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Gap Junction Protein Cx37 Interacts With Endothelial Nitric Oxide Synthase in Endothelial Cells

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Abstract

Objective—The gap junction protein connexin37 (Cx37) plays an important role in cell-cell communication in the vasculature. A C1019T Cx37 gene polymorphism, encoding a P319S substitution in the regulatory C terminus of Cx37 (Cx37CT), correlates with arterial stenosis and myocardial infarction in humans. This study was designed to identify potential binding partners for Cx37CT and to determine whether the polymorphism modified this interaction.

Methods and Results—Using a high-throughput phage display, we retrieved 2 binding motifs for Cx37CT: WHK ... [K,R]XP...and FHK ... [K,R]XXP..., the first being more common for Cx37CT-319P and the second more common for Cx37CT-319S. One of the peptides (WHRTPLPPVP) showed 77.7% homology with residues 843 to 854 of endothelial nitric oxide synthase (eNOS). In vitro binding of this peptide or of the homologous eNOS sequence to both Cx37CT isoforms was confirmed by cross-linking and surface plasmon resonance. Electrophysiological analysis of Cx37 single channel activity in transfected N2a cells showed that eNOS-like and eNOS(843–854) increased the frequency of events with conductances higher than 300 pS. We demonstrated that eNOS coimmunoprecipitated with Cx37 in a mouse endothelial cell (EC) line (bEnd.3), human primary ECs, and a human EC line transfected with Cx37-319P or Cx37-319S. Cx37 and eNOS colocalized at EC membranes. Moreover, a dose-dependent increase in nitric oxide production was observed in ECs treated with Cx37 antisense.

Conclusion—Overall, our data show for the first time a functional and specific interaction between eNOS and Cx37. This interaction may be relevant for the control of vascular physiology both in health and in disease.

Keywords

connexin37; GJA4; endothelial nitric oxide synthase; gene polymorphism

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Anna Pfenniger, and Jean-Paul Derouette, contributed equally to this study as first authors, and Brenda R. Kwak and Mario Delmar contributed equally as senior authors.

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Connexins are integral membrane proteins that oligomerize to form intercellular channels called gap junctions. Gating, permeability, and regulatory properties of a gap junction channel vary depending on the connexin isoform. The present study focuses on connexin37 (Cx37), an isoform expressed mostly in endothelial cells (ECs), monocytes, and macrophages but also found in vascular smooth muscle cells.¹ Studies on genetically modified mouse models suggest an involvement of Cx37 in atherogenesis.² Additional studies have shown atherosclerosis-related changes in Cx37 expression; in particular, Cx37 can no longer be detected in the endothelium overlaying advanced atheromas, whereas there is high expression in macrophage foam cells and in previously Cx37-deprived medial smooth muscle cells.³ Studies in humans indicate that a polymorphism at position 1019 of the *GJA4* gene, encoding human Cx37, may be a prognostic marker for atherosclerosis.¹ This C1019T polymorphism codes for a proline-to-serine substitution (P319S) in the C-terminal domain of the protein. Two separate studies showed a correlation between the Cx37-319S isoform and an increased incidence of myocardial infarction in the sampled population.^{4,5} The Cx37-319S genotype was also shown to predict survival after an acute coronary syndrome.⁶ In 3 additional studies, the alternative isoform (Cx37-319P) was found to segregate with an increased incidence of stenosis in the coronary arteries^{7,8} or the carotid arteries.⁹ Studies at the cellular level recently showed that Cx37 expression inhibited monocyte adhesion to the endothelium; this effect was more pronounced in monocytes expressing Cx37-319P than in those expressing Cx37-319S.² Overall, the data support the notion that Cx37 could play a protective role against the development of atherosclerosis, and they leave open the possibility that variations in the sequence of the C terminus of Cx37 (Cx37CT) could have functional effects relevant to pathophysiology.

Little is known about the functional role of Cx37CT. Primary sequence analysis of this region identifies several potential consensus sites for phosphorylation and for the binding of Cx37 to other molecules.^{10,11} It is thus reasonable to speculate that, as in the case of other connexin isoforms,¹² the C-terminal domain is the major regulatory domain of Cx37, and, perhaps, the P319S polymorphism alters Cx37 regulation. However, an unbiased search for potential molecular partners of Cx37 remains to be conducted.

In the present study, we have carried out a high-throughput phage display screening in search for peptidic sequences that bind to Cx37CT. Our results show that Cx37-319P preferentially binds to peptides containing the motif WHK ... [K,R] XP..., whereas Cx37-319S displays a preference for FHK ... [K,R]XXP ... motifs. Moreover, we identified a particular peptidic sequence homologous to a region of endothelial nitric oxide synthase (eNOS), an enzyme of fundamental importance to vascular biology and disease.¹³⁻¹⁵ Additional studies showed that this peptide (called eNOS-like) or the homologous sequence of eNOS (called eNOS[843-854]) could affect the function of Cx37 channels. Moreover, these results led us to demonstrate that Cx37 associates with eNOS in the native environment of ECs, thereby functionally affecting the enzyme. Overall, our data indicate a possible cross-talk between these 2 molecules and suggest a functional role for this interaction in the production of nitric oxide (NO) in the vascular endothelium.

Methods

An expanded Methods section is available in the Data Supplement, available online at <http://atvb.ahajournals.org>. In brief, production of recombinant Cx37CT, phage display, surface plasmon resonance (SPR), and cross-linking experiments on Cx37CT-319P or Cx37CT-319S were performed following methods previously described.¹⁶⁻¹⁸ Cell culture of murine neuroblastoma cells (N2a; American Type Culture Collection, Manassas, VA), a mouse EC line (bEnd.3),¹⁹ a human EC line (EA.hy926; American Type Culture Collection), and human umbilical vein ECs followed standard protocols. Transfection of EA.hy926 and N2a cells with Cx37-319P or Cx37-319S was conducted as previously described.² Cx37 channel

properties in the presence of peptides was assessed by double patch clamp on transfected N2a cells. The interaction of Cx37 with eNOS in ECs was determined by coimmunoprecipitation using specific antibodies directed against each protein, and colocalization was assessed by coimmunofluorescence. Total nitrite and nitrate production in ECs was measured in the culture supernatant using the Total Nitric Oxide Assay Kit (Assay Designs) according to the manufacturer's instructions. All results are presented as mean±SEM. Unpaired *t* test was used to compare differences between 2 groups, and ANOVA was used for comparison of multiple groups. Data were considered statistically significant at $P<0.05$.

Results

Phage Display

We analyzed the sequence of the insert retrieved from a total of 120 plaques for each Cx37CT isoform. Of the estimated 2.5×10^9 different sequences presented in the phage display, 44 were captured by Cx37CT-319P and 41 by Cx37CT-319S. Thirteen of these sequences were recovered by both baits. The corresponding peptide sequences and the number of plaques analyzed containing the same sequence are presented in Supplementary Table I. Compared with the prebound library, basic residues were more frequently found in captured peptides, whereas acidic residues showed an opposite trend (Supplementary Table II). In addition, most peptides presented amino acids [F,W]HK in positions 1 to 3 and included the motif [K,R]XP or [K,R]XXP as part of their sequence (Supplementary Table I). Motif [K,R]XP was more common in peptides captured by Cx37CT-319P, whereas [K,R]XXP was more common in those peptides captured by Cx37CT-319S.

We calculated the probability of occurrence of these motifs by chance alone, taking into account the experimental abundance of individual amino acids. The results indicate that the actual occurrence of the specific sequences was significantly higher than their expected probability (Supplementary Tables III and IV). Overall, the data support the notion that Cx37CT-319P bound with enhanced selectivity to peptides containing the motif WHK...[K,R]XP..., whereas the sequence FHK . . .[K,R] XXP ... was selected by Cx37CT-319S. Moreover, sequence alignments against the National Center for Biotechnology Information protein database (BLAST) indicated homology between one of the selected peptides and amino acids 843 to 854 of eNOS (Table), with the eNOS(843-854) sequence containing both [K,R]XP and [K,R]XXP motifs. Given the biological importance of eNOS in cells where Cx37 is naturally present, we pursued further characterization of these peptides and the C-terminal domain of Cx37.

In Vitro Binding Detected by SPR

Phage display allows screening for potential binding sequences, but it does not give information on the characteristics of the binding reaction. We therefore used SPR to quantitatively characterize binding of selected peptides to Cx37CT. Our studies focused on the peptide showing the highest homology to eNOS (eNOS-like) and a 12-mer peptide corresponding to sequence 843 to 854 of eNOS (eNOS[843-854]). Each Cx37CT isoform was bound to a separate carboxymethyl dextran matrix. Synthetic peptides were presented at concentrations varying between 1 mmol/L and $62.5 \mu\text{mol/L}$ (for eNOS-like) or between 0.5 mmol/L and $62.5 \mu\text{mol/L}$ (for eNOS[843-854]), and the time course and amplitude of binding were recorded. The perfusion of eNOS-like or eNOS(843-854) peptides elicited a concentration-dependent resonance shift indicative of binding to Cx37CTs (Figure 1). As a control, a scrambled peptide at 0.5 mmol/L was presented to Cx37CTs, which did not result in any significant resonance shift (Supplementary Figure I, available online at <http://atvb.ahajournals.org>). The binding of eNOS-like to Cx37CT-319P or Cx37CT-319S was best described by a first-rate order model with average dissociation constants (K_D) of $66 \pm 7 \mu\text{mol/L}$ and $65 \pm 9 \mu\text{mol/L}$, respectively. The binding of eNOS(843-854) to Cx37CT-319P yielded a K_D of $144 \pm 31 \mu\text{mol/L}$, whereas the

interaction of this same peptide with Cx37CT-319S was too weak to allow for proper quantitative analysis. These K_D values were not statistically different ($N=3$). As a control for specificity, both peptides were presented to mouse Cx40CT or rat Cx43CT, which did not result in any significant resonance shift (Supplementary Figure II). Overall, our data show that these peptides are able to interact specifically with a recombinant Cx37CT.

Cross-Linking of Cx37CT to Peptides

Whereas SPR analysis requires the physical constraint of the bait protein to the sensor chip matrix, the use of a cross-linker reagent allows for the study of intermolecular interactions when the purported molecular partners are free in solution. Figure 2 shows the results obtained from the cross-linking of Cx37CT to either eNOS-like (Figure 2A) or eNOS(843–854) (Figure 2B). After separation by SDS-PAGE, samples were stained with Coomassie Blue. A band of ≈ 11 kDa representing Cx37CT could be seen in all samples. In the samples incubated with the cross-linking reagent (Figure 2A and 2B, first, second, fourth, and fifth lanes from the left in each panel), some supplementary bands were observed in the range between 22 and 36 kDa, likely resulting from polymerization of Cx37CT. The second, third, fifth, and sixth lanes in Figure 2A and 2B show results obtained in the presence of peptides. Bands of low molecular mass, corresponding to monomers or multimers of the peptides, can be observed. Interestingly, in the second and fifth lanes (Figure 2A and 2B), where samples contain one isoform of Cx37CT, eNOS-like or eNOS(843–854) peptides and the cross-linker, a supplementary band can be seen above the Cx37CT band. The mobility of this band corresponds to the estimated molecular mass of a Cx37CT-peptide complex (≈ 12 kDa). The density of this band was actually higher than that of the multimers of Cx37CT, suggesting a higher affinity for the peptide-protein interaction than for oligomerization of the free Cx37CT.²⁰ A control experiment performed in the presence of ^{37,40}Gap26, a Cx37 channel-blocking peptide,² did not reveal any cross-linking of this peptide and Cx37CT (data not shown). Our results are consistent with those obtained by SPR and suggest that eNOS-like and eNOS(843–854) bind to both Cx37CT isoforms.

Effect of eNOS-Like and eNOS(843–854) on the Function of Cx37 Channels

The ability of eNOS-like and eNOS(843–854) peptides to bind Cx37CT led us to hypothesize that the peptides may also change the behavior of Cx37 channels. Gap junction currents were recorded from N2a cells transfected with Cx37-319P or Cx37-319S. To reduce macroscopic currents, cell pairs were superfused with 2 to 4 mmol/L octanol.

Frequency histograms of unitary conductances obtained from Cx37-319P or Cx37-319S cell pairs in the presence of a scrambled peptide (Figure 3A and 3D, respectively) show that, as previously described, Cx37 channels transit between various conductive states up to 400 pS, 11 with a trend for Cx37-319S channels to reside at smaller unitary conductance states.²¹ Interestingly, the presence of eNOS-like or eNOS(843–854) alters the frequency of high-conductance events (Figure 3B, 3C, 3E, and 3F). Quantitative analysis showed that for Cx37-319P, the frequency of events corresponding to channels of 300 pS or higher increased from 33% to 72% in the presence of eNOS-like and to 44% in the presence of eNOS(843–854) (Figure 3A through 3C); for Cx37-319S, the same parameter changed from 14% in control to 37% in the presence of eNOS-like and to 57% in the presence of eNOS(843–854) (Figure 3D through 3F). Examples of single channel traces with all-points histograms are shown in Supplementary Figure III. Overall, the data indicate that eNOS-like and eNOS(843–854) can alter the function of Cx37 channels, suggesting that eNOS could modify Cx37 properties and, likely, its function within the cellular environment.

Effect of Cx37 Interaction on eNOS Function

To study the possible interaction between eNOS and Cx37 in a cellular environment, we performed coimmunoprecipitation studies using the mouse EC line bEnd.3 which constitutively expresses all vascular connexins. Cx37 antibody immunoprecipitates from bEnd.3 cells contained a ≈ 140 -kDa protein that was immunoreactive to eNOS antibodies (Figure 4A, second lane of left panel). Conversely, eNOS antibodies immunoprecipitated a protein recognized by Cx37 antibodies (Figure 4A, second lane of right panel). No signal for eNOS or Cx37 was observed in control experiments, where the immunoprecipitating antibody was omitted from the procedure (Figure 4A, right lane of each panel). The subcellular localization of eNOS and Cx37 was next examined by immunofluorescence. As expected,^{19,22} a strong Cx37 signal was observed at cell-cell contacts in bEnd.3 cells. After incubation with eNOS antibodies, bEnd.3 cells showed a staining in the perinuclear region and at cell membranes. Cx37 and eNOS colocalization was observed mostly in regions of cell-cell contacts (Figure 4B). In contrast, Caveolin-1 (Cav1), a protein known to interact with eNOS, was mostly localized intracellularly in bEnd.3. When examined by confocal microscopy, this protein did not colocalize with Cx37 in bEnd.3 cells (Supplementary Figure IV).

Previous studies indicated that eNOS enzyme activity might be affected by interacting proteins.^{23,24} The colocalization and coimmunoprecipitation of eNOS and Cx37 in the EC line led us to hypothesize that the interaction may also affect the activity of eNOS. In the next series of experiments, we exposed bEnd.3 cells to Cx37 antisense. As illustrated in Figure 4C, Cx37 expression was considerably decreased in response to 50 $\mu\text{mol/L}$ Cx37 antisense (right panel), whereas the same concentration of sense oligonucleotides did not affect the expression of the protein (left panel). Constitutive NO production by bEnd.3 cells, as evaluated by the measure of the end product nitrite, was 0.403 ± 0.030 nmol/h per 10^5 cells (N=8). Interestingly, this nitrite production dose-dependently increased with increasing concentration of Cx37 antisense (Figure 4D). This effect was not due to an upregulation of eNOS expression nor to a modification in expression levels of Cx40, Cx43 or Cav1 (Figure 4E). Inducible nitric oxide synthase (iNOS), an alternative source of NO, could not be detected under all conditions compared with a 5-fold smaller amount of stimulated macrophage lysate. Moreover, Cx37 antisense did not affect eNOS or Cav1 subcellular localization (Supplementary Figure IV). On the other side, downregulation of Cx40 or Cx43 with their respective antisense oligonucleotides did not consistently modify nitrite production in bEnd.3 (Supplementary Figure V). Overall, the data suggest that the interaction with Cx37 decreases the activity of eNOS in a specific way.

As bEnd.3 cells have been transformed with polyomavirus middle T antigen, they might harbor functional differences from native ECs. We therefore carried out experiments using human umbilical vein endothelial cells (HUVECs; passages 1 to 2). Similar to the mouse cell line, Cx37 immunoprecipitation of HUVECs showed a ≈ 140 -kDa protein consistent with eNOS, which was absent in the control condition (Figure 5A). To assess the functional consequence of this interaction on eNOS, HUVECs were exposed to Cx37 antisense. Incubation with 50 $\mu\text{mol/L}$ Cx37 antisense effectively repressed Cx37 expression (Figure 5B, bottom panel) compared with the same concentration of Cx37 sense (middle panel) or the absence of oligonucleotides (top panel). Similar to bEnd.3 cells, production of nitrite by HUVECs increased in response to Cx37 antisense exposure (Figure 5C). Constitutive nitrite production from HUVECs was 0.062 ± 0.008 nmol/h per 10^5 cells (N=9). These data confirm that the interaction of Cx37 and eNOS is also present and functionally relevant in primary human ECs.

To assess the effect of the Cx37 P319S polymorphism on this interaction, we transfected the human EC line EA.hy926 with Cx37-319P or Cx37-319S. Cx37 antibody immunoprecipitates obtained from the lysates of ECs transfected with both isoforms of Cx37 contained a ≈ 140 -kDa protein that was immunoreactive to eNOS antibodies (Figure 5D, second and fifth lanes).

Control experiments using EA.hy926/empty vector transfectants, HeLa/Cx37-319P or HeLa/Cx37-319S transfectants showed the absence of an eNOS-immunoreactive protein in the precipitate (Figure 5D, first, third, and fourth lanes), further illustrating the specificity of this reaction.

Discussion

We have used phage display to identify peptides capable of binding to Cx37CT-319P and Cx37CT-319S. Our results identified 2 main consensus motifs, [K,R]XP and [K,R]XXP, in the peptidic sequences binding to Cx37CT. By comparing the sequences we obtained with the protein database, we found one particular peptide with homology to a region of the enzyme eNOS. Binding of this peptide, as well as of the corresponding sequence of eNOS to both isoforms of Cx37CT, was then assessed by various in vitro methods and in a cellular environment. In addition, functional consequences of the interaction were evaluated. Before discussing these results and their implications, we will address a few technical aspects of our study.

Technical Considerations

Phage display is a method that allows for high-throughput screening of binding sequences. However, it has limited sensitivity, and as such, it is possible to miss sequences of interest. Although the peptides found are capable of interacting with Cx37CT fragments, we likely failed to detect peptides of biological relevance that correspond to sequences of native Cx37 partners. It is also likely that some of the identified peptides lack biological relevance. In this study, we decided to focus our attention on one peptide (eNOS-like), as it opened the possibility that these 2 proteins, Cx37 and eNOS, may interact in intact cells.

Surface plasmon resonance is a powerful technique to measure binding kinetics of a peptide to a protein. To detect a resonance shift, the target (in our case each isoform of Cx37CT) was covalently bound to a matrix. The consequence of this step is that the configuration of Cx37CT might be modified and might not reflect the proper structural order of the protein when in solution (or in vivo). Moreover, SPR is limited in its ability to detect low-affinity interactions, particularly when these involve molecules of low mass. However, those limitations notwithstanding, SPR allowed us to characterize the interaction of eNOS-like to Cx37 and determine, comparatively, the interactions to the 2 Cx37 polymorphs. Cross-linking experiments are an alternative method to assess binding in vitro. They allow for studying the interaction of peptidic molecules in a soluble conformation. In our study, this method confirmed the results obtained by SPR, which indicate that both synthetic peptides, eNOS-like and the corresponding sequence of eNOS, interact with both isoforms of Cx37CT with comparable affinities. However, those experiments were performed with small peptides and therefore do not prove an interaction between Cx37 and the complete eNOS protein.

Binding Motifs

Based on the occurrences of the 2 motifs identified, we constructed a consensus motif for sequences binding to each isoform of Cx37CT. For Cx37CT-319P, the consensus motif would be WHK ... [K,R]XP ... and for Cx37CT-319S, FHK ... [K,R]XXP. ... Our results showed that the Cx37CT consensus binding motifs are rather similar to those previously identified for Cx43.¹⁷ This indicates that, though different in primary sequence, the two C-terminal domains may share higher order structures. The identification of the RXP motif led to the discovery of a peptide (RXP-E) that is able to interfere with the regulation of Cx43.¹⁷ Whether peptides known to affect the function of Cx43 could also affect the function of Cx37 (or vice versa) remains to be determined. However, eNOS-like and eNOS(843–854) did not bind to Cx43CT in vitro, suggesting a specific interaction with Cx37CT.

eNOS and Cx37CT

We have shown by 3 different methods that a peptide similar to a part of eNOS binds to Cx37CT. The homologous sequence extracted from amino acids 843 to 854 of eNOS bound to Cx37CT as well, even though with a lower affinity. These *in vitro* studies suggest a possible relationship between those 2 proteins *in vivo*. The synthesis of NO by eNOS in ECs is well known to play a vasoprotective role by different mechanisms. Thus, endothelium-derived NO controls vascular tone, inhibits leukocyte adhesion to the endothelium, inhibits platelet aggregation, and decreases endothelial permeability.^{13,14} Endothelium-derived NO also inhibits vascular smooth muscle cell migration and proliferation. Adequate levels of NO are thus important to preserve normal vascular physiology; diminished NO bioavailability can lead to endothelial dysfunction and increased susceptibility to atherosclerosis.¹⁵ Interestingly, Cx37 can also be considered a vasoprotective and antiatherosclerotic protein.¹ Although the normal endothelium displays sizable Cx37 expression throughout the vascular tree, Cx37 expression is decreased in response to factors inducing endothelial dysfunction.^{3,22,25-27} Moreover, Cx37-deficient mice show enhanced susceptibility to atherosclerosis.² The close association between Cx37 expression levels and eNOS activity led us to hypothesize that the 2 proteins might interact in ECs and affect each other's function.

In agreement with previous studies,^{11,21,28} we have shown here that human Cx37 channels in N2a cells transit between multiple conductive states up to ≈ 400 pS. As shown in Figure 3A and 3D, the frequency of high-conductance events (>300 pS) in the presence of a scrambled peptide was higher for Cx37-319P channels (33%) compared with Cx37-319S channels (14%). In analogy to Cx43,²⁹ this may reflect differences in posttranslational modification, such as phosphorylation, between the 2 polymorphic proteins. Cx37CT contains indeed multiple consensus sequences for phosphorylation by various protein kinases, including the serine at position 319. We have also shown that the frequency of high-conductance events was increased in the presence of eNOS-like (Figure 3B and 3E) or eNOS(843–854) (Figure 3C and 3F), suggesting that eNOS-Cx37 interaction could modify Cx37 channel properties. In addition, it has recently been shown that exposure to an NO donor may reduce the permeability of Cx37 gap junctions to small molecules.³⁰ The regulation of Cx37 gap junctions by eNOS could therefore implicate several pathways, either via a direct protein-protein interaction or via its product NO.

Consistent with our *in vitro* binding experiments (SPR and cross-linking), the two peptides induced similar modifications of channel properties for either polymorphic protein. This suggests that the interaction of Cx37CT and eNOS does not involve the region modified by the P319S polymorphism.

The mouse EC line bEnd.3, known to constitutively express Cx37, allowed for studying the interaction of eNOS with Cx37 in a cellular environment. Our studies revealed that eNOS coimmunoprecipitated with Cx37 in these cells. Because the use of polyomavirus middle T antigen transformation to immortalize the bEnd.3 cell line might result in aberrant protein regulation and intracellular localization, and hence in artifactual coimmunoprecipitation results, another, more physiological model was needed to confirm these results. Primary ECs, such as HUVECs, are usually avoided for this kind of study because the expression of Cx37 progressively decreases on repeated passage with virtually no remaining protein at passage 3 to 4.³¹ To overcome this limitation, we restricted the use of primary cells to HUVECs at very early passages (passage 1 or 2) for our study. As in the mouse cell line, eNOS coimmunoprecipitated with Cx37 in these primary ECs, which strengthens the relevance of this newly identified interaction. The next logical step is to clarify whether the Cx37 polymorphism affects this interaction in human cells. As it is ethically problematic to genotype cells isolated from umbilical cords, we transfected the human EC line EA.hy926 with both Cx37 polymorphic proteins. The coimmunoprecipitation of eNOS with both isoforms of Cx37

confirmed the absence of functional effect of this polymorphism on the interaction of Cx37 with eNOS.

Our functional studies demonstrated that decreasing Cx37 expression enhanced production of nitrite, the end-product of NO, in bEnd.3 cells as well as HUVECs, and that this increase was not caused by modifications in eNOS or Cav1 expression and localization. Moreover, an indirect effect of Cx37 on eNOS via other endothelial connexins could be excluded, suggesting that the eNOS-Cx37 interaction could directly modify enzyme activity. Structural studies have revealed that eNOS is a multidomain enzyme consisting of an N-terminal oxygenase domain (amino acids 1 to 492), and a reductase domain (amino acids 493 to 1205).²³ During NO synthesis, NADPH-derived electrons pass into the reductase domain flavins (flavin mononucleotide and flavin-adenine dinucleotide) and are then transferred to the heme located in the oxygenase domain that catalyzes stepwise NO synthesis from L-arginine. Calmodulin binding is known to activate NO synthesis by enabling the reductase domain to transfer electrons. The 843 to 854 binding motif resides in the eNOS reductase domain, a part that also contains a Cav1 binding domain.³² Although the possible modes of Cx37-based inhibition remain to be explored, one could imagine that, similar to Cav1, Cx37 binding to eNOS reductase compromises its ability to bind calmodulin, thereby inhibiting NO synthesis. Even though other connexins are known to interact with caveolins³³ and Cx37 was demonstrated to be expressed in caveolae-like vesicles in aortic ECs,³⁴ Cx37 did not colocalize with Cav1 in the bEnd.3 cells used for our studies.

As mentioned above, both Cx37 and eNOS play a vasoprotective role, and thus, the observation that downregulation of Cx37 leads to an increased NO production was unexpected. Several factors may contribute to this apparent discrepancy. For instance, the present study has been performed *in vitro* and thus under static conditions. A disturbed or absent flow is known to promote endothelial dysfunction and to affect NO synthesis.²⁴ Therefore, the situation may be quite different *in vivo*, where an absolutely static condition is almost never present. It is also known that proatherogenic factors, such as hypercholesterolemia and hypertension, can cause eNOS uncoupling, a deleterious condition leading to increased reactive oxygen species production.³⁵ It is conceivable that *in vivo*, the inhibitory property of Cx37 on eNOS may mostly affect reactive oxygen species production under pathological conditions rather than NO synthesis in more physiological states.

In summary, the data presented here demonstrate for the first time an interaction between Cx37 and eNOS in ECs. However, this study could not detect a difference between the interactions of each isoform of Cx37CT with the peptides corresponding to eNOS. This suggests that the interaction of Cx37CT and eNOS does not involve the region modified by the P319S polymorphism. It also indicates that the epidemiological correlation between the Cx37 polymorphism and atherosclerosis likely does not result from a modification in eNOS function. Nevertheless, this newly identified interaction may lead to new insights in vascular physiology and pathologies, such as hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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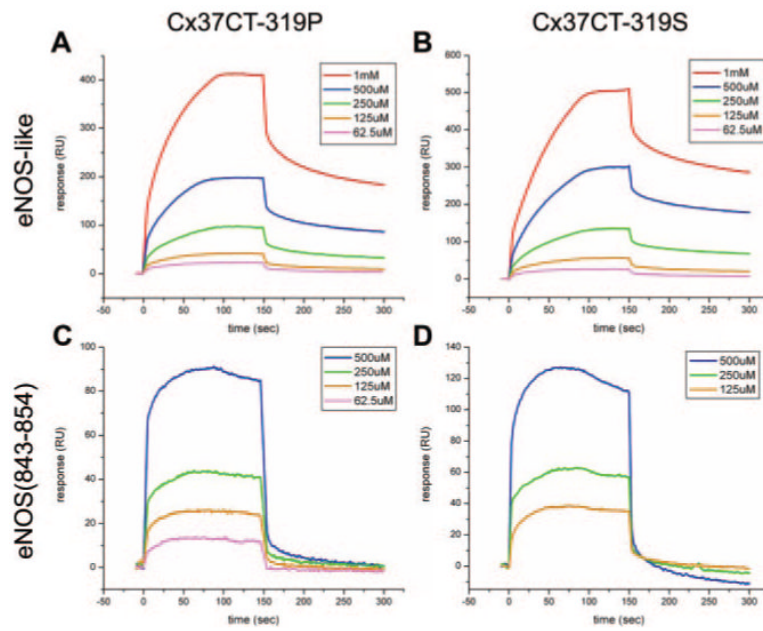


Figure 1. SPR on Cx37CT and binding peptides. Representative examples (N=3) of association and dissociation curves of peptides to Cx37CT are shown for each condition. A, Cx37CT-319P and eNOS-like. B, Cx37CT-319S and eNOS-like. C, Cx37CT-319P and eNOS(843–854). D, Cx37CT-319S and eNOS(843–854). Peptides were superfused at concentrations of 1 mmol/L (red), 500 μmol/L (blue), 250 μmol/L (green), 125 μmol/L (orange), and 62.5 μmol/L (pink).

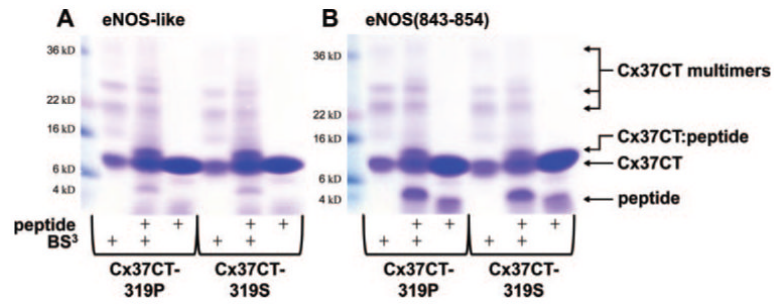


Figure 2.

Cross-linking experiment on Cx37CT and peptides. The binding in solution of eNOS-like (A) and eNOS(843–854) (B) to Cx37CT-319P or Cx37CT-319S was assessed by the incubation with the cross-linker BS³. Second and fifth lanes of each panel show an additional band at ≈12 kDa, which is absent in the other lanes, where either the peptide or the cross-linker is missing.

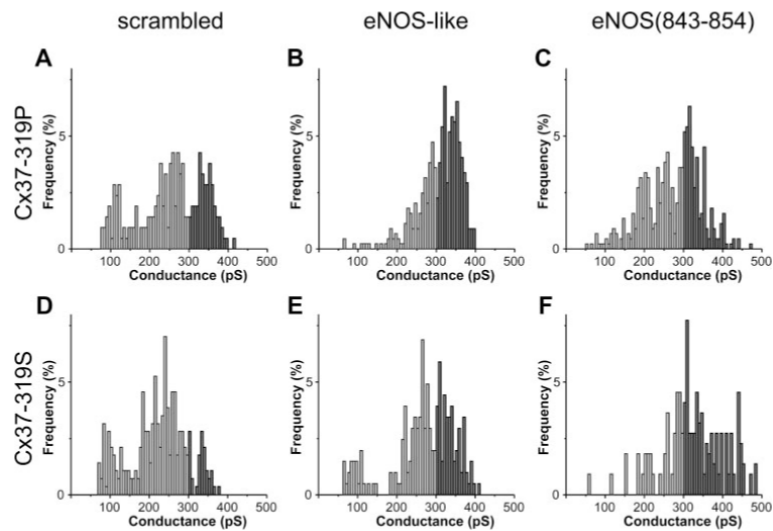


Figure 3.

Single-channel data from N2a cells transfected with Cx37-319P and Cx37-319S. All events histograms of unitary conductance in cells transfected with Cx37-319P or Cx37-319S in the presence of a scrambled peptide (A, N=4, n=211; D, N=5, n=286, respectively), eNOS-like (B, N=5, n=447; E, N=4, n=205, respectively), or eNOS(843-854) (C, N=4, n=462; F, N=3, n=276, respectively) in the pipette solution. The presence of eNOS-like or eNOS(843-854) increased the number of channel openings larger than 300 pS (shaded bars) compared with the control condition. Minimum event duration was 50 ms. Bin width=6.25 pS.

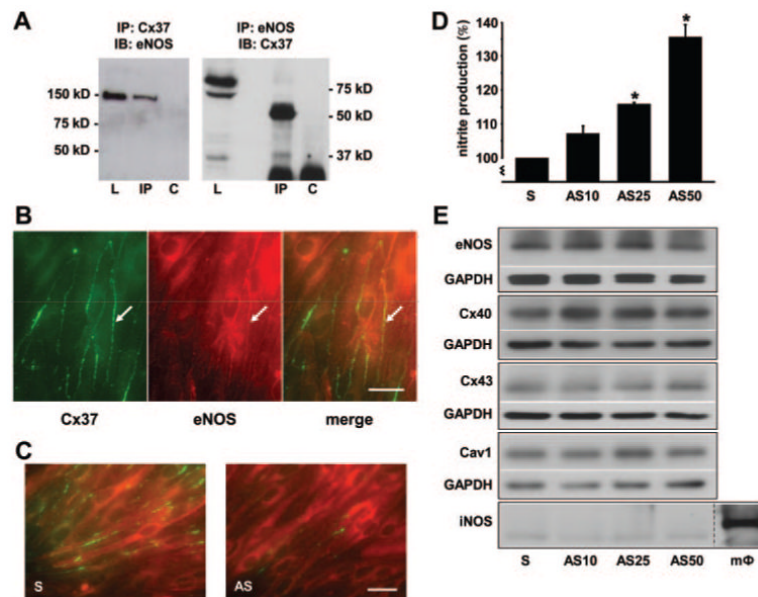


Figure 4.

Cx37 and eNOS interact in a mouse EC line. A, Immunoprecipitation of eNOS and Cx37 in bEnd.3. Left panel: eNOS immunoblot of Cx37 immunoprecipitate. Right panel: Cx37 immunoblot of eNOS immunoprecipitate. L indicates total lysate; IP, immunoprecipitate; and C, negative control. B, Cx37 and eNOS immunostaining in bEnd.3. eNOS was found perinuclearly and near membranes. Cx37 was detected at sites of cell-cell contact. Merged images show areas of colocalization (arrow). Scale bar represents 30 μm . C, Cx37 immunostaining (green) of bEnd.3 incubated with Cx37 sense (left) or Cx37 antisense (right). Cells were counterstained with Evans Blue (red). Scale bar represents 20 μm . D, Nitrite release by bEnd.3 incubated with Cx37 sense or 10, 25, or 50 $\mu\text{mol/L}$ Cx37 antisense for 48 hours. Overnight nitrite release increased with increasing dose of antisense. N=6, error bars show SEM, and $*P<0.05$. E, Lysates of bEnd.3 incubated with Cx37 antisense for 48 hours were immunoblotted against eNOS, Cx40, Cx43, Cav1, and inducible nitric oxide synthase (iNOS). m Φ : stimulated mouse macrophages.

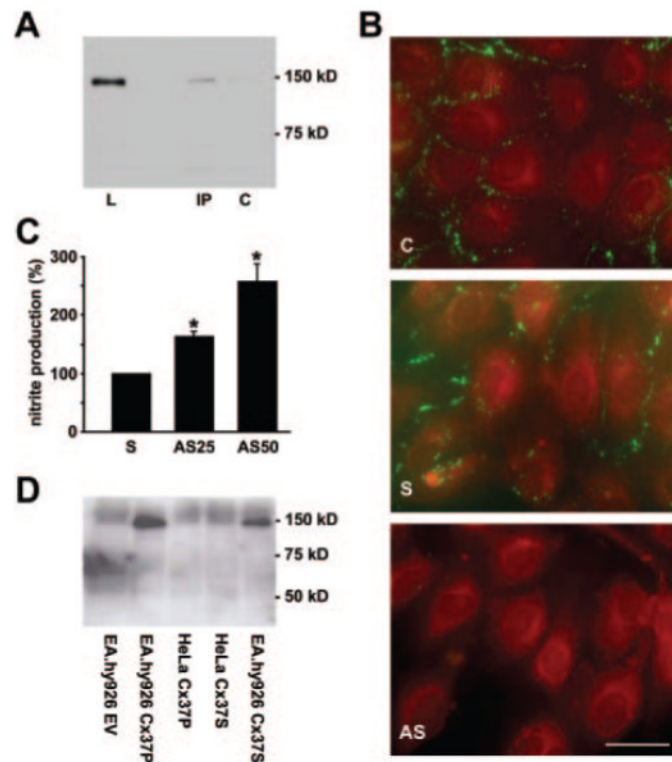


Figure 5.

Cx37 and eNOS interact in primary and transfected human ECs. A, Immunoprecipitation of eNOS with Cx37 in HUVECs. Lanes are as described in Figure 4A. B, Cx37 immunostaining (green) of HUVECs in control condition (top), incubated with Cx37 sense (middle) or Cx37 antisense (bottom). Cells were counterstained with Evans Blue (red). Scale bar represents 30 μm . C, Nitrite release by HUVECs treated with Cx37 sense or 25 or 50 $\mu\text{mol/L}$ Cx37 antisense for 48 hours. Overnight nitrite release increased with increasing dose of antisense. N=6 to 9, error bars show SEM, and * $P < 0.05$. D, Immunoprecipitation of eNOS with Cx37 in EA.hy926 (human ECs) or HeLa transfected with Cx37-319P or Cx37-319S. Cx37 antibodies pulled down a protein of ≈ 140 kDa labeled with anti-eNOS in EA.hy926/Cx37 transfectants only.

Table
Alignment of eNOS-Like Peptide With eNOS(843-854)

Peptide	Sequence
eNOS-like	<i>N-WHRTPLPPPVP-C</i>
eNOS(843–854)	<i>N-WVRDPRLPPCTL-C</i>