Regulation of cyclooxygenase-2 expression by small GTPase Rac2 in bone marrow macrophages

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Submitted 30 January 2007; accepted in final form 8 June 2007

Am J Physiol Lung Cell Mol Physiol 293: L668–L673, 2007. First published June 15, 2007; doi:10.1152/ajplung.00043.2007.—Cyclooxygenase 2 (COX-2) is induced by microbial products, proinflammatory cytokines, growth factors, and oncogenes. The Rho family includes RhoA, Rac1, Rac2, Rac3, and Cdc42 and is involved in regulation of the actin cytoskeleton organization, cell growth, vesicular cell trafficking, and transcriptional regulation. Rac2 binds to NADPH oxidase protein complex, and Rac2 null neutrophils are known to have poor phagocytic activity. We examined whether Rac2, the predominant small GTPase in hematopoietic cells, influences COX-2 expression in bone marrow-derived macrophages (BMDM). We showed that BMDM from Rac2−/− mice have reduced COX-2 expression in response to treatment with endotoxin. Despite a compensatory increase in Rac1, BMDM from Rac2−/− mice have less biologically active GTP-bound Rac in response to LPS stimulation. Signaling molecules (downstream of Rac2 and Toll-like receptor 4) such as p42/p44, p38, and pAKT were also affected in stimulation. Signaling molecules (downstream of Rac2 and Toll-like receptor 4) such as p42/p44, p38, and pAKT were also affected in stimulation.

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RHO-RELATED SMALL GTPASES are important in multiple cellular events, including actin cytoskeletal organization, cell proliferation, and survival of cell and transcriptional regulations of genes (4, 9, 13, 19, 32a, 35, 36). These small GTPases serve as molecular switches that cycle between a GTP-bound active form and a GDP-bound inactive form. There are three highly homologous forms of Rac: Rac1, Rac2, and Rac3 (5). Rac1 and Rac2 exhibit 92% amino acid identity (7, 11, 23, 28), and murine and human Rac2 are 99% identical. Unlike Rac1 and Rac3, which are widely expressed, Rac2 expression is detected with a Rac2-specific antibody only in myeloid cells, including neutrophils, monocytes, and macrophages (1, 5, 36). The small GTPase Rac has been reported to induce activation of transcription factors, such as serum response factors and nuclear factor-κB (NF-κB), resulting in transcription of multiple genes (32). Rac isoform-specific functions have been investigated recently in knockout mice and, more recently, using RNA interference knock-down technologies. Rac2 null mice are viable (5, 25) and demonstrate cellular defects in multiple hematopoietic lineages, including stem and progenitor cells, neutrophils, mast cells, T cells, and B cells (8, 9, 37). Rac2 is involved in many signal transduction pathways, including both the mitogen-activated kinases p42/p44 and p38 pathways in neutrophils and T cells (25).

Rac1 and Rac2 also have been reported to regulate macrophage ultrastructure but are not necessary for locomotion or tissue invasion (36). Rac2-deficient cells have been reported to have a compensatory increase in immunoreactive Rac1, but this only partially rescues the phenotypic expressions that are absent in myeloid cells that are deficient in Rac2 (13). These observations, together, suggest that Rac2 has a specific role in myeloid cells, which is distinct from that of Rac1, in regulating gene expression.

Macrophages play a central role in host defense against infection by many pathogens but also in regulation of immune responses and inflammation. Several cytokines, chemokines, and the key enzyme, including COX-1 and -2, are part of the inflammatory repertoire generated by activated macrophages (4, 6, 12, 22, 30). COX-1 is constitutively expressed but, under some circumstances, also can be induced by various stimuli, including shear stress. COX-2 is considered the inducible isoenzyme and is induced by various proinflammatory mediators such as lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α). Inducible COX-2 expression in alveolar macrophages, alveolar epithelial cells, and other cell lines has been linked to diseases such as asthma, pulmonary fibrosis, and lung cancer (24). COX-2 expression is regulated, in part, by the NF-κB gene activation pathway. However, it is dependent on stimulus, cell type, and activation of other transcription factors, including CREB and C/EBP-β proteins and MAPK kinases. Posttranscriptional mechanisms that involve PU.1 and YY-1 also contribute significantly to the COX-2 expression (17).

Recent studies suggest a link between activation of RhoA proteins and COX-2 expression (14, 29, 30, 31, 33), but the role of Rac2 has not been reported. It also has been suggested that Rac2 plays a significant role in phagocytosis of opsonized particles and superoxide production in bone marrow-derived macrophages (BMDM), but effects on Toll-like receptor 4 (TLR4)-mediated gene COX-2 expression have not been in-
vestigated (34). In this study, we show, for the first time, that COX-2 expression and PGE2 and PGD2 synthesis are regulated, in part, by the small GTPase Rac2. We demonstrate that Rac2 plays a significant role in COX-2 expression through activation of Rac and GTP-Rac-mediated signaling, which also involves MAPK signaling and the NF-κB activation pathway. Furthermore, Rac2 deficiency is associated with reduced expression of transcription factor PU.1, which also may contribute to COX-2 expression.

MATERIALS AND METHODS

Materials. COX-2 antibodies were obtained from Cayman Chemical (Ann Arbor, MI). Rac1 antibodies were generated as described elsewhere (2). Rac2 antibodies were a kind gift from Dr. Gary Bokoch ( Scripps Research Institute, La Jolla, CA). Other chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO) and Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of BMDM. Rac2−/− null mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Macrophages derived from mouse bone marrow (BMDM) were obtained according to our published procedure (26). After asphyxiation of mice with CO2, cellular material from femurs was aspirated and spun at 400 g at 4°C for 5 min. Bone marrow-derived cells were then cultured in DMEM with 10% FBS and 10% L929 cell-conditioned medium. These cells were allowed to mature into phenotypic macrophages by incubation in the presence of L929 cell-conditioned medium for 7 days. Cells were subsequently washed and lifted for seeding into six-well plates for subsequent experiments. We have used 1 × 106 cells for most of the time periods.

Immunoblot analysis. Cell lysate was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. COX-2 was detected using antibodies from Cayman. Phosphorylation of p38 MAPK, MAPK-activated protein kinase-2 (MAPKAPK-2), and Akt2 were detected using phosphospecific polyclonal antisera recognizing phosphorylation at threonine-180/tyrosine-182, threonine-334, and serine-473, respectively (Cell Signaling Technology, Beverly, MA). Polyclonal antisera recognizing p38, MAPKAPK-2, and Akt2 (Cell Signaling Technology) were used to probe total levels of these proteins. Antibody binding was detected using anti-Rac antibodies. Rac was separated on 12% SDS-PAGE gels and subsequently transferred to PVDF membranes for immunoblotting.

Measurement of PGE2 and PGD2. Equal numbers of wild-type and Rac2 null BMDM cells were treated with 1 μg/ml LPS for the respective time periods. Medium supernatant samples were collected at 0, 4, 6, and 16 h. These supernatants were frozen for later measurement of PGE2 and PGD2 by liquid chromatography in conjunction with mass spectrometry (LC-MS-MS). Briefly, each sample was collected and spiked with d4-PGE2 (100 ng/ml) as an internal standard, and citric acid and 10% butylated hydroxytoluene were added to prevent radical peroxidation. PGE2 and PGD2 were extracted by adding 2 ml of hexane-ethyl acetate (1:1, vol/vol) and vortex mixing for 1 min. The upper organic phases were collected after centrifugation at 3,000 rpm for 15 min at 4°C. The extraction step was repeated twice, and the organic phases were dried under nitrogen at room temperature. Samples were reconstituted in 200 μl of methanol and 10 mM ammonium acetate buffer (pH 8.5, 1×) before LC-MS-MS analysis. The HPLC system consisted of Shimadzu (Columbia, MD) LC-10Advp pumps with a Leap (Carrboro, NC) HTS PAL autosampler. PGE2 and PGD2 were separated on a Luna (Phenomenex; Torrance, CA) 3-μm phenyl-hexyl 2 × 150-mm analytical column with a linear gradient from 22–50% acetonitrile in 10 min in a 10 mM ammonium acetate buffer (pH 8.5) at a flow rate of 0.15 ml/min.

RESULTS

Expression of TLR4 in BMDM from WT and Rac2 null mice. Cell surface expression of TLR4/MD-2 complex was evaluated using phycoerythrin-conjugated MTS510 antibody and fluorescence-activated cell sorting analysis. As shown in Fig. 1A, TLR4/MD-2 protein expression on the surface of Rac2 null and WT BMDM was nearly identical. These data are in agreement with studies using cells from VAV null mice that lack VAV, a guanine nucleotide-exchange factor for Rac that also shows no change in TLR4/MD-2 expression on the cell surface (20).

Expression of Cdc42, Rac1, and Rac2 in BMDM. To evaluate the expression of Rho GTTPases, we performed Western blotting on LPS (1 μg/ml) stimulated BMDM cell lysate and detected total Rac, Cdc42, and Rac2 proteins. We examined the expression of Cdc42 and Rac1 in BMDM from WT and Rac2−/− null mice to show their response to TLR4 signaling. Figure 1B shows the effect of LPS treatment on the level of immunoreactive Cdc42 and Rac1 expression in WT and Rac2−/− null BMDM. We observed that Rac2−/− null BMDM
MD2 expression was nearly identical in Rac2 null bone marrow-derived macrophages (BMDM). A: to determine the cell surface TLR4/MD2 expression, cells were suspended in 250 μl of 1% FBS-0.1% Na3PO4-PBS and incubated with 2.5 μl of TLR4/MD2 phycoerythrin-conjugated antibody on ice for 45 min. The pattern of TLR4/MD2 expression was identical for Rac2 null and wild type (WT) BMDM, as shown. B: expression of the Rho family GTPase Cdc42 and total Rac was compared with that of immunoreactive GTP-Rac in the Rac2 null BMDM in response to LPS treatment. This suggests that although increased Rac1 is present in the Rac2 null BMDM, the total GTP-Rac that results from increased Rac1 is significantly less in response to LPS treatment (Fig. 2). These data indicate that in LPS-treated BMDM, Rac2 is the preferred substrate for activation by coupling to GTP compared with Rac1.

Phosphorylation of transcriptional activators is reduced in Rac2 null mice. After establishing that Rac is activated to GTP-Rac in response to LPS treatment, we examined the effect on the phosphorylation activity of transcriptional activators such as p38 and p42/44 MAP kinases and pAKT. As shown in Fig. 3, A and B, Rac2 null BMDM have less activation of p38 kinase activity and p42/44 compared with WT BMDM. These results in BMDM are consistent with earlier reports (25) establishing that phosphorylation of the transcriptional activators p38 and p42/44 MAPKs are reduced in Rac2-deficient neutrophils. There also were differences in the appearance of pAKT in the Rac2 null compared with WT BMDM (Fig. 3C). Whereas there was reduced pAKT activity at 30 min, levels were similar at 45 min and much greater by 60 min in the Rac2 null compared with WT BMDM. This increased pAKT in Rac2-deficient neutrophils has been observed by antibodies slightly cross-reacted with Rac1 in Rac2 null cells (at the 0- and 12-h time points), but it otherwise was not detected in Rac2 null cells. We did not examine for the presence of Rac3 because this isoform is not present in hematopoietic cells (36).

LPS mediated GTP-Rac activation in BMDM. Rac-mediated cell signaling requires GTP-Rac to activate downstream effectors. We measured total Rac and GTP-Rac activation in macrophages from Rac2 null and WT cells after LPS stimulation. For evaluation, BMDM were treated with LPS (1 μg/ml) for the indicated time periods, and the GTP-Rac was measured using a PAK pull-down assay, as previously described by our group (2). The results (Fig. 2) showed that in response to LPS treatment, there was a three- to sixfold increase in the detection of GTP-Rac in the WT compared with Rac2 null BMDM. We were able to detect only a transient increase, from 15 to 30 min, in immunoreactive GTP-Rac in the Rac2 null BMDM in response to LPS treatment. This suggests that although increased Rac1 is present in the Rac2 null BMDM (as shown in Fig. 1B), the total GTP-Rac that results from increased Rac1 is significantly less in response to LPS treatment (Fig. 2). These data indicate that in LPS-treated BMDM, Rac2 is the preferred substrate for activation by coupling to GTP compared with Rac1.

have normal Cdc42 expression, and there is a slightly higher level of total Rac in Rac2 null BMDM compared with the total Rac protein levels in WT BMDM. This observation is in agreement with other published reports (36) showing that Rac2 null hematopoietic cells have a compensatory increase in levels of Rac1. We also detected a small increase in total Rac in the WT BMDM that were treated with LPS. In contrast, total Rac in the Rac2 null BMDM was constitutively present at the 0- through 6-h time points and slightly decreased by 12 h following LPS treatment. Figure 1C shows that Rac2 is present in the WT BMDM but is not changed in response to LPS treatment. As shown in Fig. 1C, bottom, Rac2
others (25). These data indicate that the absence of Rac2 in BMDM has an effect on p38 and p42/44 MAPKs and pAKT that is similar to that observed by another group in neutrophils. One report (21) suggests that members of the Rho family of GTPases can provoke concomitant stimulation of two counteracting signaling pathways and that their balance ultimately determines the ability of these small GTPases to promote cell survival and death.

Rho GTPase Rac2 is involved in LPS-mediated COX-2 expression. To examine the possibility that Rac2 is involved in the induction of COX-2 expression, we treated WT and Rac2 null BMDM with LPS (1 μg/ml) for the indicated time periods. As shown in Fig. 4, A and B, there was a significant decrease in COX-2 expression in Rac2 null compared with WT BMDM in response to LPS treatment. Decreased expression of COX-2 enzymatic activity was confirmed by measuring the production of PGE2 and PGD2 in the...
culture supernatant of Rac2−/− null and WT BMDM. As shown in Fig. 4, C and D, respectively, there was a significant decrease in both PGE2 and PGD2 synthesis in Rac2−/− null compared with WT BMDM in response to LPS treatment. *IkBα is not degraded in Rac2 null mice. *IkBα degradation leads to translocation of the p50/RelA heterodimeric form of NF-κB to the nuclear compartment, which regulates, in part, COX-2 gene transcription. To examine the effect of Rac2 deficiency on the NF-κB activation pathway, we stimulated the BMDM with 1 μg/ml LPS and followed the degradation and resynthesis of *IkBα in Rac2−/− null and WT BMDM. As shown in Fig. 5A, we detected significant differences in the pattern for *IkBα degradation in Rac2−/− null compared with WT BMDM. There was near complete degradation of *IkBα by 6 h, and this increase is markedly attenuated in Rac2−/− null BMDM. We have determined that COX-2 expression is in-mediated signaling and subsequent COX-2 expression in BMDM. Studies involving neutrophils derived from Rac2−/− null mice have shown similar results (25).

We have investigated the effect of Rac2 deficiency on three possible mechanisms that could relate Rac2 deficiency to know mechanisms for COX-2 expression. First, we have shown that activation of NF-κB, measured as degradation of *IkBα, is significantly attenuated in the Rac2−/−/null compared with WT BMDM. Our group and other investigators have shown that activation of NF-κB is sufficient for inducing COX-2 expression in macrophages. Thus attenuation of NF-κB activation, as demonstrated by showing reductions in *IkBα degradation, could result in decreased COX-2 expression. Second, we have shown that PU.1 levels in LPS-treated BMDM are reduced in the Rac2−/− null compared with WT BMDM. PU.1 is necessary for normal macrophage function and contributes to the production of COX-2 protein (16). Decreased cellular content of PU.1 could result in an attenuation of COX-2 expression. Finally, we also have shown that Rac2 deficiency in BMDM results in decreased activation of multiple members of the MAPK family, including reductions in the appearance of phosphorylated pERK, p38 kinase, and pAkt kinases, which could be involved in COX-2 expression (3, 21). Thus Rac2 appears to be essential component of several LPS-mediated signaling events that could directly or indirectly have an impact on COX-2 expression.

In summary, we have shown that Rac2 plays an important role downstream of the TLR4 receptor complex in BMDM that are treated with LPS. There is decreased production of COX-2 protein and enzymatic synthesis of PGE2 and PGD2 in Rac2−/− null BMDM that are treated with LPS. Rac2 deficiency is associated with reduced activation of NF-κB, decreased PU.1 protein production, decreased phosphorylation of p42/44 and p38, and a different pattern of phosphorylation of pAkt in BMDM that are treated with LPS. These data indicate that Rac2 mediates signaling events in BMDM that are initiated through TLR4 and result in COX-2 expression.

DISCUSSION

Several members of the Rho families of small GTPases have been reported to be involved in the regulation of COX-2 expression. For example, levels of COX-2 protein are dramatically increased in H-Ras (V12)-transformed cells (27), and ectopic expression of constitutively active RhoA, Rac1, or Cdc42 leads to COX-2 expression in NIH3T3, Madin-Darby canine kidney epithelial cells, and H29 colon cells (3). In this study, we have examined the involvement of Rac2 in LPS-mediated signaling and subsequent COX-2 expression in BMDM. We have determined that COX-2 expression is induced by TLR4-Rac2 signaling pathway. Our data show that Rac2-deficient mouse BMDM produce less COX-2 protein and less PGE2 and PGD2 synthesis in response to LPS treatment. These data suggest that stimulation of TLR4 by LPS treatment leads to COX-2 expression by a pathway that involves the small GTPase Rac2. It is interesting to note that ARF6, which binds to Rac2, regulates the assembly of TLR4 complex involving phosphatidylinositol 4,5-bisphosphate (18). Our data show that LPS stimulation of WT BMDM leads to an increase GTP-Rac by 6 h, and this increase is markedly attenuated in BMDM from Rac2−/− null mice. However, total Rac is increased in the Rac2−/− null BMDM, from a compensatory increase in Rac1, but is not activated by LPS treatment into the GTP-Rac form that results in COX-2 expression. These data indicate that Rac2 is a preferred substrate, compared with Rac1, for activation to GTP-Rac in response to LPS treatment in BMDM. Studies involving neutrophils derived from Rac2−/− null mice have shown similar results (25).

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