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

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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−/− null mice have reduced COX-2 expression in response to treatment with endotoxin. Despite a compensatory increase in Rac1, BMDM from Rac2−/− null 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 p42/44, p38, and pAKT were also affected in BMDM from Rac2−/− null mouse macrophages. We also observed that BMDM from Rac2−/− null failed to degrade IκBα significantly and had less immunoreactive PU.1. We show that both NF-κB pathway and PU.1 are involved in normal macrophage function and play a role in macrophage COX-2 expression. In summary, these data indicate that Rac2 regulates COX-2 expression in BMDM.

small guanosine triphosphatases; Rac1; Cdc42

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 immune reactive 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 isomerase 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 PGE$_2$ and PGD$_2$ 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 CO$_2$, 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 subsequently washed and lifted for seeding into six-well plates for subsequent experiments. We have used 1 × 10$^6$ 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, serine-473 and threonine-567, respectively (Cell Signaling Technology, Beverly, MA). Polyclonal antisera recognizing p38, was detected using antibodies from Cayman. Phosphorylation of p38, threonine-180/tyrosine-182, threonine-334, and threonine-567, respectively (Cell Signaling Technology). 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 × 10$^6$ cells for most of the time periods.

Flow cytometry. Cell suspensions were prepared, counted, and stained with antibodies following standard procedures. BMDM were washed twice with cold 0.1% NaN$_3$-PBS and harvested. After being suspended in 1 ml of cold 0.1% NaN$_3$-PBS, each sample was divided into two 500-μl aliquots. For determination of the cell surface TLR4/MD-2 expression, cells were suspended in 250 μl of 1% FBS-0.1% NaN$_3$-PBS and incubated with 2.5 μl of TLR4/MD-2 phycocerythrin-conjugated antibody (MTS510; Santa Cruz Biotechnology) on ice for 45 min. Cells were subjected to flow cytometric analysis on a FACSCalibur flow cytometer (Becton Dickinson).

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 previous 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). The expression of Cdc42, Rac1, and Rac2 in BMDM. To evaluate the expression of Rho GTPases, 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...
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 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.

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 p38 and p42/44 phosphorylation in 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...
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

Fig. 3. Western blot showing the phosphorylation of transcriptional activators p38, p42/44, and Akt kinases. BMDM were treated with LPS (1 μg/ml) for the designated time periods and subjected to electrophoresis and Western blotting. Cell lysates from WT and Rac2−/− null BMDM were probed with phosphospecific antibodies against p38 (A), p42/44 (B), and Akt (C). Also shown are the total p38, p42/44, and Akt in the same samples. These Western blots are representative of 3 separate experiments.

Fig. 4. Decreased COX-2 protein production and prostanoid synthesis by Rac2−/− null BMDM in response to LPS treatment. A: BMDM from WT and Rac2−/− null mice were stimulated with LPS (1 μg/ml) for various time periods and subjected to SDS-PAGE and Western blotting using anti-COX-2 antibodies and control anti-β-actin antibodies. This blot is representative of 4 separate experiments giving similar results. B: densitometric analysis of COX-2 expression in the experiments shown in A. Data are means ± SE (n = 4 for each condition). C: PGE2 levels released in the medium after BMDM from Rac2−/− null and WT mice were treated with LPS (1 μg/ml) for the designated time periods. Data are means ± SE of 3 separate experiments. Values at 16 h are significantly different at P < 0.01 level. D: PGD2 levels released in the medium after BMDM from Rac2−/− null and WT mice were treated with LPS (1 μg/ml) for the designated time periods. Data are means ± SE of 3 experiments. Values at 16 h are significantly different at P < 0.005 level.
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 30–45 min and only partial reconstitution by 90 min after LPS treatment in WT BMDM. In contrast, Rac2−/− BMDM had higher levels of IkBα at the zero time point and all subsequent time points following LPS treatment. This suggests that NF-κB activation in BMDM in response to LPS treatment is dependent on Rac2 by a mechanism that appears to be related to augmentation in the levels of immunoreactive IkBα in the cytoplasm.

Production of PU.1 in response to LPS treatment is reduced in Rac2−/− null BMDM. PU.1 is an Ets family transcription factor that is expressed in macrophages and has been shown by our group to contribute to COX-2 gene expression in macrophages (16). We observed that WT macrophages have an induction in PU.1 protein levels in response to LPS treatment in a time-dependent manner (Fig. 5B). This increase is markedly attenuated in PU.1 protein production in BMDM from Rac2−/− null mice in response to LPS treatment.

**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 treated Rac2−/− null BMDM that are treated with LPS. Rac2 deficiency is associated with reduced activation of NF-κB, decreased PU.1 protein production, reduced 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.

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, reduced 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.

**GRANTS**

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**REFERENCES**


