgG1 isotype control antibody (R&D Systems). The samples were incubated on ice for 40 minutes, washed with 1 mL of staining buffer, and centrifuged at 400g for 5 minutes at 4°C. The cell pellets were resuspended in 100 μL of staining buffer containing a 1:500 dilution of secondary antibody (Alexa Fluor 488, Invitrogen). The samples were incubated on ice in the dark for 25 minutes, washed with 1 mL of staining buffer, and centrifuged at 400g for 5 minutes at 4°C. The samples were then fixed in 2% parafomaldehyde and resuspended in 200 μL of staining buffer. Cell surface ICAM-1 expression on ±15 000 cells was analyzed using a flow cytometer (Coulter EPICS XL, Beckman Coulter), and data analysis was performed using FlowJo software (version 7.2.5, Tree Star Inc.).

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism software (version 5.0, GraphPad Software). Continuous data are expressed as the mean±SD. Normality of the data was determined using the method of Kolmogorov and Smirnov. A 1-way ANOVA with post hoc pairwise comparisons among all of the groups was performed using the Bonferroni correction for data determined to have a normal distribution, otherwise, a nonparametric Kruskal-Wallis test with post hoc pairwise comparisons among all of the groups was performed using the Dunn post test. Data examining the effect of treatment at various time points were analyzed using a 2-way ANOVA.

Results

Hypoxia Stimulates Egfl7 Expression

Figure 1 depicts the effect of exposure to hypoxia on HCAEC Egfl7 expression after 3 and 6 hours of hypoxia. Exposure to 3 hour of hypoxia significantly increased Egfl7 expression (P<0.05) with a further increase after 6 hours of hypoxia (P<0.001) compared with the normoxic control expression level (n=6 per group).

Egfl7 Inhibits Basal ICAM-1 Expression

Exposure to Egfl7 at varying doses from 0.1 to 100.0 ng/mL for 6 hours reduced total cellular ICAM-1 expression in HCAECs, with ICAM-1 expression dropping maximally to 58±2% of control when a dose of 100 ng/mL was used (P<0.01; n=4 per group; Figure 2A). Cell surface expression of ICAM-1 was significantly attenuated by incubation with Egfl7 (100 ng/mL) for 48 hours (43±6.7% ICAM-1–positive cells versus 71±7.2% ICAM-1–positive control cells; P=0.001; n=4 per group; Figure 2B).

Egfl7 Attenuates H/R-Induced ICAM-1 Expression

Exposure to 24 hours of hypoxia followed by a 12-hour reoxygenation period resulted in increased ICAM-1 expres-
Egfl7 Inhibits NF-κB Nuclear Translocation and Preserves IκB-α

NF-κB is active as a transcription factor when it is in the nucleus and not sequestered by IκB (the α-isof orm being the most prevalent) in the cytoplasm. Exposure to 12 hours of hypoxia followed by a 6-hour of reoxygenation period resulted in significant nuclear translocation of NF-κB (P<0.05 compared with control; n=4 per group; Figure 5A) that was inhibited by treatment with Egfl7 (100 ng/mL) during the reoxygenation period (P<0.05 compared with H/R alone; n=4 per group). Furthermore, H/R alone resulted in a significant decrease in IκB-α level (P<0.05 compared with control; n=4 per group; Figure 5B), which was partially abrogated by treatment with Egfl7 (100 ng/mL) during the reoxygenation period (P<0.05 compared with H/R alone; n=4 per group).

Discussion

Accumulating evidence suggests that Egfl7 may play a protective role in maintaining blood vessel integrity and specifically may have the potential to modulate the endothelial response to injury.8–10,12–15 Endothelial injury that occurs after insults, such as ischemia/reperfusion, results in the expression of inflammatory cytokines and cell adhesion molecules, such as ICAM-1, which allow circulating neutrophils to migrate to sites of endothelial injury where they subject local tissues, including the endothelium, to further injury.1–5 Furthermore, ischemia/reperfusion injury is one mechanism of injury that has been implicated in the development of transplant coronary disease after heart transplantation.7 Given the potential of Egfl7 to be a novel regulator of the coronary endothelial response to injury, we sought to determine the role that it plays in regulating the coronary endothelial response to H/R injury in isolated human coronary artery endothelial cells.
We have made the following novel observations: (1) isolated HCAECs exposed to hypoxia demonstrate an increase in Egfl7 production; (2) incubation with Egfl7 suppress both total cellular and cell surface expression of ICAM-1; (3) incubation with Egfl7 during the reoxygenation period suppresses ICAM-1 production stimulated by H/R injury; (4) incubation with Egfl7 does not adversely affect cell viability; and (5) incubation with Egfl7 inhibits H/R injury–induced NF-κB nuclear translocation and partially preserves IκB-α levels, suggesting that it inhibits NF-κB activation.

**Effect of Hypoxia on Egfl7 Production**

Our study demonstrated that hypoxia stimulates HCAEC Egfl7 production in a seemingly dose-dependent manner. This observation is in keeping with those reported by Gustavsson et al, who reported a 40% increase in Egfl7 gene expression in the brains of neonatal rats that had been subjected to 3 hours of hypoxia. In addition, elegant studies by Xu et al, in both animal and isolated cell culture models of hyperoxic injury, demonstrated that endothelial cell Egfl7 expression is downregulated by hyperoxic injury, suggesting that Egfl7 expression is modulated according to environment.

**Figure 3.** HCAEC ICAM-1 protein expression in response to H/R injury+/-Egfl7. Cell surface and total cellular ICAM-1 protein expression in response to H/R injury+/-Egfl7 100 ng/mL during the reoxygenation period. A, Total cellular ICAM-1 expression induced by H/R injury was significantly attenuated by incubation with Egfl7 during the reoxygenation period (n=4 per group; H/R alone 144±13% of control, †P<0.05 vs control; H/R+Egfl7 56±15% of control, $P<0.01 vs H/R alone). β-Actin was detected as a loading control. B, Upregulated cell surface expression of ICAM-1 induced by H/R injury was significantly attenuated by incubation with Egfl7 during the reoxygenation period (n=4 per group; MFI: 24-hour hypoxia/12-hour reoxygenation 5.37±0.92 versus 24-hour hypoxia/12-hour reoxygenation with Egfl7 3.81±0.21; *P<0.05). MFI indicates mean fluorescence intensity; H, hypoxia; R, reoxygenation.

**Figure 4.** HCAEC cell viability assessed by XTT assay. Incubation with Egfl7 (100 ng/mL) for 24 hours did not significantly affect cell viability vs control (103±2% of control, *P=0.15 vs control; n=4 per group). Although 24 hours of hypoxia significantly decreased cell viability (80±4% of control, ‡P<0.05 vs control), coincubation with Egfl7 did not significantly affect this decrease in viability (74±7% of control, VP=0.18 vs H alone). Similarly, H/R injury significantly decreased cell viability (90±5% of control, †P<0.05 vs control); however, incubation with Egfl7 during the reoxygenation period did not significantly affect this decrease in viability (86±3% of control, $P=0.49 vs H/R alone). H indicates hypoxia; R, reoxygenation.
Effect of Egfl7 on ICAM-1 Production

We determined that exposure to Egfl7 led to decreased basal total cellular and cell surface ICAM-1 expression. ICAM-1 is basally expressed in endothelial cells; however, cell surface levels normally remain low. On endothelial activation by stimulation from injury or exposure to inflammatory mediators, ICAM-1, as well as other cell adhesion molecules such as VCAM-1, P-selectin, and E-selectin, are translocated to the cell surface. These adhesion molecules play a critical role in interacting with circulating neutrophils, causing them to roll on and eventually adhere to endothelial cells. Neutrophils can then transmigrate between activated endothelial cells to sites of injury, where they further propagate tissue injury and inflammation. Our finding that Egfl7 suppresses basal endothelial ICAM-1 levels suggested that it may be capable of reducing the intensity of acute cell surface expression of adhesion molecules on injury by limiting the basal intracellular stores of ICAM-1. We sought to examine this prospect in a cell culture model of H/R injury.

Our study demonstrated the important confirmatory finding that Egfl7 inhibited total cellular and cell surface expression of ICAM-1 expression in response to H/R injury when cells were exposed to Egfl7 during the reoxygenation phase of the injury. Fukushima et al have reported previously that inhibition of ICAM-1 and/or P-selectin using antibody therapy immediately before reperfusion in a rat model of myocardial I/R injury was successful in attenuating infarct size and reducing the accumulation of leukocytes in the ischemic area. Thus, our finding that Egfl7 limits HCAEC expression of ICAM-1 after H/R injury leads us to hypothesize that it may also be protective against myocardial I/R injury in keeping with the findings of Fukushima et al.

Effect of Egfl7 on NF-κB Activation

Our study demonstrated that Egfl7 inhibits nuclear translocation of NF-κB that occurs on H/R injury in HCAECs. It is well established that NF-κB plays a key role in regulating the expression of endothelial cell adhesion molecules, such as ICAM-1. H/R injury results in the production of reactive oxygen species, and these intermediates are involved in NF-κB activation and binding of activated NF-κB to its associated DNA binding site. In unstimulated and uninjured cells, NF-κB is sequestered in the cytoplasm in an inactive state by IκB isoforms, IκB-α being the most abundant isoform. On stimulation, IκB is phosphorylated and degraded, which releases NF-κB and allows it to translocate to the nucleus to act as an active transcription factor. Nuclear translocation of NF-κB in H/R-injured endothelial cells has been implicated in the enhanced transcription-dependent expression of cell adhesion molecules, because the promoter regions of the genes for these molecules contain NF-κB binding sites that are essential for the expression of these proteins on endothelial cells. Thus, our finding that Egfl7 inhibits nuclear translocation of NF-κB on H/R injury suggests that it inhibits NF-κB activation, which may be the mechanism by which Egfl7 inhibits ICAM-1 expression. Furthermore, we found that Egfl7 partially maintains levels of IκB-α in H/R-injured HCAECs, suggesting that stabilization of IκB-α protein may be one of Egfl7’s downstream effects.
that protects against NF-κB activation. However, because we were unable to completely block the degradation of IkB-α in our H/R studies with Egfl7, there may be another significant pathway that mediates Egfl7’s inhibitory effect on NF-κB nuclear translocation.

Study Limitations
Our study provides evidence that Egfl7 inhibits coronary endothelial ICAM-1 expression in an in vitro model of I/R injury, likely through inhibition of NF-κB. Because the physiological level of Egfl7 is not currently known, we may have used a supraphysiologic dose of Egfl7 that may not be entirely representative of what occurs in vivo. The potential beneficial effects of Egfl7 on attenuating I/R injury are tentative and require further study. Although our study demonstrated attenuated ICAM-1 expression in response to Egfl7, it does not exclude effects on other cell adhesion molecules and inflammatory signaling pathways.

Summary
In conclusion, Egfl7 inhibits HCAEC expression of ICAM-1 both basally and subsequent to H/R injury. Mechanistically, Egfl7 prevented NF-κB nuclear localization and augmented IkB-α protein levels, suggesting that it inhibits NF-κB activation, a key step in the inflammatory activation of endothelial cells. Egfl7 may be protective against H/R injury incurred during events such as heart transplantation or after percutaneous coronary intervention or coronary artery bypass grafting for acute myocardial infarction. Augmentation of Egfl7 may be a novel therapeutic strategy to modulate the inflammatory events that prime the endothelium for the future development of atherosclerosis, in-stent restenosis, and/or transplant vasculopathy.

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Disclosures
None.

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