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Hemoglobin Induces the Expression of Indoleamine 2,3-Dioxygenase in Dendritic Cells Through the Activation of PI3K, PKC, and NF-κB and the Generation of Reactive Oxygen Species

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ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme in the kynurenine (Kyn) pathway of tryptophan (Trp) metabolism. IDO is immunosuppressive and is induced by inflammation in macrophages and dendritic cells (DCs). Previous studies have shown the serum Kyn/Trp levels in patients with hemolytic anemia to be notably high. In the present study, we demonstrated that hemoglobin (Hb), but not hemin or heme-free globin (Apo Hb), induced IDO expression in bone marrow-derived myeloid DCs (BMDCs). Hb induced the phosphorylation and degradation of IκBα. Hb-induced IDO expression was inhibited by inhibitors of PI3-kinase (PI3K), PKC and nuclear factor (NF)-κB. Hb translocated both RelA and p52 from the cytosol to the nucleus and induced the intracellular generation of reactive oxygen species (ROS). Hb-induced IDO expression was inhibited by anti-oxidant *N*-acetyl-L-cysteine (NAC) or mixtures of SOD and catalase, however, IDO expression was enhanced by 3-amino-1,2,4-triazole, an inhibitor of catalase, suggesting that the generation of ROS such as O_2^-, H_2O_2, and hydroxyl radical is required for the induction of IDO expression. The generation of ROS was inhibited by a PKC inhibitor, and this action was further enhanced by addition of a PI3K inhibitor. Hb induced Akt phosphorylation, which was inhibited by a PI3K inhibitor and enhanced by a PKC inhibitor. These results suggest that the activation of NF-κB through the PI3K-PKC-ROS and PI3K-Akt pathways is required for the Hb-induced IDO expression in BMDCs. J. Cell. Biochem. 108: 716–725, 2009.

KEY WORDS: INDOLEAMINE 2,3-DIOXYGENASE; HEMOGLOBIN; BONE MARROW-DERIVED MYELOID DENDRITIC CELLS; PKC; PI3K; NF-κB; REACTIVE OXYGEN SPECIES; INDUCIBLE NITRIC OXIDE SYNTHASE; HEME OXYGENASE-1; TRYPTOPHAN

Indoleamine 2,3-dioxygenase (IDO)-initiated tryptophan (Trp) metabolism along the kynurenine (Kyn) pathway regulates T-cell responses in some dendritic cells (DCs) such as plasmacytoid DCs or $CD8^+$ DCs in mouse spleen cells [Grohmann et al., 2003; Mellor and Munn, 2004]. Two mechanisms of the IDO-induced inhibition of T-cell responses have been proposed; the local depletion of Trp required for cell proliferation and the induction of apoptosis or growth arrest by Trp metabolites [Grohmann et al., 2003; Mellor and Munn, 2004]. IDO is induced by inflammation as well as immune responses such as tumor immunity or infectious responses. The IDO expression is induced in DCs by various stimuli such as IFN-γ, toll-like receptor (TLR)-ligation by LPS or CpG oligodeoxynucleotides, or CD80/CD86-ligation by CTLA-4 expressed on regulatory T cells [Grohmann et al., 2003; Mellor and Munn, 2004].

Hemoglobin (Hb) in red blood cells is the most abundant hemoprotein in the body, and its levels are maintained via constant synthesis and degradation. If, however, excessive concentrations of Hb exceeding the capacity of metabolic pathways are released into plasma, they contribute, as heme-free globin (apohemoglobin, Apo Hb) and/or free heme, to the pathogenesis of toxic injures such as atherosclerosis [Jeney et al., 2002; Grinshtein et al., 2003; Wagener et al., 2003; Kumar and Bandyopadhyay, 2005; Tsemakhovich et al., 2005]. Haptoglobin, an acute phase protein, forms a stable complex

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with Hb, and the complex is then removed from the bloodstream by the mononuclear phagocyte system through the Hb scavenger receptor, CD163, which is expressed on the surface of monocytes/macrophages and DCs [Kristiansen et al., 2001; Maniecki et al., 2006]. Even after haptoglobin binding capacity is exhausted by severe hemolysis, Hb can continue to interact with CD163 in the absence of haptoglobin in a low affinity pathway of Hb removal [Moestrup and Møller, 2004; Schaer et al., 2006b, 2007].

Free heme serves as a double-edged sword in cell biology. Although it is an essential element for forming hemoproteins and regulating the gene expression of Hb and myoglobin at low or physiological concentrations, it can also become highly toxic by oxidative stress and inflammation at non-physiologically high concentrations [Jeney et al., 2002; Grinshtein et al., 2003; Wagener et al., 2003; Kumar and Bandyopadhyay, 2005]. Hemopexin is a heme-binding plasma glycoprotein which forms the second line of defense against Hb-mediated oxidative damage during intravascular hemolysis [Hvidberg et al., 2005]. Recently, the low density lipoprotein (LDL) receptor-related protein (LRP/CD91), a multifunctional scavenger receptor expressed in various types of cells such as macrophages and DCs, has also been identified as a novel heme–hemopexin receptor [Hvidberg et al., 2005].

Both heme–hemopexin complex and free Hb or Hb-haptoglobin complex taken up by LRP/CD91 and CD163, respectively, in monocytes/macrophages induce heme oxygenase-1 (HO-1) [Moestrup and Møller, 2004; Hvidberg et al., 2005; Schaer et al., 2007]. HO-1 is the rate limiting enzyme in the degradation of heme and catabolizes heme into three products, namely carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron [Otterbein et al., 2003; Wagener et al., 2003; Kumar and Bandyopadhyay, 2005]. These metabolites are suppressive in various immune responses [Otterbein et al., 2003].

It has recently been shown that serum levels of Kyn/Trp are remarkably high in patients with hemolytic anemia [Weiss et al., 2004]. These results suggest the possibility that IDO is induced for the protection of cytotoxicity mediated by Hb and/or hemin, the oxidized form of heme. However, as far as we know, there have been no studies demonstrating that Hb induces the expression of IDO. In the present study, we showed that Hb, but not hemin or heme-free globin (Apo Hb), induced the expression of IDO protein and enzyme activity in bone marrow-derived myeloid DCs (BMDCs).

MATERIALS AND METHODS

REAGENTS

Hb from bovine blood, hemin, *N*-acetyl-L-cysteine (NAC), superoxide dismutase (SOD), catalase, 3-amino-1,2,4-triazole (3-AT) and SP600125 were obtained from Sigma–Aldrich (St. Louis, MO). Native Hb is easily oxidized in air, and the Hb used in our study was shown to be predominantly ferrihemoglobin (Met Hb) by an absorption spectra assay. PD98059 and LY294002 were from Cell Signaling Technology Inc. (Beverly, MA), Ro31-8220 was from BIOMOL International (Plymouth Meeting, PA), and SB203580 and BAY11-7082 were from CALBIOCHEM (San Diego, CA). Anti-mouse IDO polyclonal antibodies and anti-human monoclonal antibodies were kindly donated by Dr. O. Takikawa. LPS from *Klebsiella pneumoniae* LEN-1 (O3: K1$^-$) was kindly donated by Prof. T. Hasegawa (Aichi Medical University School of Medicine).

ANIMALS

The C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All procedures were performed with the approval of the Animal Experimentation Committee, Graduate School of Medicine, Nagoya University in accordance with the Guidelines for Animal Experimentation of Nagoya University.

PREPARATION OF BMDCs

BMDCs were generated as described previously [Hara et al., 2008]. Briefly, bone marrow cells were cultured in RPMI1640 medium (10% fetal bovine serum, 0.3 mg/ml glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol) containing 0.3% GM-CSF supernatant (from murine GM-CSF-producing chinese hamster ovary cells, a gift from Dr. T. Sudo, Toray Silicon, Tokyo, Japan). The DC culture medium was exchanged every 2 days to remove nonadherent cells. Loosely adherent clustering cells were collected on day 6 and then were used as immature DCs.

WESTERN BLOTTING

Western blotting was performed as described previously [Hara et al., 2008]. Briefly, each cell lysate (20–40 μg) was subjected to SDS–PAGE using a 10% separation gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was incubated with primary antibodies overnight at 4°C. Subsequently, the membrane was incubated with either horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibodies for 1 h at room temperature. The specific protein bands were visualized using the Enhanced Chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA) according to the recommendations of the manufacturer and presented with relative protein levels.

ENZYME ASSAY OF IDO ACTIVITY

IDO activity was determined as previously described [Takikawa et al., 1988]. Briefly, BMDCs were homogenized and centrifuged at 10,000 rpm for 10 min. The supernatant (100 μl) was mixed with an equal volume of reaction buffer (100 mM potassium phosphate buffer, pH 6.5, 40 mM ascorbate, 20 μM methylene blue, 200 μg/ml catalase and 800 μM Trp) and incubated at 37°C for 50 min to permit IDO to convert Trp to *N*-formylkynurenine. The reaction was stopped by adding 40 μl of 30% (w/v) trichloroacetic acid (TCA), and the mixture was subsequently incubated at 50°C for 30 min to hydrolyze *N*-formylkynurenine produced by IDO to Kyn. After centrifugation at 10,000 rpm for 15 min, the amount of Kyn in the supernatant was measured by high-pressure liquid chromatography (HPLC).

ASSAY OF Kyn

The concentration of Kyn was determined by HPLC. HPLC was performed as previously described [Takikawa et al., 1988], with minor modifications. Before the HPLC assay, the culture medium was deproteinized by treatment with 86% methanol (1:6, v/v). A sample (100 μl) was injected into a 5 μm endcapped Purospher RP-18 column (COSMOSIL PACKED COLUMN for HPLC,

150 mm × 4.6 mm) and analyzed at a flow rate 1.0 ml/min. The mobile phase was 10 mM acetic ammonium (pH 6.5) and 10% methanol. Kyn was detected by a UV-detector at a wavelength of 360 nm.

CELL FRACTIONATION

The BMDCs were resuspended in lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail) and incubated on ice for 15 min. IGEPAL®CA-630 (10%) was then added to the homogenate to a final concentration of 3.8%, and the mixture was vortexed and centrifuged at 3,000 rpm for 5 min. The pellet containing nuclei was resuspended in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA-2Na and 1% Triton-X 100) and centrifuged at 15,000 rpm for 40 min. The supernatant (nuclear extract) was either used immediately or stored at −80°C.

IMMUNOFLUORESCENT STAINING

BMDCs were incubated with 20 μM Hb on slides. The slides were then fixed with 4% paraformaldehyde phosphate buffer solution for 1 h at room temperature and permeabilized with 1% Triton-X 100 in PBS. The slides were treated with primary rabbit polyclonal antibodies against RelA (Cell Signaling Technology Inc., Beverly, MA) and p52 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, and then incubated with FITC-labeled goat anti-rabbit antibody (MBL, Nagoya, Japan). The nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen Life Technologies, Carlsbad, CA).

MEASUREMENT OF INTRACELLULAR ROS GENERATION

BMDCs were stimulated with Hb, and 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen Life Technologies) was added for the last 15 min of incubation. The BMDCs were washed with PBS and analyzed by an EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA).

PREPARATION OF APO Hb

Apo Hb was prepared by the acid-acetone method as previously described [Ascoli et al., 1981]. Briefly, Hb solution was added dropwise under vigorous stirring into acid-acetone (25 μl of 2 M HCl in 10 ml of acetone) cooled at −20°C. At the end of the addition, the acid-acetone was slightly reddish, and globin precipitated as a white material. The suspension was centrifuged at 5,000 rpm for 10 min at 4°C. The final colorless precipitate of Apo Hb was dissolved in water by raising the pH to 9.5–10 of 1 M NaOH and then neutralized.

STATISTICAL ANALYSIS

Statistical comparisons among the groups were assessed by a one-way analysis of variance (ANOVA). When F ratios were significant ($P < 0.05$), Scheffe's post-hoc tests between the two groups were performed, and $P < 0.05$ were considered a statistically significant post-hoc difference. Statistical analyses were performed with the StatView software program (Abacus Concept Inc.).

RESULTS

INDUCTION OF IDO EXPRESSION IN BMDCs AND THP-1 CELLS STIMULATED WITH Hb

BMDCs were stimulated with 20 μM Hb for the indicated time or with 1 μg/ml LPS for 24 h as a control. Hb induced the expression of IDO protein at 24 h and inducible nitric oxide synthase (iNOS) protein at 8–24 h although the expression levels were lower than those induced by LPS (Fig. 1A). IDO induced by Hb showed a low but definite enzyme activity in the assay using cellular extracts (Fig. 1B). IDO expression was also induced in human monocytic THP-1 cells stimulated with 10–20 μM Hb for 24 h (Fig. 1C). THP-1 cells secreted Kyn in a concentration-dependent manner upon stimulation with 10–40 μM Hb for 24 h (Fig. 1D). These results demonstrated that Hb induced the expression of active IDO in both mouse BMDCs and human monocytic THP-1 cells.

REQUIREMENT OF BOTH Apo Hb AND HEME FOR THE INDUCTION OF IDO EXPRESSION IN BMDCs

The abilities of various concentrations of Hb or hemin to induce IDO expression were comparatively studied. All the concentrations (10–40 μM) of Hb induced the expression of IDO and iNOS proteins in BMDCs (Fig. 2A), although 10–80 μM of hemin did not induce the expression of these proteins. However, these concentrations of Hb or hemin induced high levels of HO-1 expression (Fig. 2A). Heme is released from Met Hb, and Apo Hb is concomitantly generated [Bunn and Jandl, 1968; Jeney et al., 2002; Grinshtein et al., 2003]. Apo Hb has been shown to be cytotoxic [Tsemakhovich et al., 2005], although the anti-inflammatory action of Apo Hb to specifically inhibit LPS activity has also been shown [Yang et al., 2002]. We demonstrated that purified Apo Hb did not induce the expression of IDO in BMDCs (Fig. 2B). Therefore, we conclude that both Apo Hb and heme are required for the induction of IDO expression.

REQUIREMENT OF PI3K AND PKC ACTIVATION FOR THE INDUCTION OF IDO EXPRESSION BY Hb IN BMDCs

The signal pathways required for the induction of IDO expression were studied using PD98059 (an ERK inhibitor), SP600125 (a JNK inhibitor), SB203580 (a p38 inhibitor), LY294002 (a PI3-kinase (PI3K) inhibitor), and Ro31-8220 (a pan-PKC inhibitor) in BMDCs stimulated with 20 μM Hb for 24 h (Fig. 3). Hb-induced IDO expression was inhibited by 10 μM LY294002 or 2 μM Ro31-8220. Ten micromolar LY294002, 10 μM SB203580, or 1.5–2.0 μM Ro31-8220 inhibited the expression of iNOS, however, HO-1 expression was only slightly inhibited. Therefore, we concluded that the activation of PI3K and PKC is required for the induction of IDO expression by Hb and that the signals required for IDO and iNOS are different.

REQUIREMENT OF NF-κB ACTIVATION FOR THE INDUCTION OF IDO EXPRESSION BY Hb IN BMDCs

Previous studies have shown that both PI3K and PKC activate NF-κB via the phosphorylation and degradation of IκBα or the phosphorylation of RelA [Ozes et al., 1999; Ramashkova and Makarov, 1999; Duran et al., 2003; Chen and Greene, 2004]. NF-κB activation is also required for the induction of IDO expression in

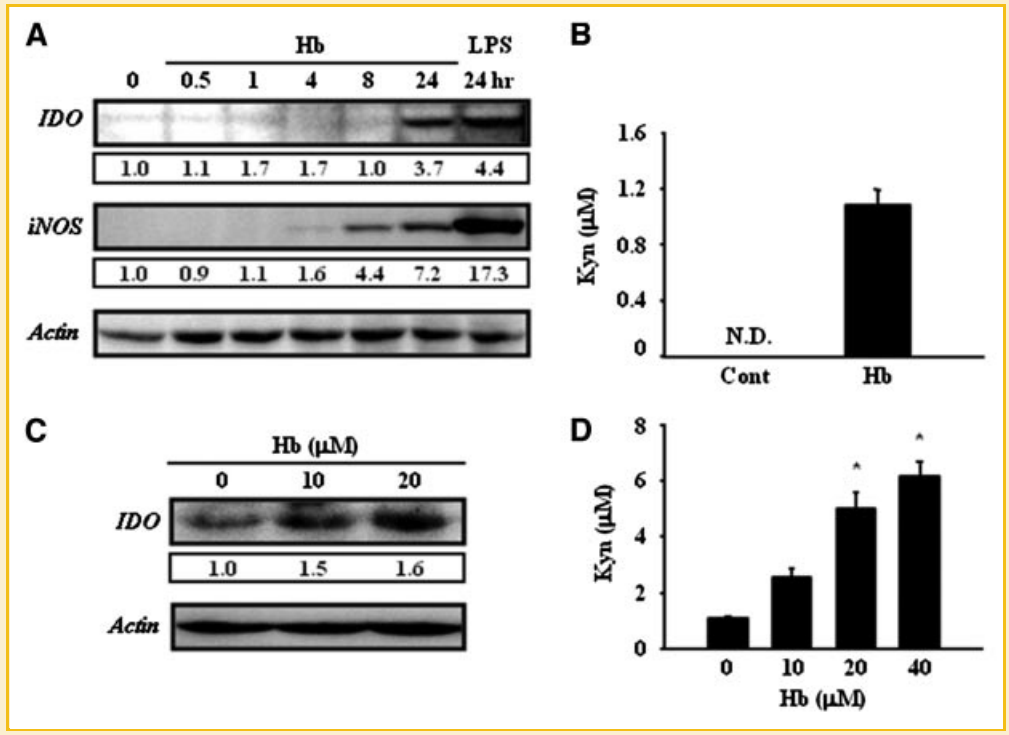

Fig. 1. The induction of IDO expression in BMDCs and THP-1 cells by Hb. A: BMDCs (7×10^6 cells) or (C) 2×10^6 THP-1 cells were stimulated with (A) 20 μM Hb or 1 μg/ml LPS for the indicated time (0, 0.5, 1, 4, 8, 24 h) or (C) 10–20 μM Hb for 24 h. The expression of IDO and iNOS proteins was determined by Western blotting. B: BMDCs were stimulated with 20 μM Hb for 24 h. IDO activity was determined via Kyn formation using cellular extracts as described under Materials and Methods Section. ND, not detectable. D: THP-1 cells were stimulated with 10–40 μM Hb for 24 h. Kyn concentration in the culture supernatant was measured by HPLC. The results shown are representative of three independent experiments. Significantly different from the data at concentration zero of Hb ($^*P < 0.05$).

human monocytic THP-1 cells stimulated with LPS [Fujigaki et al., 2006]. Therefore, we tested whether Hb induced the expression of IDO via the activation of NF-κB. As shown in Figure 4A, Hb induced the phosphorylation and degradation of IκBα within 15 min and most of the IκBα was degraded within 1 h. Thereafter, we tested the effects of BAY11-7082, an inhibitor of NF-κB in the canonical pathway, on the Hb-induced IDO expression in BMDCs. BAY11-7082 (10 μM) clearly suppressed the induction of IDO expression by 20 μM Hb (Fig. 4B). These data suggest that Hb induces IDO expression through the activation of PI3K, PKC, and NF-κB.

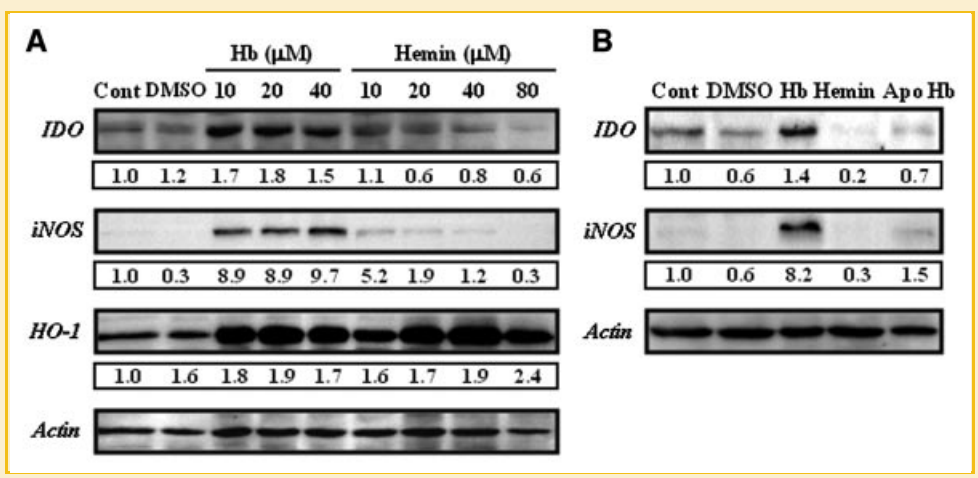

Fig. 2. Requirement of both Apo Hb and heme for Hb-induced IDO expression in BMDCs. BMDCs (7×10^6 cells) were stimulated with (A) 10–40 μM Hb or 10–80 μM hemin (in DMSO) or (B) 20 μM Hb, 80 μM hemin or 20 μM Apo Hb for 24 h. The expression levels of IDO, iNOS, and HO-1 proteins were determined by Western blotting. The results shown are representative of three independent experiments.

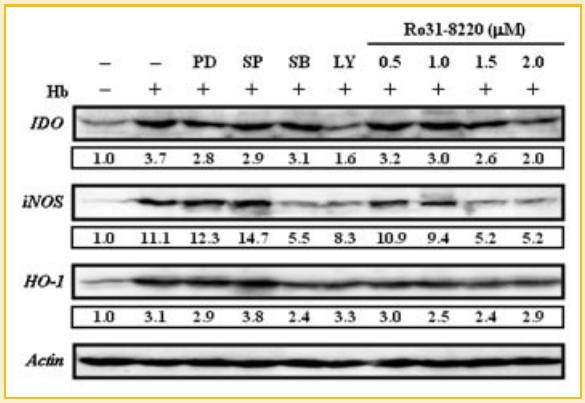

Fig. 3. Requirement of PI3K and PKC activation for the induction of IDO expression by Hb in BMDCs. BMDCs (7×10^6 cells) pretreated with various inhibitors such as 10 μM PD98059 (PD), 10 μM SP600125 (SP), 10 μM SB203580 (SB), 10 μM LY294002 (LY) or 0.5–2.0 μM Ro31-8220 for 30 min were incubated with 20 μM Hb for 24 h. The expression levels of IDO, iNOS and HO-1 proteins were determined by Western blotting. The results shown are representative of three independent experiments.

It has recently been shown that IDO expression is induced through the activation of the non-canonical pathway of NF-κB in plasmacytoid DCs by ligation of GITR-ligand or CD40 [Grohmann et al., 2007; Puccetti and Grohmann, 2007; Tas et al., 2007]. Akt, a downstream signaling molecule of PI3K, also activates the non-canonical pathway of NF-κB [Gustin et al., 2006]. Therefore, we compared the Hb-mediated activation of NF-κB along the canonical pathway (RelA/p50) and non-canonical pathway (RelB/p52). The expression of p52 protein was increased in BMDCs stimulated with 20 μM Hb for 1–8 h and decreased thereafter (Fig. 4C). The expression of p52 was induced in nuclear extracts of BMDCs cultured with Hb for 1 h, and further increased until 4 h (Fig. 4D). RelA expression was also induced in nuclear extracts of BMDCs cultured with Hb for 1 h and decreased within 4 h. Immunofluorescent staining also showed that Hb translocated RelA from the cytosol to the nucleus within 1 h and translocated p52 to the nucleus at 4 h (Fig. 4E). Therefore, both RelA and p52 were translocated to the nucleus, although the latter was detected in the nucleus much longer than the former. Our results suggest that the activation of NF-κB via the canonical and non-canonical pathways contributes to the induction of IDO expression in BMDCs stimulated with Hb.

REQUIREMENT OF ROS GENERATION FOR THE INDUCTION OF IDO EXPRESSION BY Hb IN BMDCs

We subsequently examined whether ROS generation was required for Hb-induced IDO expression in BMDCs. The intracellular generation of ROS was assayed using H_2DCFDA, which primarily detected H_2O_2. The intracellular generation of ROS was induced in BMDCs stimulated with 20 μM Hb for 2 h (Fig. 5A). Antioxidant NAC (2–20 mM) inhibited the induction of IDO expression by Hb in a concentration-dependent manner (Fig. 5B). Hb-induced IDO expression was inhibited by 200–1,000 U/ml catalase in the presence of 200 U/ml SOD but not by SOD alone (Fig. 5C). 3-AT (10–20 mM), an inhibitor of catalase, enhanced the induction of IDO expression by 20 μM Hb (Fig. 5D). These results suggest that the generation of ROS such as O_2^-, H_2O_2, and hydroxyl radical is required for the induction of IDO expression by Hb in BMDCs.

REQUIREMENT OF PI3K-MEDIATED PKC ACTIVATION FOR ROS GENERATION AND NF-κB ACTIVATION BY Hb IN BMDCs

Previous studies have shown that PKC stimulates NADPH oxidase in the generation of ROS [Dang et al., 2001; Frey et al., 2002]. Therefore, we tested whether Hb stimulated PKC in ROS generation in BMDCs and how PI3K influenced Hb-mediated ROS generation. Five micromolar Ro31-8220 inhibited ROS generation in BMDCs stimulated with Hb, indicating the necessity of PKC activation for Hb-mediated ROS generation in BMDCs (Fig. 6A, upper panel). Ten micromolar LY294002 also inhibited the induction of ROS generation by Hb (Fig. 6A, middle panel). The addition of Ro31-8220 and LY294002 inhibited the induction of ROS generation much more strongly than Ro31-8220 or LY294002 alone (Fig. 6A, lower panel). These results suggest that PI3K activates PKC and the activated PKC transduces the signals for NADPH oxidase activation, which subsequently generates ROS.

It has been shown that the Akt is a downstream molecule of the PI3K signal pathway and activates NF-κB through IκBα phosphorylation and degradation [Ozes et al., 1999; Ramashkova and Makarov, 1999]. Therefore, we investigated whether Akt was activated by Hb in BMDCs. Hb induced Akt phosphorylation, and Akt phosphorylation was inhibited by LY294002 (Fig. 6B). Unexpectedly, Ro31-8220 enhanced Hb-mediated induction of Akt phosphorylation (Fig. 6B). These results suggest that PI3K, but not PKC, is located upstream from Akt.

DISCUSSION

The present study shows that Hb induces IDO expression in BMDCs. Previous studies have reported that the serum levels of Kyn/Trp are notably high in patients with hemolytic anemia of inflammation [Weiss et al., 2004]. Hemolytic anemia results in an increased ATP concentration in plasma because high concentrations of ATP are accumulated in red blood cells. ATP enhances the induction of IDO in human DCs stimulated with IFN-γ [Marteau et al., 2005]. Therefore, it is suggested that both Hb and ATP released from red blood cells through hemolysis may cooperatively induce the expression of IDO in DCs in order to down-regulate inflammation induced by Hb.

We showed that Hb, although not hemin or Apo Hb, induced the expression of IDO in BMDCs. These results are consistent with a previous report that Met Hb, but not Oxy Hb or hemin, induced the production of inflammatory cytokines such as IL-6 and IL-8 in endothelial cells [Liu and Spolarics, 2003]. Peiró et al. [2003] also showed the importance of Apo Hb in a study which demonstrated that human Oxy Hb glycosylated at elevated levels activated NF-κB and activator protein-1 in cultured human aortic smooth muscle. These findings suggest that the Hb induction of IDO expression may be caused by the differences of the signals via CD163 and CD91. Cross-linking of CD163 also induces the production of inflammatory mediators such as NO, TNF-α, IL-1β, IL-6, and GM-CSF in human

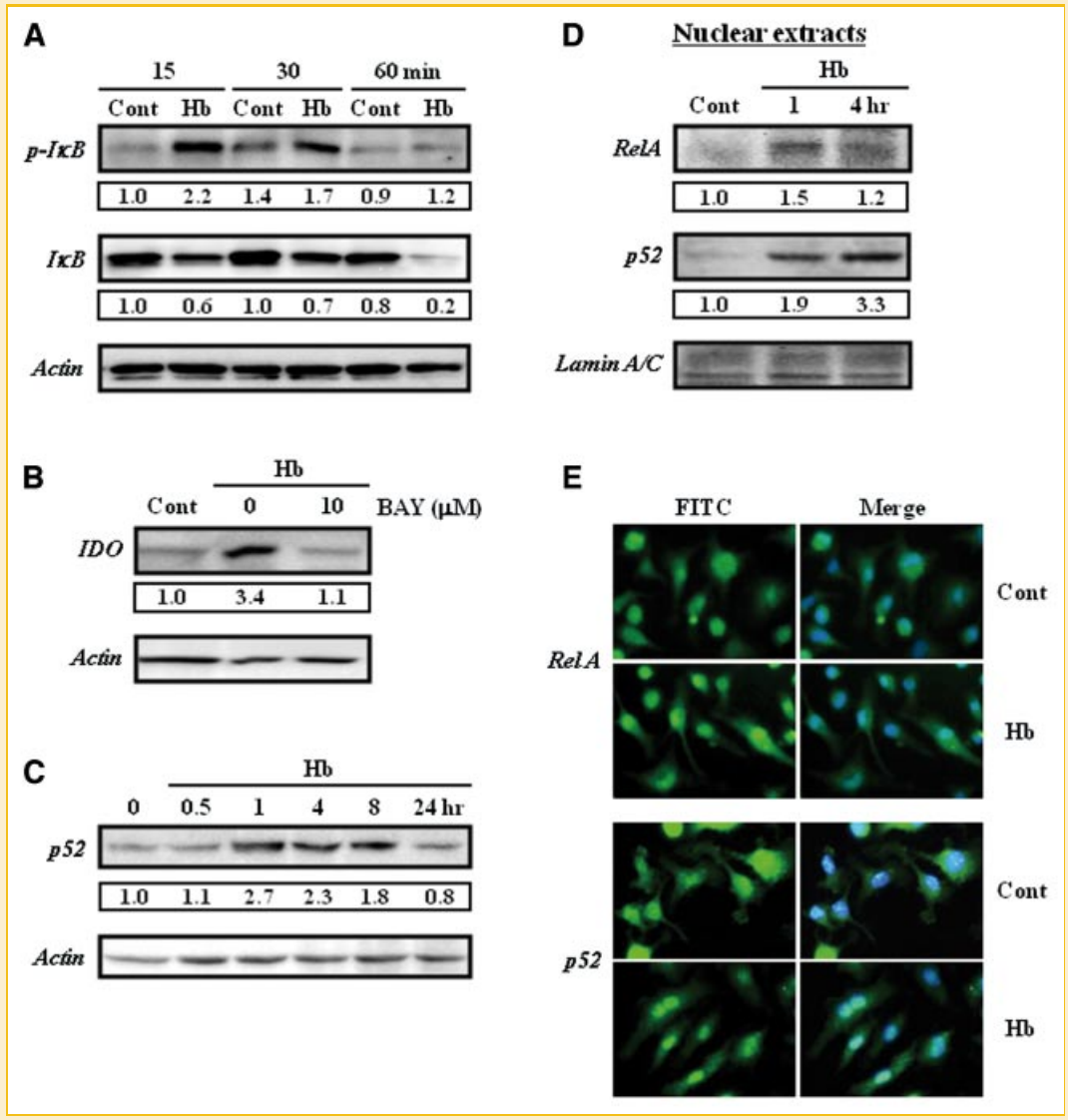

Fig. 4. Requirement of NF-κB activation for the induction of IDO expression by Hb in BMDCs. A: BMDCs (7×10^6 cells) were stimulated with 20 μM Hb for the indicated time. The phosphorylation and degradation of IκBα were determined by Western blotting. B: BMDCs (7×10^6 cells) pretreated with 10 μM BAY11-7082, an NF-κB inhibitor, for 30 min were incubated with 20 μM Hb for 24 h. C,D: BMDCs (7×10^6 cells) were stimulated with 20 μM Hb for the indicated time, and (C) whole cell lysates and (D) nuclear extracts were prepared. The expression of (B) IDO or (C) p52 and (D) translocation of RelA and p52 were determined by Western blotting. Lamin A/C was used for a marker of nucleus. E: Nuclear translocation of RelA after 1 h incubation and p52 after 4 h incubation of BMDCs with 20 μM Hb were determined by immunofluorescent staining. RelA and p52 were stained with FITC-labeled antibodies and the nucleus with DAPI. The results shown are representative of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and rat macrophages [Polfliet et al., 2006; Van den Heuvel et al., 1999]. However, CD163 expression is strongly induced by anti-inflammatory mediators, such as glucocorticoids and IL-10 [Van den Heuvel et al., 1999; Philippidis et al., 2004; Maniecki et al., 2006; Polfliet et al., 2006; Schaer et al., 2006a, 2007]. CD163-mediated Hb-haptoglobin uptake provides anti-inflammatory effects via HO-1 induction, macrophage activation, and the induction of IL-10 production [Philippidis et al., 2004; Schaer et al., 2006a]. It has recently been published that CD163 and CD91 are co-expressed in human macrophages and DCs [Maniecki et al., 2006]. Our study suggests that, via CD163, Hb transduces the signals required for the expression of immunosuppressive IDO.

The present study shows that PI3K and PKC are required for the induction of IDO. Both the regulatory β-subunit of casein kinase and PKC are involved in the CD163 signaling mechanism that results in the secretion of proinflammatory cytokines [Ritter et al., 2001]. The activation of PI3K and JNK are involved in the LPS-induced pathway leading to IDO expression in BMDCs [Jung et al., 2007]. However, our results show that Hb requires a novel pair of signal transduction molecules for the induction of IDO expression in BMDCs. In addition, we used BAY11-7082 to demonstrate that the activation of NF-κB along the canonical pathway was necessary for the induction of IDO expression. This function was also confirmed by the observation that Hb induced the phosphorylation and

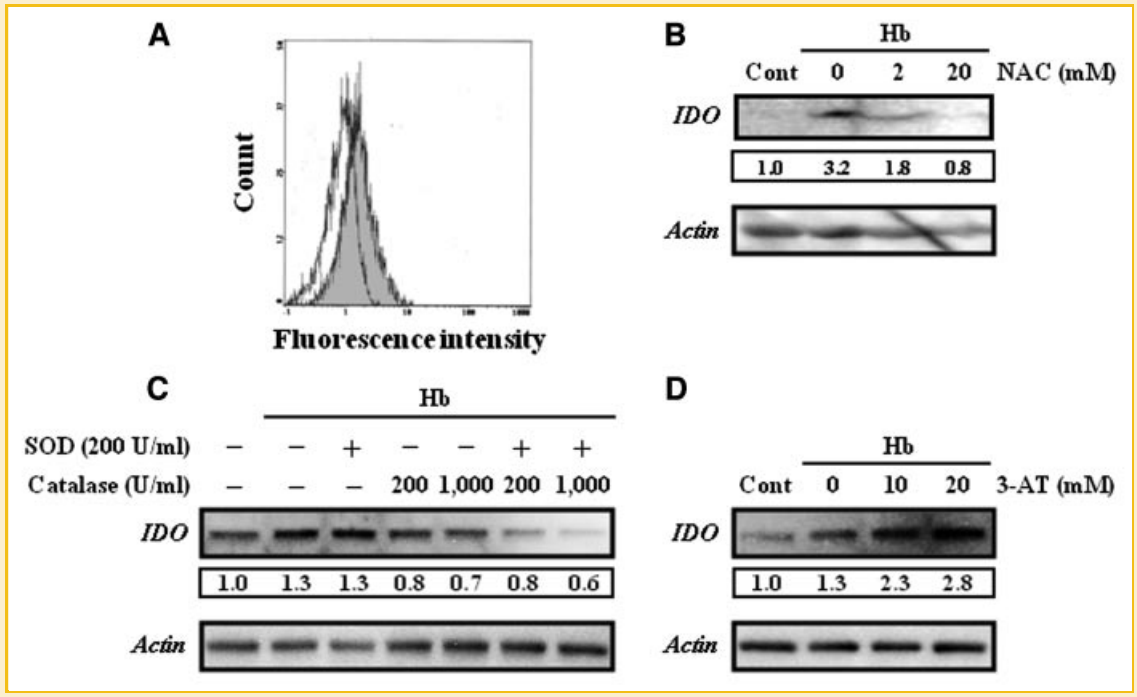

Fig. 5. Requirement of ROS generation for the induction of IDO expression by Hb in BMDCs. A: BMDCs (7×10^6 cells) were stimulated with 20 μM Hb (gray-filled curve) for 2 h and 20 μM H_2DCFDA was added for the last 15 min of incubation. ROS generation was detected by flow cytometry. BMDCs (7×10^6 cells) pretreated with (B) 2–20 mM NAC, (C) 100 U/ml SOD and/or 200–1,000 U/ml catalase or (D) 10–20 mM 3-AT for 30 min were incubated with (B) 40 μM or (C,D) 20 μM Hb for 24 h. B–D: The expression of IDO was determined by Western blotting. The results shown are representative of three independent experiments.

degradation of IκBα in BMDCs. Therefore, we concluded that Hb induces IDO expression through the activation of PI3K, PKC, and NF-κB. This result is consistent with the ability of Met Hb to induce the activation of NF-κB in human endothelial cells and with the requirement of NF-κB activation for the induction of IDO expression by LPS in THP-1 cells [Liu and Spolarics, 2003; Fujigaki et al., 2006]. The present study shows that NF-κBs (RelA and p52) along the canonical and non-canonical pathways are translocated into the nucleus by Hb in BMDCs. Other studies have shown that lymphotoxin and LPS induce NF-κB-p52 generation by a co-translational mechanism [Mordmüller et al., 2003; Dejardin, 2006]. Our results suggest that NF-κBs along the canonical and non-canonical pathways cooperate in inducing IDO expression in BMDCs stimulated with Hb.

The present study demonstrates that ROS generation is another factor required for the induction of IDO expression by Hb in BMDCs. The generation of ROS was strongly inhibited by the mixture of SOD and catalase and by a catalase inhibitor. Therefore, the generation of ROS such as O_2^-, H_2O_2 and hydroxyl radical may be required for the induction of IDO expression by Hb in BMDCs. Antioxidants such as *t*-butyl hydroquinone, ebselen, or 2-ME also inhibit IFN-γ-mediated induction of IDO protein expression in human macrophages [Thomas et al., 2001]. Consequently, ROS generation is an important factor in enhancing the induction of IDO protein expression.

Our findings demonstrated the role of ROS generation and the activation of PI3K, PKC, and NF-κB in the induction of IDO expression in BMDCs. Hb-mediated ROS generation was inhibited by a PKC inhibitor, and the inhibition was enhanced by addition of a PI3K inhibitor. PKC has been shown to activate NADPH oxidase for ROS generation [Rahman et al., 1999; Dang et al., 2001; Frey et al., 2006]. Therefore, our observations suggest that PI3K transduces the signal for the activation of PKC. This result is consistent with the signal pathway for the generation of ROS in vascular endothelial cells stimulated with TNF-α [Frey et al., 2006]. It has been shown that NF-κB is activated by PKC through the ROS-dependent or -independent activation of the IκBα kinase (IKK) [Bubici et al., 2006]. ROS-mediated IKK activation may be required for the activation of NF-κB in the induction of IDO by Hb in BMDCs. On the other hand, we show that Hb-mediated Akt phosphorylation is inhibited by PI3K but enhanced by a PKC inhibitor, suggesting that Akt is also the downstream molecule of PI3K for NF-κB activation and competes with PKC for the PI3K signal. Other studies have shown that both PI3K and PKC activate NF-κB via the phosphorylation and degradation of IκBα or the phosphorylation of RelA [Ozes et al., 1999; Ramashkova and Makarov, 1999; Duran et al., 2003; Chen and Greene, 2004]. PI3K may therefore activate NF-κB through the PKC-ROS pathway and the Akt pathway upon stimulation with Hb in BMDCs.

The heme protein IDO initiates oxidative metabolism of Trp along the Kyn pathway, and this action requires reductive activation of Fe^{3+}. O_2^- has been believed to be responsible for this activation. However, it has recently been shown that cytochrome b_5, not O_2^-, is a major reductant of IDO in human cells [Maghzal et al., 2008]. Therefore, IDO does not possess antioxidative activity such as O_2^- depletion. IDO is primarily an immunosuppressive enzyme that locally depletes the Trp required for cell proliferation and produces

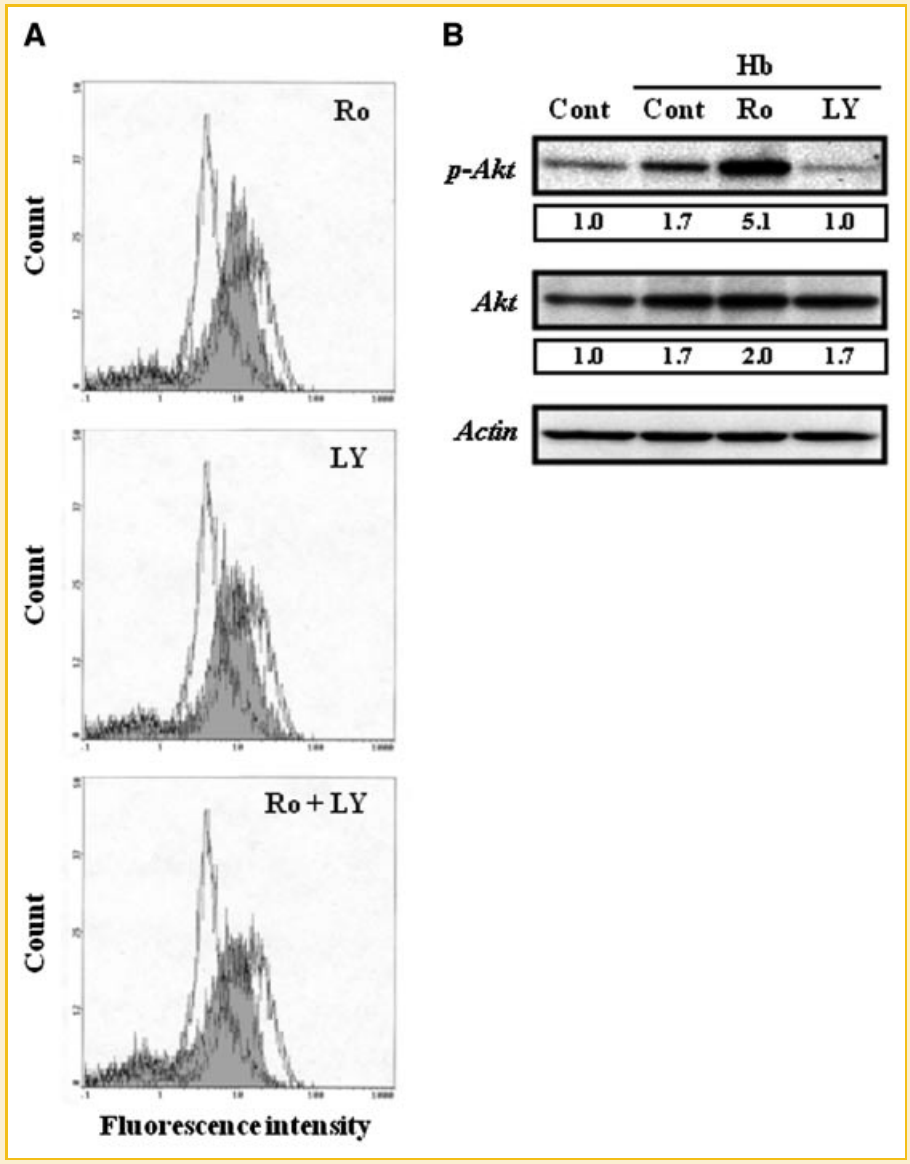

Fig. 6. Requirement of PI3K-mediated PKC activation for ROS generation and NF-κB activation by Hb in BMDCs. A: BMDCs (3×10^6 cells) were pretreated with or without various inhibitors such as (upper panel) 5 μM Ro31-8220 (Ro), (middle panel) 10 μM LY294002 (LY) or (lower panel) 5 μM Ro31-8220 plus 10 μM LY294002 for 30 min (gray-filled curve) and were stimulated with 20 μM Hb for 30 min. Twenty micromolar H_2DCFDA was added for the last 15 min of incubation. ROS generation was detected by flow cytometry. The mean fluorescence intensities of the control group and the Hb stimulation group were 3.69 and 7.72, respectively. The mean fluorescence intensities of the Hb stimulation group in the presence of Ro31-8220, LY294002, and Ro31-8220 plus LY294002 were 5.16, 6.03, and 4.29, respectively. B: BMDCs (7×10^6 cells) pretreated with 5 μM Ro31-8220 or 10 μM LY294002 for 30 min were stimulated with 20 μM Hb for 10 min. The phosphorylation of Akt was determined by Western blotting. The results shown are representative of three independent experiments.

metabolites that induce T-cell apoptosis [Mellor and Munn, 2004]. On the other hand, HO-1 is primarily an antioxidative enzyme that protects cells against oxidative damage by degrading heme and producing antioxidative metabolites such as bilirubin [Otterbein et al., 2003; Wagener et al., 2003; Kumar and Bandyopadhyay, 2005]. Therefore, IDO and HO-1 protect cells against heme-mediated cytotoxicity via different actions.

In conclusion, Hb induced the expression of IDO protein and activity through ROS generation and the activation of PI3K, PKC, and NF-κB in BMDCs. These results suggest the possibility that IDO may be induced in various cells such as macrophages, DCs, or vascular endothelial cells exposed to Hb in hemolysis and may control the induction of inflammation by toxic heme.

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