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Lack of a significant role of P-Rex1, a major regulator of macrophage Rac1 activation and chemotaxis, in atherogenesis

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Abstract

Background—Rho GTPases are known to play important roles in regulating multiple cellular processes that include cell polarization and migration. Among these Rho GTPases, Rac has been shown to be essential for F actin formation and cell migration. P-Rex1 is a Guanine Nucleotide Exchange Factor (GEF) that was previously found to mediate the activation of Rac2, but not Rac1, in mouse neutrophils.

Objectives—Here we examined the role of P-Rex1 in mouse macrophages and atherogenesis.

Methods and Results—PBD (p21 binding domain) pull down assay was performed to compare the Rac1 activation in WT and P-Rex1 deficient macrophage. In addition, transwell assay was conducted to compare chemotaxis of WT and P-Rex1 deficient macrophage. We found that P-Rex1 is a major Rac1 regulator in mouse macrophages as its deficiency significantly compromises macrophage chemotaxis, superoxide production (SOD), and Rac1 activation in response to chemoattractants. The potential role of P-Rex1 in atherogenesis is also investigated by transferring P-Rex1 deficient bone marrow cells to LDLR deficient mice. Contrary to our prediction, P-Rex1deficiency did not alter atherogenesis, suggesting chemoattractant-induced macrophage migration may not have a significant role in atherogenesis.

Conclusions—P-Rex1 is one of major GEFs in macrophage regulating Rac1 activation and chemotaxis.

Keywords

P-Rex1; Rac1; GEF; chemotaxis; atherosclerosis

Rho GTPases belong to the Ras GTPase superfamily. Rho GTPases play important roles in regulations of many cell functions, including cell migration, secretion, phagocytosis, transcription, and the production of reactive oxygen species[(1–4)]. Rac is a member of the Rho GTPase family and has three isoforms, Rac1-3. Although Rac1 and Rac2 are expressed at similar levels in mouse neutrophils, Rac1 accounts for more than 90 % of Rac proteins in

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mouse macrophages[5]. Rho GTPases cycle between GDP-bound inactive form and GTP-bound active form, which is necessary for exerting their biological function. Because of their important biological functions, their activities are under tight regulation. One group of the Rho GTPase regulators is the guanine nucleotide exchange factor (GEF), which dictates the GDP dissociation and facilitates the GTP loading of Rho GTPases. Until now, over 70 unique Rho GEFs have been identified[6–9]. Some of Rho GEFs have been characterized on their biological functions in cell migration, such as Tiam1[10], Dbl[11] and Vav[12–14], but the biological significance of other GEFs is still limited.

P-Rex1 is a GEF of Rho GTPase whose activity is regulated by Gβγ and phosphatidylinositol 3,4,5-trisphosphate (PIP3)[15]. Its close homologs were also cloned, including P-Rex2[16,17], and P-Rex2B[18]. The P-Rex family members appear to preferentially activate the Rac proteins. The in vivo functions of the P-Rex GEFs have also been investigated using mouse models of gene disruption. Inactivation of P-Rex1 led to a partial impairment in superoxide production and chemotaxis in mouse neutrophils[19,20]. These partial effects appear to be attributed to the selective effect of P-Rex1-defcieicy on the activation of Rac2 rather than Rac1[19,20]. The function of P-Rex2b was investigated using siRNA and it was found to be involved in the endothelial cell migration by S1P [18].

In the current study, we investigate the role of P-Rex1 in regulation of macrophage Rac1 activity and chemotaxis. In addition, we also investigated the effect of P-Rex1-deficiency on a chronic inflammation paradigm, atherosclerosis, in which macrophages and their chemoattractants have been shown to play an important role. Although P-Rex1-deficiency significantly impaired mouse macrophage chemotaxis, phagocytosis, and superoxide production which might be explained by defective Rac1 activation, it surprisingly had little effect on atherogenesis in a bone marrow transfer atherosclerotic model.

RESULTS

We and other group previously showed that P-Rex1 was a key regulator of Rac2 activity in mouse neutrophils. In the current study, its functions in macrophages were investigated. First, the involvement of P-Rex1 in regulating macrophage chemotaxis was determined. Thioglycolate-elicited peritoneal macrophages from the WT and P-Rex1 deficient mice were prepared and transwell migration assays were carried out. Two macrophage chemoattractants were used. They are C5a and macrophage chemoattractant protein (MCP)-1. C5a induced more than 3 fold increase in chemotactic activity, while MCP-1 induced 2.5 fold for wildtype macrophages. For P-Rex1-null macrophages these two chemoattractants only induced above half a fold increase in chemotactic activity (Fig. 1A). These results indicate that P-Rex1-deficiency leads to more than 80% reduction in macrophage chemotactic activity in response to these two potent macrophage chemoattractants. Thus, we conclude that P-Rex1 has an important role in regulating chemoattractant-induced macrophage.

We also determined SOD production in response to C5a in WT and P-Rex1 deficient macrophages. As shown in Figure 1B, C5a-induced SOD production was significantly impaired by P-Rex1deficiency. This result also indicates that P-Rex1 is an important regulator of macrophage functions.

Because P-Rex1 has been shown to regulate Rac GTPases in neutrophils, it is reasonable to believe that the observed suppression of macrophage chemotaxis and SOD production by P-Rex1-deficiency is due to impairment in Rac activation. To test this hypothesis, we performed the PBD (p21 binding domain of PAK1) pull-down assay with purified p21 binding domain of PAK1, which has a high affinity for GTP-bound Rac. As shown in Figure

2, C5a could induce more than one fold increase in the level of active Rac ten seconds after stimulation in WT macrophages. However, C5a failed to induce significant Rac1 activation in macrophages lacking P-Rex1. These results suggest that P-Rex1 is a primary GEF that is required for chemotactic ligands to induce Rac1 activation in mouse macrophages, even though P-Rex1 has only a marginal effect on Rac1 activation in mouse neutrophils.

Knowing that macrophage chemoattractants and their receptors have been shown to play an important role in the initiation and progression of atherosclerosis, we decided to investigate if P-Rex1-deficiency has an effect on atherogenesis. To determine the effect of P-Rex1-deficiency on atherogenesis, we transferred the bone marrows from WT and P-Rex1 mice into lethally irradiated LDLR-deficient mice via periorbital vein injection. After four weeks recovery, the recipient mice were fed with high fat diet for 17 weeks before atherosclerotic lesion analyses. To our surprise, we did not observed any significant difference in the lesion area on the cross sections on the aortic roots between mice receiving the wildtype bone marrow and those receiving the P-Rex1-null bone marrow (Fig. 3A). Neither was there any significant difference in aortic lesion areas determined by *en face* oil-red-o staining (Fig. 3B & C). We also examined the lipoprotein cholesterol profile in plasma from wildtype and P-Rex1-null mice. Most of the cholesterol was in the VLDL and LDL fractions, and no significant differences were observed between the genotypes (Fig. 3D).

Next, we examined the macrophage content in the lesions. The cross sections of atheromas at aortic arches were stained with the anti-Moma-2 antibody. Moma-2 is a maker for mature macrophages and foam cells. As shown in the two representative images from the mice receiving wildtype bone marrow and P-Rex1-null bone marrow (Fig. 4A), the macrophage contents appear to be comparable. We also calculated the Moma-2 staining area relative to the total lesion area and found that there was no significant difference between samples from the mice receiving the wildtype bone marrow and those receiving the P-Rex1-null bone marrow (Fig. 4B). These results suggest that P-Rex1-deficiency may not affect the recruitment of macrophages to the atherosclerotic lesions.

DISSCUSSION

In the previous studies, P-Rex1 has been shown to be a major regulator of small GTPase Rac2 in mouse neutrophils[18]. In the current study, we showed that P-Rex1 is the major regulator of Rac1 in chemotactic signaling of macrophages. Consistent with the role of Rac in regulation of leukocyte chemotaxis, P-Rex1-deficiency significantly impairs macrophage chemotaxis and SOD production. Although we have tested only two chemoattractants (C5a and MCP-1), other macrophage chemoattractants, including PAF-1, S1P, LPC, and LPA, may also regulate these processes through P-Rex1, which need to be further tested. Nevertheless, the findings described in this report elucidate an important regulatory mechanism for Rac1 in macrophages. However, we do not know why the same GEF has very different roles in regulating two highly homologous small GTPases in neutrophils and macrophages. One possibility is that a different GEF that is expressed in neutrophils, but not macrophages, has a higher affinity for Rac1 than P-Rex1. This GEF would preferentially regulate Rac1 whereas P-Rex1 preferentially regulates Rac2 in neutrophils. In macrophages, P-Rex1 becomes the primary regulator of Rac in the absence of the other GEF. This idea is consistent with observations that P-Rex1 is capable of regulating both Rac1 and Rac2 in *in vitro* and overexpression assays and that P-rex1 has a higher affinity for Rac2 than Rac1[19].

Despite of the strong effects on Rac1 activation and chemotaxis of macrophages, PRex1 deficiency surprisingly failed to produce significant effects on atherogenesis. This seemly discrepancy could be due to many reasons. It is no question that macrophages and their chemoattractant signaling play important roles in initiation and progression of

atherosclerosis. The studies using mouse models lacking MCP-1 or CCR2 have clearly cemented the importance of the macrophage chemoattractant and its receptor in both macrophage infiltration into the intima and atherosclerotic lesion formation[21–24]. There are two possible explanations to our observation of the lack of significant effects of PRex1-defcieicny on atherogenesis. One is that there may be a different mechanism in the macrophages that are recruited into the intima as macrophages are known to be heterogeneous. The alternative explanation is that macrophage chemotaxis may not have a significant role in atherogenesis. MCP-1 signaling primarily regulates activation of endothelial cells, the interaction between monocytes and endothelial cells and/or transendothelial cell extravasations rather than migration of monocytes. Considering that the recruited macrophages do not need to migrate far into the intima for them to become foam cells during atherosclerosis, it is not difficult to envision that there may not be a need for chemokine-induced long distance migration of macrophages during atherogenesis. Based on the observation that similar numbers of macrophages were found in the peritonea elicited by thioglycolate between wildtype and P-Rex1-null mice, which suggests that P-Rex1-deficiency has little role in peritoneal macrophage infiltration even though it blocks the chemotaxis of the same cells, we believe that the second explanation is more plausible.

METHODS

Animals

Generation of P-Rex1-defeicnt has been previously described[19]. P-Rex1 null mice have been backcrossed with C57BL/6J for more than eight generations. Heterozygotes of P-Rex1 null mice were crossed to generate littermate of WTs and homozygotes of P-Rex1 null, which were used for experiments. LDLR-null mice in the C57BL/6J background purchased from Jackson Laboratory. For analysis of atherogenesis, mice were fed with high fat diet (Harlan Teklad) for 17 weeks. All animal procedures used in this study followed institutional guidelines of Yale University.

Chemotaxis of peritoneal macrophage

Peritoneal macrophage was elicited by peritoneal injection of 1ml 3% thioglycollate 72 hours before harvesting. Harvested peritoneal macrophages were loaded into the upper chambers of 24-well transwell plates (Costar, 5uM pore size). The lower chambers were filled with the same medium, but supplemented with 10 nM C5a (Sigma) and 10nM MCP-1(Pepro Tech) for macrophage migration. The plates were incubated at 37°C for 4 hours. For peritoneal macrophage migration assays, cells attached to the lower surfaces of inserts were trypsinized and collected for counting and FACS analysis after staining with the anti-F4/80 antibody. Chemotaxis index was calculated based on migrated macrophage in the presence and absence of chemotactic ligands.

PBD pull down assay

The pull down assays for determining the activity of small GTPases were carried essentially as previously described. In brief, peritoneal macrophages were harvested as above and incubated on ice for 30min before stimulation with 10nM C5a for 10 or 30 seconds. At the specified time, lysis buffer [50mM Tris–HCl (pH 7.3), 10mM MgCl2 and 0.2M NaCl, 2% NP40, 10% glycerol, 2mM orthvanadate] containing recombinant GSTPAK-PBD was added into the cell suspension to stop stimulation. The cell lysates were then cleaned by centrifugation before pre-washed glutathione beads were added. The mixtures were rotated at 4 °C for 1 hour. Beads were then washed three times with lysis buffer before 2× SDS sample buffer was added and boiled at 100 °C for 5min. Bound GTPases were detected by Western analysis with an anti-Rac1 antibody.

Bone marrow transplantation

Recipient LDLR-null mice were lethally irradiated with 550 Rads twice at a 4-hour interval. Twenty four hours later, the mice were transplanted via periorbital vein injection with bone marrow cells (2 million/per recipient) from WT and P-Rex1 null mice. The bone marrow cells were prepared by flushing femurs with 1× DPBS and lysing red blood cells with the RBC lysis buffer (8.3g NH_4Cl, 1.0g $KHCO_3$, 1.8ml 5% EDTA in 1000ml dH_2O). Four weeks after bone marrow transfer, recipient mice were fed with high fat diet for 17 weeks for atherosclerotic lesion analysis.

Atherosclerotic lesion analysis

Mice were anesthetized, and blood samples were collected via heart puncture for lipoprotien profile analyses by FPLC, as previously described ( ). Then the cardiovascular system was perfused with 20ml 1× DPBS (Cellgro), through the heart apex, followed by 5ml fixatives (4% PFA, 5% sucrose, 20uM EDTA in 1× DPBS, PH 7.4). The aorta from the aortic root to iliac artery was dissected and cut open longitudinally *in situ* and immerged in the fixative for 12 hours before being rinsed with 1×PBS and stained with Oil-Red-O (FisherBiotech). The aorta was then spread, and the lesion area was quantified using the imaging analysis software, ImagePro from Media Cybernetics. The heart and proximal portion of aortic root was also fixed and embedded in OCT for lesion area quantification on cross sections of the aortic root as previously described (25).

Immunohistochemistry

For Moma-2 staining, frozen sections were fixed with acetone/methanol (v:v 1:1) for 30min before transferred to 1× TBS(8.766g/L NaCl, 6.055g/L Tris, PH:7.4) for 10min at RT. Sections were then immerged in 0.3% H_2O_2, rinsed, and transferred into 10% normal rabbit serum (Vector Laboratories), followed by staining with Rat-anti-Mouse Moma-2 antibody, biotinylated second antibody, and HRP-avidin conjugates using the ABC kit from Vector Laboratories). Finally the sections were developed with DAB (Dako North American) and counter-stained with hematoxylin (Thermo Electron Corporation). The macrophage (Moma-2 positive) area and lesion area were quantified by using MetaMorph Software.

Superoxide Assay

Mouse peritoneal macrophage superoxide production was measured by isoluminol chemiluminescence assay[19]. Briefly, Harvested macrophages were suspended in Hank's buffer (Gibco) at 10×10^6/ml on ice. 50ul cell suspension were added into the 96 well plate after mixing with 40 μl HRP solution and 80 μl isoluminol. 10uM C5a was added into the reaction system followed by reading chemiluminescence by a luminometer. Assay was performed in triplicate.

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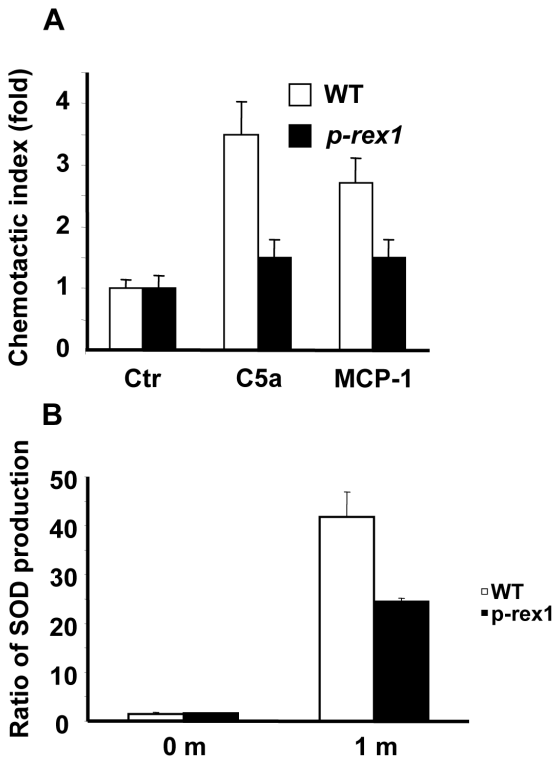

Figure 1. P-Rex1 deficiency leads to impaired macrophage chemotaxis

Peritoneal macrophages from WT and P-Rex1 deficient mice were elicited by thioglycolate. (A). Macrophage chemotaxis in response to C5a and MCP-1 were determined using a transwell assay. Chemotaxis index was calculated based on number of transmigrated macrophage in the presence and absence of ligands. n=3, $p<0.01$ between WT and P-Rex1 KO macrophage in presence of C5a and MCP-1. Error bars mean standard deviation. (B) Superoxide production was performed by using isoluminol chemiluminescence assay. 0 m and 1m stand for before and 1m after C5a stimulation. $p<0.01$. Error bars means standard deviation.

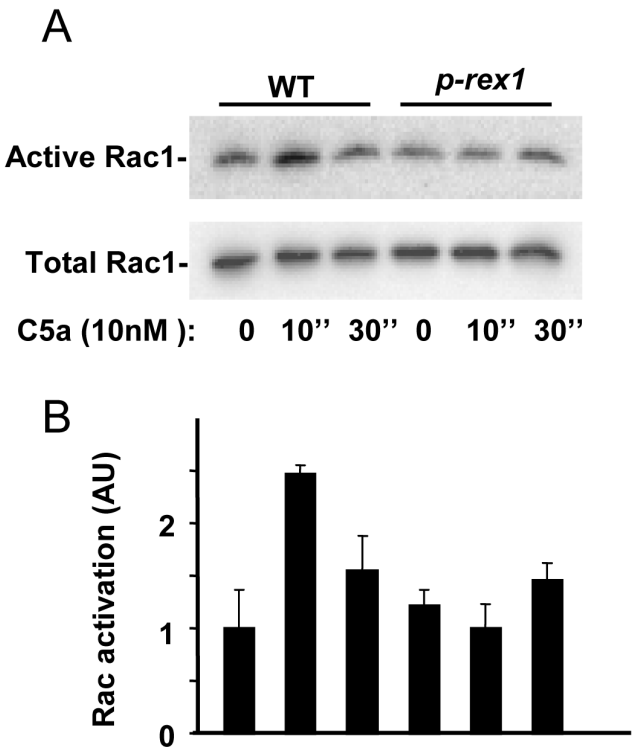

Figure 2. P-Rex1 deficiency leads to impaired Rac1 activation in mouse macrophages

Peritoneal macrophages from WT and P-Rex1-deficient mice were stimulated with C5a for indicated time. PBD pull-down assays were performed, and Rac1 was detected by an anti-Rac1 antibody (A). Densitometry was performed to quantify the levels of active and total Rac1, and the relative levels of active Rac1 normalized against total Rac1 levels are shown (B). n=3, $p<0.01$ on Rac1 activity between WT and P-rex1 KO macrophage at 10 second. Error bars means standard deviation.

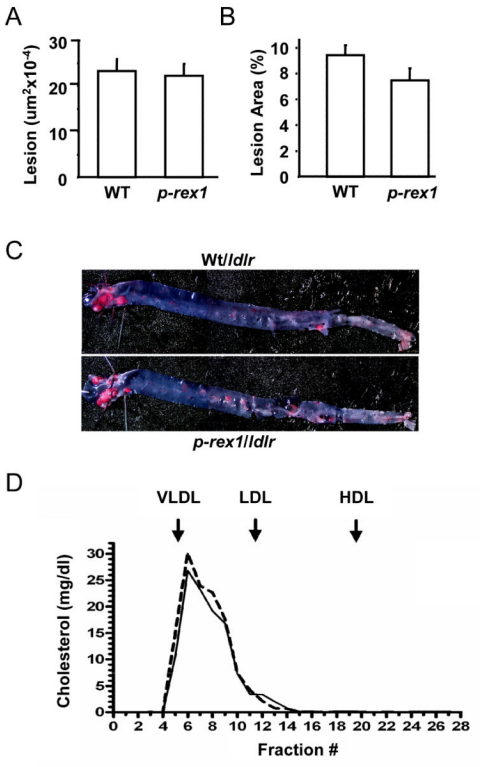

Figure 3. P-Rex1 deficiency does not affect atherogenesis

A and B) Quantification of atherosclerotic lesion area on the cross sections of aortic root (A) and on the surface of aorta (B) of *ldlr*−/− mice receiving wildtype or P-Rex1-null bone marrows. p>0.05, n=8, error bars denote SEM. C) Representative images of *en face* oil-red-o staining of aortas. D) Lipid profiles of LDLR-deficient mice receiving WT and P-Rex1 deficient bone marrows.

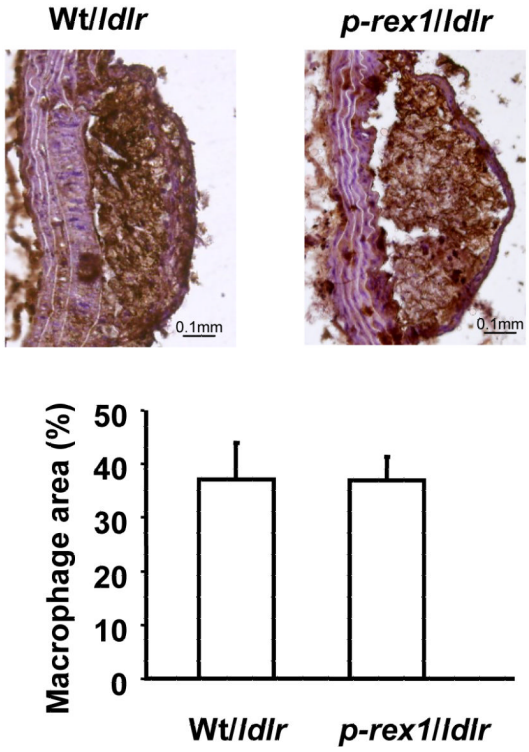

Figure 4. Macrophage contents in the lesions

A) Representative images of Moma-2 staining of aortic cross sections from LDLR-deficient mice receiving WT and P-Rex1 deficient bone marrows. B) The macrophage area relative to the lesion area was quantified. $p>0.05$, $n=7$, error bars denote SEM.