Oxidized Phospholipids Alter Vascular Connexin Expression, Phosphorylation, and Heterocellular Communication

Brant E. Isakson, Gerhard Kronke, Alexandra Kadl, Norbert Leitinger, Brian R. Duling

Objective—In endothelial cells (EC) and vascular smooth muscle cells (VSMC) from atherosclerotic mice, connexin (Cx) expression becomes distorted. Lipoprotein-derived phospholipid oxidation products (OxPAPC) play a critical role in atherosclerosis, and we hypothesized that they may act as trigger molecules causing the changes in connexin expression.

Methods and Results—We applied OxPAPC to murine carotid arteries in vivo and vascular cell cocultures. OxPAPC applied to carotids induced an upregulation of both Cx37 and Cx43 in the VSMC. In EC, Cx43 was upregulated and Cx37 was downregulated, whereas Cx40 in EC remained constant. In the vascular cell coculture, OxPAPC caused similar changes in Cx37 and Cx40 but caused a decrease in Cx40 in EC and an elevation of Cx40 in VSMC. In the coculture model, OxPAPC treatment led to the selective disappearance of Cx40 at the myoendothelial junction. Biocytin dye transfer between EC and VSMC coupling was dramatically reduced by OxPAPC. The decrease in dye transfer after OxPAPC treatment was correlated with an increase in tyrosine 265 phosphorylation of Cx43, especially at the in vitro myoendothelial junction.

Conclusions—We conclude that OxPAPC could be responsible for the changes in connexin expression previously reported in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: phospholipids ■ connexins ■ gap junctions ■ atherosclerosis ■ phosphorylation
In Vivo Application of OxPAPC

Application of lipids onto the carotid artery was performed as previously described. Briefly, C57/BL6 mice (12 to 15 weeks; n=3 mice with OxPAPC, n=3 mice with DMPC, and n=3 control mice) were anesthetized, and the left common carotid artery was exposed and freed from surrounding tissue. Ice-cold Pluronic F127 gel (60 µL; Molecular Probes) containing 50 µg of either OxPAPC or DMPC was applied onto the vessel. On gelling of the solution, the skin wound was closed by sutures. Twenty-four hours after surgery, animals were euthanized and perfusion-fixed with 4% paraformaldehyde. Carotid arteries were isolated and embedded in paraffin, and 5-µm sections were cut and prepared for immunocytochemistry with antigen retrieval (Vector). Confocal settings for in vivo sections were constant for all experiments at 750 V PMT, 4.8× gain, and 4% offset with laser power at 20%.

Vascular Cell Coculture

Vascular cell cocultures were made according to methods previously described.17 Primary cultures of mouse VSMC were plated on the underside of a Transwell insert with a pore diameter of 0.4 µm. On VSMC confluence, the Transwell insert was inverted, and primary cultures of EC were plated on the top side of the Transwell insert for at least 2 days after confluence before being used. When lipids were added to the vascular cell coculture, they were added in 7.5% FBS for 8 hours. In all experiments in which the vascular cell coculture was used, cells from at least 4 different mice were used, and each experiment was repeated in triplicate.

Immunocytochemistry/Immunoblot

For transverse views of the vascular cell coculture, the cultures were fixed with 2% paraformaldehyde before being embedded in OCT. Approximately 10-µm sections were cut perpendicular to the Transwell insert and prepared for immunocytochemistry before being imaged. Confocal settings for the vascular cell coculture were constant for all experiments at 700 V PMT, 4.5× gain, and 7% offset with laser power at 20%. For immunoblot analysis, the vascular cell cocultures were snap-frozen with liquid nitrogen, and EC and VSMC with laser power at 20%. For immunoblot analysis, the vascular cell cocultures were snap-frozen with liquid nitrogen, and EC and VSMC were removed with Laemmli sample buffer (Bio-Rad) and run on a 12.5% SDS-PAGE. Antibodies used for identification of connexins were specifically downregulated in EC and upregulated in VSMC. Immunoblot boxes represent a molecular weight range of 50 kDa at the top to 25 kDa on the bottom. In each paradigm, the lipids were added to the top (EC) and bottom (VSMC) of the Transwell insert. In response to OxPAPC, Cx37 is diminished in EC and increased in VSMC.

Biocytin Dye Transfer

Biocytin is a low-molecular-weight (373 Da), uncharged molecule that passes through gap junctions, regardless of connexin composition. As previously described, EC were loaded with biocytin (Molecular Probes), and biocytin transfer to VSMC was assessed by cutting transverse sections of the vascular cell coculture and exposing the biocytin with fluorophore-conjugated streptavidin. The pixel intensity in the XZ direction was measured every 1 µm down the length of the Transwell pore. The gap junction inhibitor 18-glycyrrhetinic acid (18-ga; 50 µmol/L; Sigma) was used as previously described.17

Statistics

Significance was set at P<0.05 and determined by 1-way ANOVA (Bonferroni post hoc test); error bars are ±SE.

Results

In Vivo Changes in Connexin Expression

In untreated, control carotids (supplemental Figure IA, available online at http://atvb.ahajournals.org) and in carotids treated with DMPC (supplemental Figure IB), Cx37 was present in EC and VSMC. Treatment with OxPAPC eliminated Cx37 expression in EC and caused a more diffuse stain in VSMC (supplemental Figure IC) without affecting Cx40 expression in either EC or VSMC (supplemental Figure ID to IF). OxPAPC induced upregulation of Cx43 in both EC and VSMC of carotids (supplemental Figure IG or IH versus II). In carotids treated with OxPAPC, the changes in connexin expression were correlated with upregulation of heme oxygenase-1, which is known to be upregulated by OxPAPC (supplemental Figure ID to IF).

In Vitro Changes in Connexin Expression

We applied increasing concentration of the lipids to the top (EC) and bottom (VSMC) of the Transwell inserts used to create a vascular cell coculture. In the EC monolayer, there was a dose-dependent loss of Cx37 and Cx40 (Figures 1A and 2A). In contrast, there was an increase in Cx37 expression in the VSMC monolayer (Figure 1B). Cx40 in VSMC was upregulated by OxPAPC in parallel with the changes in Cx37 (Figure 2B). Lastly, OxPAPC induced an increase in Cx43 expression in both EC and VSMC, manifested on immunoblot as an increase in the unphosphorylated lower band and phosphorylated upper bands (Figure 3A and 3B). Changes in EC and VSMC connexin expression in the 2 models after OxPAPC treatment in comparison to published changes in connexin expression in atherosclerotic mice are summarized in the Table.

OxPAPC-Induced Changes in Gap Junction Composition and Permeability at the MEJ

To examine the changes at the MEJ resulting from altered connexin expression induced by OxPAPC, we immunostained transverse sections of the vascular cell coculture with antibodies against Cx37 (Figure 4A), Cx40 (Figure 4B),...
or Cx43 (Figure 4C). At a concentration of 100 μg/mL OXPAPC, Cx37 and Cx40 expressions were confined to VSMC monolayers (Figure 4A and 4B). Cx43 was the only connexin found in both the EC and VSMC extensions (Figure 4C) at points of heterocellular contact, the in vitro MEJ. The absence of Cx40 in EC extensions represents a distinct change in connexin organization of gap junctions linking the EC and VSMC at the in vitro MEJ whether in control12 or DMPC-treated vascular cocultures (data not shown). Changes in EC and VSMC connexin expression in the 2 models after OXPAPC treatment are summarized in the Table.

We hypothesized that phosphorylation of the Cx43 at the in vitro MEJ after OXPAPC treatment could provide an explanation for the elimination of biocytin dye transfer.18 We used a phosphospecific antibody against the tyrosine 265 residue on Cx43 to detect phosphorylation of Cx43 in vitro (Figure 4D and 4E), and biocytin movement was inhibited by 18 α-GA, demonstrating that the coupling was via gap junctions. Treatment of vascular cell cocultures with OXPAPC inhibited coupling between EC and VSMC (Figure 4F) and eliminated any effect of 18 α-GA on Cx43 expression. The loss of coupling with OXPAPC was not due to retraction of EC and VSMC in vitro (Figure 5A and 5B) or in vivo (Figure 5D and 5E). After OXPAPC application, phosphorylation of Cx43 was evident in cellular extensions of both EC and VSMC in vitro (Figure 5C) and EC and VSMC in vivo (Figure 5F).

**Discussion**

OXPAPC is thought to play a role in the pathogenesis of atherosclerosis. Our data show that OXPAPC induces changes in vascular connexin expression that parallel changes seen in advanced atherosclerosis. Data from the present experiments and from the literature are summarized in the Table.

**Endothelial Cells**

Mouse carotids and vascular cell cocultures exposed to OXPAPC showed a pattern identical to that seen in advanced atherosclerotic mice (14 weeks; Table).12 The expression of Cx40 took on a different pattern because the protein remained constant in the initial stages of atherosclerosis and after...
OXPAPC treatment but was decreased in the advanced atherosclerotic plaques of mice and in the vascular cell coculture. These differences were not due to cell culture because there was a change in connexin expression in 2 different in vivo states. It is possible that Cx37/Cx43 and Cx40 may be differentially regulated.

Yeh et al\textsuperscript{26} characterized the effect of hypercholesteremia on aortic EC and found that Cx40 and Cx37 were both decreased and that a return to normal mouse chow diet rapidly brought Cx37 expression up to normal levels, whereas Cx40 levels remained decreased. Simon et al\textsuperscript{27} demonstrated that injections of lipopolysaccharide into mice caused significant Cx40 downregulation in aortic EC compared with Cx37. These reports demonstrate that Cx40 and Cx37 may be differentially regulated during the inflammation process, ie, their expression is independent of the expression of the other connexins. This is in contrast to connexin expression levels in EC (ie, lacking inflammatory stimuli), which have been demonstrated previously to have coregulated connexins, ie, the up or down connexin expression is dependent on the expression of other connexins.\textsuperscript{22,28,29} How OXPAPC may decouple connexin coregulation is potentially of great interest in understanding EC response to inflammatory mediators. As the effects of OXPAPC on nuclear transcription factors become more clear (eg, Kronke et al\textsuperscript{24}), a more precise idea of the differential control of connexin expression will likely become more evident.

The discrepancy between in vivo and in vitro findings regarding Cx40 is not easily explained; however, EC that migrate off of aortic explants, similar to the ones used in these experiments, are angiogenic and have a phenotype that resembles microvasculature EC (eg, Li and Olsen\textsuperscript{30}) more.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{OXPAPC reorganizes connexins and alters gap junction permeability between EC and VSMC in vitro. Cx37 (A), Cx40 (B), and Cx43 (C) were examined between EC and VSMC on transverse sections of vascular cell cocultures. Each transverse section was double stained with VE-cadherin (VE-cad) antibodies to mark EC and EC extensions (green) and connexin antibodies (red). Cx43 was the only connexin found in the cellular extensions. Bar in A is 10 \(\mu\)m and applies to all images. We examined whether the Cx43 formed functional gap junctions. In J to L, biocytin transfer from EC through Transwell insert pores to VSMC at 1-\(\mu\)m intervals was determined by streptavidin pixel intensity. Biocytin transfer was assessed in control conditions (CT; J), after application of DMPC (K), and after application of OXPAPC (L). Purple lines represent biocytin transfer without 18-\(\alpha\)-glycyrrhetinic acid (\(-\alpha\)-GA), and gray lines represent transfer with 18-\(\alpha\)-GA (\(+\alpha\)-GA). In both J and K, \(+\alpha\)-GA significantly inhibited biocytin transfer from EC to VSMC, whereas in L with OXPAPC, it had no effect. OXPAPC reduced coupling between EC and VSMC. *Significant difference (\(P<0.05\)) between \(+\alpha\)-GA and control.}
\end{figure}
than the macrovasculature EC (eg, the carotids). It is therefore possible that the changes in Cx40 expression demonstrated on the vascular cell coculture may be more representative of what may happen in the microcirculation in response to circulating OxPAPC. More research on the effect of OxPAPC on microvascular connexin expression is required.

Vascular Smooth Muscle Cells

Similar to that seen in EC, the changes in Cx37 expression in VSMC were consistent among all experimental conditions. In regard to Cx40, in each of the in vivo models (atherosclerosis and OxPAPC), expression was not evident, and the upregulation of the protein was only seen in VSMC in vitro. It is possible that different VSMC differentiation states may account for this discrepancy (eg, Nicosia and Villaschi).

Cx43 was the most peculiar in VSMC as OxPAPC-treated carotids and vascular cell cocultures responded with an increase in expression, whereas in advanced atherosclerotic mice the expression of Cx43 was downregulated. This difference represents the only distinct change in connexin expression between OxPAPC and atherosclerotic mice. In untreated aorta and early atherosclerotic mice, Cx43 is highly expressed in VSMC, and therefore the difference between the advanced atherosclerotic mice and the OxPAPC-treated mice may represent a distinct pathological state. In rat aorta, levels of Cx43 in VSMC have been demonstrated to be highly responsive to levels of Cx43 in EC (eg, Simon et al and Liao et al), and therefore the possibility of some type of heterocellular interaction governing these responses in response to inflammatory mediators is intriguing.

Myoendothelial Junctions

Changes in heterocellular communication between EC and VSMC due to atherosclerosis have not been studied at the functional gap junction level. Here we demonstrate that the addition of OxPAPC fundamentally alters coupling between EC and VSMC. It was previously reported that at the in vitro MEJ, connexins formed heterotypic gap junctions composed of Cx40 and Cx43 heteromeric hemichannels (in EC) and Cx43 homomeric hemichannels (in VSMC) that were permeable to biocytin. The addition of OxPAPC initially appeared to have left Cx43 at the in vitro MEJ intact, indicating that the 2 cell types were likely coupled. Indeed, with just Cx43 linking the 2 cell types, both biocytin and Cy3 have been shown to be permeable through the gap junctions at the MEJ. However, our work demonstrated a significant inhibition of the dye transfer moving from EC to VSMC. For this reason, we concluded that it was possible that although Cx43 may be present and form a functional gap junction, the gap junction may not be functionally “open.” Because connexins can be phosphorylated by a multitude of activated signaling cascades, and phosphorylation generally closes gap junctions, we were interested in the phosphorylation state of the Cx43 linking the EC and VSMC.

With the addition of OxPAPC, there was a change in the phosphorylation state of residual Cx43 protein, especially at the in vitro MEJ. Phosphorylation of Cx43 can occur at several serine and tyrosine sites on the carboxy tail of Cx43 and has been shown to be associated in most instances with reduced gap junctional coupling (for review, see Lampe and Lau). In particular, phosphorylation of tyrosine 265 on Cx43 has been associated with decreased cell coupling in multiple cell types through a v-src kinase signaling cascade. Because there is evidence that OxPAPC induces activation of v-src, it is conceivable that v-src mediates the OxPAPC-induced phosphorylation of Cx43.

Taken together, the altered heterocellular communication between the 2 cell types raises the possibility of poorly coupled vascular tissue during atherogenesis and after OxPAPC treatment. This observation has important implications for disease states associated with elevated levels of oxidized phospholipids, in which vascular heterocellular communication might be disturbed. It remains to be determined whether these OxPAPC-induced disturbances in cellular communication within the vascular wall provide a link between hyperlipidemia and hypertension, as observed in patients suffering from the metabolic syndrome. It might be possible that pharmacological intervention to increase heterocellular coupling by altering the phosphorylation state of Cx43 may be effective in slowing the progress of the diseases.

In summary, a correlation exists between EC and VSMC after OxPAPC treatment and connexin expression in advanced atherosclerotic mice, especially in regard to Cx37 and Cx43. These changes affected coupling between EC and VSMC in our vascular cell coculture so that all gap junction-mediated communication between the 2 cell types in vitro was significantly inhibited. We suggest that phospholipid oxidation products such as OxPAPC, which accumulate during the development of atherosclerotic lesions, may affect gap junction-mediated communication in the vascular wall and detrimentally contribute to the development of the disease.

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**Disclosures**

None.

**References**

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Online Figure I: OxPAPC induces changes in connexin expression in murine carotid vessels. Antibodies against Cx37 (A-C), Cx40 (D-F) or Cx43 (G-I) were used to detect changes in connexin protein expression in carotids under control conditions (CT; A, D, G), carotids treated with DMPC (B, E, H), and carotids treated with OxPAPC (C, F, I). Green is autofluorescence of the elastic lamina, red is the connexin protein. Bar in A is 20 µm and is indicative for all images; asterisks indicate lumen.