RAGE-Dependent Activation of the Oncoprotein Pim1 Plays a Critical Role in Systemic Vascular Remodeling Processes

Jolyane Meloche, Roxane Paulin, Audrey Courboulin, Caroline Lambert, Marjorie Barrier, Pierre Bonnet, Malik Bisserier, Mélanie Roy, Mark A. Sussman, Mohsen Agharazii, Sébastien Bonnet

Objective—Vascular remodeling diseases (VRD) are mainly characterized by inflammation and a vascular smooth muscle cells (VSMCs) pro proliferative and anti-apoptotic phenotype. Recently, the activation of the advanced glycation end products receptor (RAGE) has been shown to promote VSMC proliferation and resistance to apoptosis in VRD in a STAT3-dependent manner. Interestingly, we previously described in both cancer and VRD that the sustainability of this pro proliferative and antiapoptotic phenotype requires activation of the transcription factor NFAT (nuclear factor of activated T-cells). In cancer, NFAT activation is dependent of the oncoprotein Moloney murine leukemia virus (Pim1), which is regulated by STAT3 and activated in VRD. Therefore, we hypothesized that RAGE/STAT3 activation in VSMC activates Pim1, promoting NFAT and thus VSMC proliferation and resistance to apoptosis.

Methods/Results—In vitro, freshly isolated human carotid VSMCs exposed to RAGE activator Ne-(carboxymethyl)lysine (CML) for 48 hours had (1) activated STAT3 (increased P-STAT3/STAT3 ratio and P-STAT3 nuclear translocation); (2) increased STAT3-dependent Pim1 expression resulting in NFATc1 activation; and (3) increased Pim1/NFAT-dependent VSMC proliferation (PCNA, Ki67) and resistance to mitochondrial-dependent apoptosis (TMRM, Annexin V, TUNEL). Similarly to RAGE inhibition (small interfering RNA [siRNA]), Pim1, STAT3 and NFATc1 inhibition (siRNA) reversed these abnormalities in human carotid VSMC. Moreover, carotid artery VSMCs isolated from Pim1 knockout mice were resistant to CML-induced VSMC proliferation and resistance to apoptosis. In vivo, RAGE inhibition decreases STAT3/Pim1/NFAT activation, reversing vascular remodeling in the rat carotid artery-injured model.

Conclusion—RAGE activation accounts for many features of VRD including VSMC proliferation and resistance to apoptosis by the activation of STAT3/Pim1/NFAT axis. Molecules aimed to inhibit RAGE could be of a great therapeutic interest for the treatment of VRD. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Keywords: apoptosis / oncogenes / vascular biology / vascular muscle

Vascular remodeling disease (VRD) such as hypertension, atherosclerosis and postangioplasty restenosis are characterized by increased media wall thickness and neointimal formation. This histological remodeling has been attributed in part to an increase in vascular smooth muscle cell (VSMC) proliferation and resistance to apoptosis.1 This pro proliferative and antiapoptotic phenotype is mediated by a variety of growth factors (PDGF), cytokines, and chemokines5-6 stimulating prosurvival factors like survivin, which by inhibiting apoptosis7 and promoting mitotic progression enhances neointimal formation.8 We recently described that the sustainability of this phenotype is due to the activation of the transcription factor NFAT (nuclear factor of activated T-cell).1 Once activated, NFAT promotes inflammation by stimulating cytokine production10 and VSMC proliferation by decreasing K+ channel,1 such as Kv1.5, which promotes [Ca2+] and apoptosis resistance, in part by increasing Bcl2 expression. This results in subsequent mitochondrial membrane potential (ΔΨm) hyperpolarization, and trapping of proapoptotic factors, such as cytochrome c, in the mitochondria.11 The mechanisms accounting for NFAT activation in VRD remain undetermined.

Advanced glycation end products (AGEs) are now recognized as bioactive circulating molecules able to enhance proliferative pathways through their receptor RAGE.12 Circulating AGEs are particularly elevated in diabetic13 and hypertensive subjects14,15 and contribute to vascular remodeling processes associated with inflammation.16-18 Although AGEs are part of a wide family of molecules, only a few have been characterized and identified in human tissues. These include Ne-(carboxymethyl)lysine (CML), pentosidine, pyrraline, and immidazole,19 with only CML being able to
increase RAGE signaling. Finally, because CML is the most commonly encountered AGE in vivo,20 it is present in atherosclerotic lesions,21,22 and its binding to RAGE correlates with carotid artery diameter in normoglycemic patients,23 we decided to focus our study on the role of CML/RAGE interaction in VRD.

Although RAGE activation in neointimal formation has been established,24 the mechanism by which RAGE promotes VRD remains unknown. Recently, RAGE activation has been associated with an increase in STAT3 pathway.25 STAT3 has been shown to be implicated in VSMC proliferation.26–28 In cancer, STAT3 promotes the expression of the Proviruses integration site for Moloney murine leukemia virus (Pim1),29 a proto-oncogene encoding a serine/threonine protein kinase.30 Overexpression of Pim1 is linked to the development and progression of several cancers by increasing cell proliferation/survival and resistance to apoptosis.31–33 Furthermore, it has been demonstrated that Pim1 could also be implicated in VRD.34 Interestingly, Pim1 activation enhanced NFATc1–4 activity in rat PC12 cells and lymphoid cells.35 Our team recently showed that the STAT3/Pim1/NFATc2 axis is responsible for abnormal SMC proliferation in pulmonary arteries.27 Thus, the RAGE-dependent activation of STAT3 in VSMCs could trigger Pim1/NFAT axis activation and promote VRD.

The present study demonstrated, indeed, that RAGE activation triggers STAT3 accounting for a Pim1-dependent NFAT activation promoting VSMC proliferation and resistance to apoptosis. Similarly to RAGE inhibition, Pim1 silencing prevented and reversed RAGE-dependent proproliferative and antiapoptotic phenotype seen in cultured VSMCs stimulated by CML. In vivo, RAGE inhibition decreases STAT3/Pim1/NFAT activation preventing vascular remodeling in carotid artery postangioplasty.

Methods

An expanded Methods section is available at http://atvb.ahajournals.org.

Cell Culture

Primary cultured human carotid artery smooth muscle cells (CASMCs) from 2 healthy donors were used (less passage 6), see the expanded Methods section, available at http://atvb.ahajournals.org. Mouse carotid artery smooth muscle cells were freshly isolated from Pim1 knockout (KO) mice and proper control littersmates. Final concentration of 1 μg/mL of CML-bovine serum albumin (BSA) was used as RAGE agonist, and NFAT competitor peptide, VIVIT, was used at 4 μmol/L. CASMCs were transfected by CaPO4 precipitation with 20 nmol/L small interfering RNA (siRNA) oligonucleotides.

ELISA Assay

CML auto-antibody and direct CML ELISA assays (Circulex) were performed following manufacturer’s instructions. Serum antibodies anti-CML levels were studied in human subjects with history of cardiovascular disease (n=15) and controls (n=44). Cardiovascular disease was defined as history of coronary artery revascularization, myocardial infarction with abnormal wall motion by ultrasound, ischemic stroke, and symptomatic peripheral vascular disease. The protocol was approved by the institutional review board and patients provided informed consent. No sex-based or racial/ethnic-based differences were present.

Table. Ne-(Carboxymethyl)lysine Levels in Controls and in Patients with Cardiovascular Disease

<table>
<thead>
<tr>
<th></th>
<th>Control (n=44)</th>
<th>CVD (n=15)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57±8</td>
<td>56±11</td>
<td>ns</td>
</tr>
<tr>
<td>Male (%)</td>
<td>68%</td>
<td>73%</td>
<td>ns</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>40.0±3.8</td>
<td>38.7±2.4</td>
<td>ns</td>
</tr>
<tr>
<td>Ab anti-CML (ng/L)</td>
<td>25.7±26.5</td>
<td>54.4±51.4</td>
<td>0.012</td>
</tr>
<tr>
<td>CML-Albumin ratio (μg/g)</td>
<td>0.64±0.64</td>
<td>1.43±1.43</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Values are means±SD; CML indicates Ne-(carboxymethyl)lysine; CVD, cardiovascular disease; ns, not significant.

Whole Cell Patch Clamping

Whole cell patch clamping was performed on cells voltage-clamped at a holding potential of −70 mV. Currents were evoked by 200 ms test pulses from −70 to +70 mV with 20mV steps as previously described.1 To quantify the effects of CML-BSA on voltage-gated K+ current (Kv), 4-aminopyridine 1 mmol/L (4-AP), a Kv channel blocker, was used.1

Confocal Microscopy/Immunofluorescence, Immunoblotting

Sections of carotids and human CASMCs were used for immunofluorescence staining (see the expanded Methods section, available at http://atvb.ahajournals.org).

Nuclear Translocation Assays

STAT3 and NFATc1 activity were estimated by the percentage of cells presenting a P-STAT3 or NFATc1 nuclear pattern.

Luciferase Assay

Cells were lysed, and luminescence was detected using the Luciferase Assay System (Promega) according to the manufacturer’s instructions. Luminescence counts were standardized to protein content.

Carotid Artery (Balloon) Injury Model

Three groups of rats were studied. Sham operated and rats with angioplasty received either negative control siRNA (siCRM) or siRAGE (both 10 μmol/L).

Statistics

Data are presented as mean±SD with Mann-Whitney U test for human serum CML levels and mean±SEM for all other studies. Normality of our data were assessed by the Shapiro-Wilk normality test. All our data were normally distributed (P>0.05). For comparison between 2 means, we used unpaired Student t-test. For comparison between 2 means, we used 1-way ANOVA followed by Tukey-Kramer tests. Probability values less than 0.001 (***) , 0.01 (**), and 0.05 (*) were considered as statistically significant.

Results

CML-Circulating Levels are Increased in Patient With Vascular Diseases

It was recently described that plasma CML levels are significantly higher in patients presenting an increased carotid diameter or an acute myocardial infarction.23,36 In order to extend these observations, we studied CML plasma levels in a cohort of patients with cardiovascular disease as defined by a history of coronary artery revascularization (60%), ischemic stroke (20%), and symptomatic peripheral vascular disease (33%). Plasma levels of antibodies anti-CML were measured by ELISA assay.37 There is a 52% increase (P=0.012) in CML plasma levels in patients with cardiovascular diseases compared with control patients (Table). This result suggests
that CML is implicated in many vascular dysfunctions; thus, a better understanding of its role may be of great therapeutic interest.

CML Triggers STAT3 Activation Through a RAGE-Dependent Mechanism in CASMCs

To determine whether CML triggers STAT3 activation, CASMCs were exposed to CML-BSA (1 μg·mL⁻¹) (dose previously used and shown to have biological effects) for 15, 30, 60 minutes and 48 hours. After a 15-minute exposure, CML-BSA–treated cells showed a sustained 1.5-fold increase in STAT3 activation measured by immunoblots (ie, Y705-phosphorylated STAT3/total STAT3 ratio normalized to smooth muscle actin) compared with control CASMCs (n=3, P<0.05; Figure 1A). This finding was confirmed by a STAT3 nuclear translocation assay on CASMCs (n=50 CASMC/experiment for 4 experiments, P<0.01). CML-BSA induces a 80% increase in STAT3 activation and RAGE blockade (by siRNA 20 nmol/L) decreases the activation by 60%. We also confirmed that STAT3 activation was mediated by RAGE by immunoblot, showing that CML-BSA induces a 1.6-fold increase and RAGE blockade decreases STAT3 activation (n=4, P<0.01). CML-BSA triggers Pim1 and NFATc1 expression (800-fold increase; qRT-PCR, n=4, P<0.001). RAGE and STAT3 inhibition by siRNA induce a 150- and 300-fold decrease in Pim1 and NFATc1 expression, respectively (qRT-PCR).
CML-BSA–Dependent STAT3 Activation Increases Pim1 and NFATc1

Because STAT3 has been demonstrated as a putative regulator of the NFAT activator Pim1 in cancer cells, and as Pim1/NFAT is implicated in VRD, we measured whether the CML-BSA–dependent STAT3 activation in CASMCs increases Pim1-dependent NFATc1 (the NFAT isoform expressed in CASMC) expression and activation. Using both qRT-PCR and immunoblot, we demonstrated that CML-BSA significantly increases Pim1 and NFATc1 mRNA (300- and 800-fold increase, n=4, P<0.01, respectively; Figure 1C) and protein levels (both 2.4-fold increase; n=4, P<0.05; Supplemental Figure IB). Interestingly, the timing of Pim1/NFATc1 protein activation occurs 30 minutes post-CML-BSA exposure, while STAT3 activation occurred within 15 minutes, suggesting, as in cancer, a possible implication of STAT3 in Pim1/NFAT regulation. This was confirmed by qRT-PCR, measuring Pim1 and NFATc1 mRNA levels in with RAGE and STAT3 siRNAs. Both siRAGE and siSTAT3 decreased Pim1 and NFATc1 expression compared with siSCR (140- and 300-fold decrease, n=4, P<0.001, respectively; Figure 1C). (Note that RAGE, STAT3, and Pim1 siRNAs efficiencies were confirmed; see Supplemental Figure IIA, IIB, and IIC, available at http://atvb.ahajournals.org.) NFATc1 activation, measured by nuclear translocation and luciferase assays, confirmed this finding (n=50 CASMC/experiment for 4 experiments, P<0.001 and n=5, P<0.05, respectively; Figure 2A).

Because the Akt/GSK3β pathway can also activate Pim1/NFAT in CASMC, we studied whether CML-BSA increases the Akt/GSK3β pathway. CML-BSA does not increase Akt expression or activation as measured by immunoblot (ie, PS473-Akt/Akt ratio, n=4, P<0.05 and yellow staining in nuclear translocation assay, n=50 CASMCs/experiment for 4 experiments, P<0.05) compared with siSCR (Figure 1B and Supplemental Figure IA).

CML-BSA Enhances CASMC Proliferation and Decreases Apoptosis Through a RAGE/Pim1/NFATc1-Dependent Mechanism

We previously showed that NFATc1 activation in VRD accounts for the sustainability of the proproliferative and antiapoptotic phenotype of CASMCs by decreasing whole cell K+ current, depolarizing CASMC membrane potential, and increasing [Ca2+]i and mitochondrial membrane potential (ΔΨm) hyperpolarization.1 To determine whether these effects were mediated by the CML-BSA–dependent activation of Pim1/NFAT through RAGE, we measured K+ current (patch clamp) [Ca2+]i (FLUO3), proliferation (Ki67 and PCNA); ΔΨm (TMRM); and apoptosis (TUNEL and annexin-V) in CASMCs treated with CML-BSA in presence of either Pim1 siRNA, NFAT inhibitor (VIVIT) (VIVIT efficiency is shown in Supplemental Figure IID, and the control peptide is not shown in graph because it has no effect as previously described,41 Supplemental Figure IIE), siRAGE, or siSTAT3.

Using whole cell patch clamping, we demonstrated that CML-BSA decreases voltage-gated K+ current (n=at least 7 per group, P<0.05; Figure 2B cell capacity were not different between CASMCs and average around 30pF), which is restored when RAGE is inhibited. As shown in Supplemental Figure IVA, available at http://atvb.ahajournals.org, CML-BSA has less 4-AP-sensitive current than control or siRAGE-treated CASMCs (n=5 per group, P<0.05). Because 4-AP is a voltage-dependant potassium channel blocker, the current diminution is a consequence of a decrease of cell membrane potassium channels (Kv1.5, for example) which confirms our hypothesis because NFAT is responsible of diminution of K channels transcription.11 Representative currents of each conditions are shown in Supplemental Figure IVB.

Decrease of K+ current causes an increase of CASMCs [Ca2+]i (1.7-fold increase, n=50 CASMC/experiment for 5 experiments, P<0.001; Figure 2B and Supplemental Figure IVA) which stimulates cell proliferation (25% increase, n=50 CASMC/experiment for 5 experiments, P<0.001) (Ki67, Figure 2B and PCNA, Supplemental Figure IIIC, Pim1, NFATc1, RAGE, or STAT3 inhibition reversed these effects (at least 30% decrease) (n=50 CASMC/experiment for 5 experiments, P<0.005) (Figure 2B and Supplemental Figure IIIC). The fact that either siPim1, VIVIT, siRAGE, or siSTAT3 normalized [Ca2+]i and proliferation in CML-BSA–treated CASMCs with the same efficiency, suggests that their effects are not additive and that indeed the calcium-dependent proliferation is mediated by the RAGE/STAT3/Pim1/NFAT axis.

CML-BSA significantly increased ΔΨm hyperpolarization (greater red staining) (Figure 2D). Once again, RAGE, STAT3, Pim1, and NFATc1 inhibition (siRNA and VIVIT peptide) normalized ΔΨm compared, respectively, to siSCR (for RAGE, STAT3, and Pim1) and to CML-BSA–treated cells (for NFAT) (1.9-fold increase, n=50 CASMC/experiment for 5 experiments, P<0.001) (Figure 2C). ΔΨm normalization by RAGE/STAT3/NFAT inhibition reverses the resistance to serum starvation (0.1% FBS for 24 hours) induced apoptosis measured by AnnexinV and TUNEL (n=50 CASMC/experiment for 5 experiments, P<0.05) (Figure 2C and Supplemental Figure IIID, respectively). This finding demonstrates that, as for proliferation, apoptosis resistance in CML-BSA–treated CASMCs is due to the activation of the RAGE/STAT3/NFAT axis.
Pim1 KO Mice Are Resistant to RAGE-Induced VSMC Proliferation and Resistance to Apoptosis

To demonstrate that RAGE-dependent VSMC proliferation and resistance to apoptosis rely on Pim1/NFATc1 activation, we freshly isolated CASMCs from Pim1 KO mice and littermate control mice. VSMCs were then exposed to CML-BSA for 48 hours. As in humans, in control VSMCs, CML-BSA triggers STAT3 activation, ie, P-STAT3 nuclear translocation assay, which promotes cell proliferation and inhibits apoptosis in human carotid artery smooth muscle cells.

Figure 2. Nε-(carboxymethyl)lysine-bovine serum albumin (CML-BSA) enhances Pim1 and nuclear factor of activated T-cells (NFAT)c1 expression and activation, which promotes cell proliferation and inhibits apoptosis in human carotid artery smooth muscle cells. A, CML-BSA also promotes NFATc1 activation, ie, translocation to the nucleus, through a RAGE/STAT3/Pim1-dependent mechanism (over 80% increase; immunofluorescence, n=50 CASMC/experiment for 4 experiments, P<0.001), also measured by luciferase assay (n=5, P<0.05). B, CML-BSA decreases K⁺ current (n=7 per group, P<0.01), which promotes intracellular calcium entry measured by FLUO3-AM by immunofluorescence (1.7-fold increase, n=50 CASMC/experiment for 5 experiments, P<0.001), which enhances cell proliferation. Cell proliferation was measured by activation of proliferation factor Ki67 (25% increase, n=50 CASMC/experiment for 5 experiments, P<0.001). To calculate the percentage of proliferation: cells positive for proliferation factors activated divided by total number of cell (visualized by 4,6-diamidino-2-phenylindole (DAPI)). C, CML-BSA hyperpolarizes mitochondrial membrane potential measured by tetramethylrhodamine methyl ester (1.9-fold increase, n=50 CASMC/experiment for 5 experiments, P<0.001) which blocks cell death. CML-BSA inhibits apoptosis in CASMCs put in 0.1% of fetal bovine serum to stimulate apoptosis in normal cells (starvation) (n=50 CASMC/experiment for 5 experiments, P<0.05). Apoptosis was measured by calculating the percentage of positive AnnexinV cells, divided by total number of cell (visualized by DAPI). CML-BSA induced apoptosis resistance is mediated by RAGE/STAT3/Pim1/NFAT pathway, showed by an increase of apoptosis when either one of these effectors is blocked (by VIVIT 4 μmol/L or siRNA 20 nmol/L).
translocation (n=30 cells per condition per experiment for 3 experiments, P<0.001; Supplemental Figure VA, available at http://atvb.ahajournals.org) in both wild type (WT) and Pim1 KO mice. CML-BSA also triggers a RAGE-dependent NFATc1 activation in Pim1 WT-CASMCs (nuclear translocation assay) (n=30 cells per condition per experiment for 3 experiments, P<0.05) (Supplemental Figure VB) promoting calcium release and proliferation (n=30 CASMC/experiment for 3 experiments, P<0.05; Supplemental Figure VC), as well as mitochondrial membrane hyperpolarization and resistance to apoptosis (n=30 CASMC/experiment for 3 experiments, P<0.05; Supplemental Figure VD). In Pim1 KO-CASMC, CML-BSA failed to activate NFATc1 and to promote proliferation and resistance to apoptosis (Supplemental Figure V).

Taken together, these findings demonstrated that CML-BSA triggers NFATc1 expression through STAT3 and NFATc1 activation through Pim1. Lack of NFATc1 activation in Pim1 KO-CASMCs demonstrated that the RAGE-dependent proliferation and resistance to apoptosis in CML-BSA–stimulated CASMCs rely mainly on the Pim1/NFATc1 axis.

CML-BSA Promotes the RAGE/STAT3/NFATc1-Dependent Proproliferative and Antiapoptotic Phenotype Through the Activation of a Positive Feedback Loop Increasing RAGE Expression

RAGE expression is described in VRD as dependant of the amount of circulating CML.35,45 We confirmed this topic by studying RAGE mRNA level (qRT-PCR) in CASMCs exposed to increasing doses of CML-BSA. As shown in Figure 3A, CML-BSA promotes RAGE expression in a dose-dependent manner (n=4, P<0.01). To elucidate the mechanism explaining how CML enhances RAGE expression, we measured RAGE protein expression in CASMCs exposed to CML-BSA for 15, 30, and 60 minutes. RAGE expression increases after 60 minutes of CML-BSA exposure (n=4, P<0.01; Supplemental Figure IC). Considering that STAT3 is activated at 15 minutes (Figure 1A), NFAT at 60 minutes (Supplemental Figure IB), and that both STAT3 and NFAT are transcription factors, we speculated that either STAT3 or NFAT could be implicated in the regulation of RAGE at the transcriptional level. RAGE expression was measured in presence of siSTAT3 or NFAT inhibitor (VIVIT). STAT3 and NFATc1 inhibition decreased by 15-fold CML-BSA–dependent RAGE mRNA level. This suggests the effective NFATc1 implication in the transcriptional regulation of RAGE (n=4, P<0.001; Figure 3A). Further experiments are required to elucidate the exact mechanisms and will be the purpose of a future study.

RAGE Inhibition In Vivo Prevents Vascular Remodeling Processes Through the Inhibition of the Pim1/NFATc1 Axis

Vascular remodeling was observed before sacrifice using echography Doppler by measuring carotid wall thickness and internal diameter of the vessel. Injured rats treated with siRNA directed against RAGE showed less remodeling, ie, a thinner artery wall and a larger internal diameter of the vessel (n=5 per group, P<0.05; Supplemental Figure VIA) compared with rats treated with a control siRNA. Animals were euthanized 14 days after injury. We confirmed that the injury increased the amount of CML-BSA in the carotid by Western blot (n=4, P<0.01; Supplemental Figure VIB). Using H&E staining on harvested carotid, paraffin embedded, we measured neointimal cross-sectional area in control injured carotid arteries and injured carotids transfected with siSCRM (Figure 3B). As expected, injured carotids transfected with a siRNA directed against RAGE showed 55% less neointimal thickening (P<0.05) as compared with siSCRM (n=5 rats per group; Figure 3B). In vivo, RAGE inhibition was confirmed at the RNA level by qRT-PCR and at the protein level by immunofluorescence (n=5 rats per group, P<0.05) (Figure 3C). Immunofluorescent staining of smooth muscle actin (in green) indicates that neointimal formation is mainly constituted of smooth muscle cells, confirming the impact of the RAGE/STAT3/Pim1/NFATc1 axis on SMC, which enhance their proliferation and progressive obstruction of the lumen. Triple staining with either NFATc1 or P-STAT3 (in red), α-smooth muscle actin (green) and 4,6-diamidino-2-phenylindole (DAPI) showed that STAT3 and NFATc1 activation are increased in CASMCs of siSCRM injured carotids (showed by the colocalization in yellow of P-STAT3/DAPI or NFATc1/DAPI) compared with sham. siRAGE injured carotids at the opposite showed a decreased NFATc1 and STAT3 activation compared with siSCRM treated ones (n=50 CASMC/rat, 5 rats per group, P<0.001; Figure 4A and Supplemental Figure VIC). Immunostaining for Ki67 (in red), α-smooth muscle actin (green), and DAPI showed that carotids injury is associated in saline or control siRNA with a increase of CASMC proliferation compared with sham (measured by the the nuclear localization of Ki67) (n=50 CASMC/rat, 5 rats per group, P<0.01; Figure 4A and Supplemental Figure VIC). Immunostaining for Ki67 (in red), α-smooth muscle actin (green), and DAPI showed that carotids injury is associated in saline or control siRNA with a increase of CASMC proliferation compared with sham (measured by the the nuclear localization of Ki67) (n=50 CASMC/rat, 5 rats per group, P<0.01; Figure 4B). In siRAGE injured carotids the proliferation rate of CASMCs is decreased compared with siSCRM-treated injured carotids, showing that interruption of proliferative signal progression by RAGE inhibition have a powerful nonproliferative effect on CASMCs in vivo, mainly by inhibiting the activation of the STAT3/Pim1/NFATc1 axis. Furthermore, triple staining with TUNEL (in red), α-smooth muscle actin (green), and DAPI showed that siRAGE carotids presented significantly more apoptotic CASMCs than any other group (n=50 CASMC/rat, 5 rats per group, P<0.001). These results suggest that localized RAGE inhibition decreases postinjury carotid artery remodeling in rats, mainly by inhibiting the STAT3/Pim1/NFATc1 axis.

Discussion

We showed for the first time that RAGE activation in both humans’ and rodents’ CASMCs triggers the activation of the
STAT3/Pim1/NFAT axis accounting for CASMC proliferation and resistance to apoptosis. Moreover, using Pim1 KO mice we showed that the RAGE-dependent CASMC proliferation and resistance to apoptosis rely on the activation of NFATc1 by Pim1, a mechanism also found in neoplastic processes.

As in cancer cells, Pim1/NFATc1 activation hyperpolarized mitochondrial membrane potential (suppressing CASMC apoptosis) and increased [Ca\textsuperscript{2+}], (promoting CASMC proliferation). These finding are in accordance with previous published studies (including ours) showing that Pim1 (through Bad phosphorylation\textsuperscript{46}) and NFAT (through pyruvate kinase regulation\textsuperscript{41}) hyper-

Figure 3. Enhanced advanced glycation endproducts receptor (RAGE) expression stimulates vascular remodeling. A, Nε-(carboxymethyl)lysine-bovine serum albumin (CML-BSA) enhances RAGE expression in a dose-dependent manner (measured by qRT-PCR normalized with 18S; n=4, \(P<0.01\)). Furthermore, RAGE expression in enhanced through a positive feedback loop including STAT3 and nuclear factor of activated T-cells (NFAT) because their blockade (siSTAT3 20 nmol/L and VIVIT 4 \(\mu\)mol/L, respectively) reduces RAGE expression (qRT-PCR, n=4, \(P<0.001\)). B, After H&E staining, neointima cross-sectional areas were measured. RAGE blockade showed decreased neointima area compared with injured carotids (55% decrease, n=5 per group, \(P<0.05\)). C, RAGE expression is enhanced in the injured vascular wall and siRAGE efficiency are shown by immunofluorescence, which corresponds to RAGE protein expression (n=5 per group, \(P<0.001\)) and qRT-PCR (n=5 per group, \(P<0.05\)).
Moreover, we extensively published in the past, in both cancer and VSMCs, that NFAT activation decreases $K^+$ channels, including Kv1.5, depolarizing VSMCs and opening the voltage-gated calcium channels thus increasing $[Ca^{2+}]_i$.

Thus, by blocking Pim1/NFAT axis, RAGE inhibition restores $K^+$ current, $[Ca^{2+}]_i$, and depolarizes $\Delta\Psi_m$. In vivo, we demonstrated that localized RAGE inhibition after 30 minutes of siRNA instillation prevented carotid artery postinjury remodeling in rats by decreasing the activation of Pim1/NFATc1 axis and thus decreasing CASMC proliferation and resistance to apoptosis. Although this finding could be seen as methodologically surprising, such approach has shown similar results in the past as shown in Lipskaia et al47 confirming the translational potential of this intervention.
transcription factor HIF-1 (hypoxic-inducible factor 1) which we previously showed to be implicated in VRD. The sustained activation of NFAT, which could explain many features seen in VRD including HIF-1 associated metabolic disorders and inflammation. Thus, we offer new therapeutic perspectives, not only for vascular diseases, but also possibly for other diseases sharing common features with VRD including cancer.

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**Disclosures**

None.

**References**


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SUPPLEMENTAL METHODS

Cell culture
Human carotid artery smooth muscle cells (CASMC) isolated from 2 healthy donors (organ transplant patient) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% of an antibiotic-antimycotic mixture (GIBCO 15240, Invitrogen) and maintained in a humid atmosphere, at 37°C with 5% CO₂. Cells were used between passages 3 and 8 as previously described. Cells were transfected by CaPO₄ precipitation with 20 nM siRNA oligonucleotides. Twenty-four hours post-transfection, the cell medium was changed, and 48 h post-transfection, cells were treated as indicated. All siRNAs were obtained from Applied Biosystems. As a control oligonucleotide, ‘Silencer Negative Control #2 siRNA’ was used. Cells were treated with CML-BSA (Circulex) (1 µg/ml) for different time laps (15, 30, 60 minutes and 48 hours) before analysing.

ELISA assay
CML auto-antibody and direct CML ELISA assays (Circulex) were performed following manufacturer’s instructions. Human plasmatic levels of CML-BSA were measured by anti-CML ELISA assay in a blind study was performed on 59 patients. In order to extend these observations, we studied CML plasma levels in a cohort of patients with cardiovascular disease as defined by a history of coronary artery revascularization (60%), ischemic stroke (20%) and symptomatic peripheral vascular disease (33%). Patients were recruited through the outpatient clinics of Centre Hospitalier Universitaire de Québec – Hôtel Dieu de Québec Hospital. Blood samples were obtained between 8-10 AM after an overnight fast. The samples were immediately centrifuged and kept at -80°C until analysis. The protocol was approved by the institutional review board and patients provided informed consent.

Confocal microscopy/Immunofluorescence
Rat carotid arteries were fixed with 4% paraformaldehyde. Immunofluorescence was performed on 5 µm carotid slices. CASMC were fixed with 1% paraformaldehyde and permeabilized with 0.2% Triton X-100. P-STAT3 (1/250), RAGE (1/250) antibodies were purchased from Cell Signaling; NFATc1 (1/250) from Abcam. Alexa Fluor (1/1000) 488 and 594 were used as secondary antibodies. For proliferation measurements, PCNA antibodies (1/400) from DAKO and Ki67 antibodies (1/250) from Millipore were used. For apoptosis assays, TUNEL Apoptag kit from Millipore and AnnexinV from Clontech was performed following manufacturer’s instructions. FLUO3-AM and TMRM reagents were purchase from Invitrogen and applied at the concentration of 5µM on live cells. Nuclei were stained with diamidino-phenylindole (DAPI, DAKO Cytomation, Carpinteria, CA).

Nuclear translocation assays
To measure the activity of transcription factors STAT3 and NFATc1, a ratio was calculated by dividing the amount of cells that had staining of P-STAT3 and NFATc1 in
the nucleus (colocalisation with DAPI) on the total amount of cells. Ratios were transformed into percentage for statistical analysis.

**Immunoblotting**

Twenty-five micrograms (25 µg) of protein were loaded on SDS-PAGE (8% wt/vol), and then transferred electrophoretically to PVDF membranes. After blocking, primary antibodies were used: RAGE (1:1000, Cell Signaling), phospho-Akt (1:1000, Cell Signaling), Akt (1:1000, Cell Signaling), phospho-STAT3 (1:1000, Cell Signaling), STAT3 (1:1000, Cell Signaling), NFATc1 (NFAT2, 1:1000, Abcam), Pim1 (1:1000, Cell Signaling) and α-smooth muscle actin (1:300, Santa Cruz). HRP-conjugated secondary antibodies were used (Promega). Expression was normalized to actin to correct for loading differences.

**Quantitative real time polymerase chain reaction**

RNAs were isolated from CASMC with trizol. Reverse transcription was performed with the High capacity cDNA Reverse Transcription kit from Applied Biosystem. cDNA was used for quantitative RT-PCR Taqman. Taqman primers and probes were all purchased from Applied Biosystems and 18S were used as an internal control.

**Carotid artery (balloon) injury model**

Male Sprague Dawley (strain 001, Charles River) rats (350g) were used. Under anaesthesia (2% isoflurane), a neck incision was made. A 20 mm section of the left common carotid artery was isolated and temporarily occluded to prevent retrograde blood loss. After proximal arteriotomy, a 2F Fogarty embolectomy catheter was introduced to perform an antegrade balloon inflation along a 15 mm segment of the common carotid artery. The catheter, containing either siSCRM (10µM) or siRAGE (10µM) was maintained in place for 30 minutes to assure efficient transfection. The lumen was then flushed with heparinized saline, the arteriotomy was closed with 9-0 Prolene sutures and the perfusion was restored. Hematoxylin and Eosin (H&E) staining was performed on carotid sections to measure vascular neointima cross-sectional area 14 days post injury (5 rats per group were studied).

**Statistics**

Data are presented as mean±SD with Mann Whitney U test for human serum CML levels and mean±SEM for all other studies. Normality of our data was assessed by the Shapiro-Wilk normality test. All our data were normally distributed (P>0.05). For comparison between 2 means, we used unpaired Student t test. For comparison between 2 means, we used 1-way ANOVA followed by Tukey-Kramer tests. P values less than 0.001 (***) , 0.01 (**) and 0.05 (*) were considered as statistically significant.
Supplemental References


Supplemental Figure I

A  CML increases STAT3 activation

Merged P-STAT3 (red)/DAPI (blue)
Colocalization P-STAT3/DAPI (yellow)

B  CML increases NFATc1 and Pim1 expression

Pim1 normalized to sm-actin

NFATc1 normalized to sm-actin

C  RAGE expression is increased over time

RAGE normalized to sm-actin
Supplemental Figure II

A  *RAGE siRNA efficiency in vitro*

<table>
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<th>Control</th>
<th>CML-BSA</th>
<th>siSCRIM + CML-BSA</th>
<th>siRAGE + CML-BSA</th>
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RAGE normalized to sm-actin

B  *STAT3 siRNA efficiency*

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<th>Control</th>
<th>CML-BSA</th>
<th>siSTAT3 + CML-BSA</th>
<th>siRAGE + CML-BSA</th>
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STAT3 normalized to sm-actin

C  *Pim1 siRNA efficiency*

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D  *VIVIT efficiency*  

<table>
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<th>NFATc1 mRNA expression</th>
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E  Control peptide does not have any effect

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<th>Ki67</th>
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Supplemental Figure III

A  Akt is not enhanced by CML

B  CML enhances Survivin’s expression

C  CML enhances proliferation measured by PCNA

D  CML inhibits apoptosis measured by TUNEL
Supplemental Figure IV

A  CML-BSA decreases 4-AP sensitive current and increases $[Ca^{2+}]_i$

4-AP sensitive current (at 70mV)

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<th>Condition</th>
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<tr>
<td>Control (n=5)</td>
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<tr>
<td>CML-BSA (n=4)</td>
<td>110 ± 3</td>
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<tr>
<td>siRAGE+CML-BSA (n=4)</td>
<td>50 ± 1</td>
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</table>

** B  *P* < 0.01

** C  *P* < 0.01

B  Patch clamp representative currents

Baseline

4-AP

Control

CML-BSA

siRAGE + CML-BSA

200pA

100ms

FLUO3-AM

Control  CML-BSA  CML-BSA + VIVIT

CML-BSA + siSCRM  CML-BSA + siRAGE  CML-BSA + siSTAT3  CML-BSA + siPim1
Supplemental Figure V

A  STAT3 is activated in K.O. Pim1 mice
   P-STAT3 nuclear translocation assay

B  NFATc1 is not activated in K.O. Pim1 mice
   NFATc1 nuclear translocation assay

C  K.O. Pim1 mice are resistant to CML-induced proliferation
   Intracellular Calcium

D  CML treated K.O. Pim1 mice are not resistant to apoptosis
   Mitochondria hyperpolarization

   AnnexinV
Supplemental Figure VI

A  Vascular remodeling measured by echography

B  CML-BSA is increased in injured carotid

C  Pim1 expression is decreased by RAGE inhibition

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For A, the graphs show the comparison of lumen diameter and wall thickness between injured and siRAGE conditions.

For B, the graph illustrates the increase in CML-BSA normalized to sm-actin in injured carotid compared to control.

For C, the graph shows the decreased Pim1 mRNA expression with RAGE inhibition.
**SUPPLEMENTAL FIGURE LEGEND**

**Supplemental figure I**

(A) Representative immunofluorescences of P-STAT3 translocation assay. (B) Pim1 expression and NFATc1 protein activation occur 30 minutes post CML-BSA (1µg/mL) exposure measured by immunoblot (2.4 fold increase; \(n=4\), \(p<0.05\)). Pim1 and NFAT activation are maintained through time, showed by a 2.75 fold increase after 1 hour. (C) CML-BSA induces a positive feedback loop, increasing RAGE expression over time. In fact, 30 minutes after CML-BSA exposition, RAGE expression is increased and increases more after 1 hour (1.8 fold increase; immunoblot, \(n=4\), \(p<0.05\)).

**Supplemental figure II**

(A) RAGE siRNA efficiency is demonstrated by immunoblot. CML-BSA induces a 2 fold increase in RAGE expression and RAGE siRNA reduces its expression of 2.75 fold \((n=3, p<0.01)\). (B) STAT3 siRNA efficiency is demonstrated by immunoblot. STAT3 total expression is usually constant, but when cells are treated with STAT3 siRNA, its expression is decreased (2.7 fold decrease, \(n=3\), \(p<0.001\)). (C) Pim1 siRNA efficiency was measured by qRT-PCR as siPim1 causes a 300-fold decrease in Pim1 expression fold \((n=3, p<0.001)\). (D) VIVIT, NFAT’s inhibitor peptide, also decreases NFATc1 expression, showed by qRT-PCR, since NFATc1 autoregulates itself \((n=3, p<0.01)\). (E) VIVIT peptide is used to block NFAT activation and we confirmed that the control peptide has no effect as cells treated with CML-BSA and cells treated with CML-BSA and the control peptide have the same proliferation rate \((n=3)\).

**Supplemental figure III**

(A) NFATc1 activation by CML-BSA is not due to Akt pathway showed by immunoblot \((n=3)\). P-Akt/Akt ratio does not change between cells treated with CML-BSA. Furthermore, this ratio is below the one found in control cells (not treated) (B) CML-BSA enhances Survivin expression through RAGE/STAT3 axis showed by qRT-PCR \((n=3, p<0.01)\). Indeed, CML-BSA enhances Survivin expression by 175 fold and RAGE or STAT3 inhibition (by siRNA 20nM) decreases Survivin expression by at least 60 fold. (D) PCNA (proliferating cell nuclear antigen) is another method used to measure cell proliferation. CML-BSA induces an increase of 60% in proliferation \((n=50 CASMC/experiment for 5 experiments, p<0.001)\) and when RAGE (siRNA), STAT3 (siRNA) or NFAT (VIVIT 4µM) is blocked, proliferation is restored to normal rates \((n=250, p<0.05)\). Proliferation ratio was calculated by dividing positive cells for PCNA (i.e. PCNA in the nucleus) by the total amount of cells (visualised by DAPI) by immunofluorescence. (E) TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) is another method to measure apoptosis and we demonstrated that CML-BSA inhibits apoptosis in CASMC (decrease of 25%) through a RAGE/STAT3/Pim1/NFAT pathway since their blockade restore apoptosis \((n= n=50 CASMC/experiment for 5 experiments, p<0.05)\). Apoptosis ratio was measured by diving cells which had TUNEL staining (immunofluorescence) by the total amount of cells (DAPI).

**Supplemental figure IV**
(A) CML-BSA decreases 4-AP (1mM) sensitive current at 70mV (n=3, *p<0.05*), which corresponds to a decrease of voltage gated Kv channels. Decrease in Kv channels accounts for the increase of intracellular calcium, showed by FLUO3-AM. (B) Representative currents in different conditions using whole cell patch clamping.

**Supplemental figure V**

(A) CML-BSA stimulates STAT3 activation measured by translocation assay (*n=30 cells per condition per experiment for 3 experiments, *p<0.001*) in both wild type and Pim1 K.O. CASMC since STAT3 is upstream of Pim1. (B) Since Pim1 is responsible of NFATc1 activation, Pim1 K.O CASMC treated with CML-BSA (1µg/mL) did not have activated NFATc1 (*n=30 cells per condition per experiment for 3 experiments, *p<0.05*). (C) Wild type CASMC treated with CML-BSA have increase calcium, measured by FLUO3-AM (*n=30 cells per condition per experiment for 3 experiments, *p<0.001*), and proliferation, measured by Ki67 and K.O Pim1 CASMC are resistant to CML-BSA-induced proliferation (*n=30 cells per condition per experiment for 3 experiments, *p<0.001*). (D) Wild type CASMC treated with CML-BSA have hyperpolarised mitochondrial membrane, measured by TMRM (*n=30 cells per condition per experiment for 3 experiments, *p<0.001*), and decrease apoptosis and K.O Pim1 CASMC are not resistant apoptosis, measured by AnnexinV (*n=100, *p<0.05*).

**Supplemental figure VI**

(A) By echography, we measured carotid lumen diameter and carotid wall thickness in rats with carotid injury, which had either siSCRM or siRAGE. We demonstrated that RAGE blockade increases lumen diameter (*n=5 rats per group, *p<0.01*) and reduces wall thickness (*p<0.001*), which would result in restoring blood flow. (B) By western blot, we demonstrated an increase of CML-BSA in injured carotid (*n=4, *p<0.01*). (C) Pim1 mRNA levels in carotid of the different groups were measured by qRT-PCR. Pim1 is increased in injured carotid and showed a 5-fold decreased when RAGE was blocked (*n=5 per group; *p<0.01*).