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Catalase induces the expression of inducible nitric oxide synthase through activation of NF-κB and PI3K signaling pathway in Raw 264.7 cells

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Abstract

It has been reported that macrophages produce substantial amounts of nitrite and nitrate after addition of catalase, but the mechanism associated remains unclear. In present study, we investigated whether catalase modulates the expression of inducible nitric oxide synthase (iNOS), an enzyme that produces nitric oxide. Exposure of Raw 264.7 macrophages (Raw cells) to catalase induced high expression of iNOS mRNA as well as protein with enzymatic activity. Data of mechanical analyses, such as iNOS promoter-driven luciferase assay and actinomycin D chase experiments demonstrated that the induction was due to increased iNOS transcription and post-transcriptional iNOS mRNA stability. Of interest, catalase-induced iNOS protein expression was abrogated through inactivation of NF-κB pathway by MG132 or BAY 11-7085 and PI3K pathway by LY294002 or wortmannin, respectively. In particular, blockage of PI3K pathway by LY294002 down-regulated iNOS transcription and steady-state iNOS mRNA levels as well as iNOS mRNA stability induced by catalase, suggesting regulation of PI3K pathway in catalase-induced iNOS expression at the levels of iNOS transcription, steady-state mRNA status, and mRNA stability. Additional cell culture works in different types of cells indicated that iNOS expression by catalase might be cell type-specific, based on the facts that catalase induced iNOS expression in BV2 microglial macrophage-like cells, but not in HT-29 or A549, human colon or lung cancer epithelial-like cells. Together, these results demonstrate for the first time that catalase induces iNOS expression in Raw cells, which seems to be associated with the increase of iNOS transcription and mRNA stability as well as the activation of NF-κB and PI3K signaling pathways.

Keywords: Catalase; iNOS; NF-κB; PI3K; Raw 264.7 cells; Classification: Molecular and cellular pharmacology

1. Introduction

Nitric oxide (NO) is an important signaling molecule that involves in the regulation of vascular homeostasis, neurotransmission, and immune defense against infectious agents [1]. NO is generated from L-arginine and molecular oxygen by the action of NO synthase (NOS) [2]. In mammals, three isoforms of NOS (type I, II, and III) have been identified and molecularly cloned [2,3]. The neuronal (type I, named as nNOS) and endothelial (type III, named

Abbreviations: AP-1, activator protein-1; AT, 3-amino-1,2,4-triazole; ERKs, extracellular-regulated protein kinases; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN-Y, interferon-Y; iNOS, inducible nitric oxide synthase; IRF, IFN-Y responsive factor; JAK-2, Janus activated kinase-2; JNKs, c-Jun N-terminal kinases; NF-IL6, nuclear factor-interleukin 6; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PI3K, phosphatidylinositol 3 kinase; PKC, protein kinase C; PTX, pertussis toxin; ROS, reactive oxygen species

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as eNOS) enzymes are constitutively expressed and thought to be involved in the maintenance of physiological functions in neuronal and endothelial systems, respectively. The third member of the NOS family is iNOS (type II), which is an inducible enzyme. The inducibility of iNOS was first identified in murine macrophages stimulated with bacterial lipopolysaccharide and IFN-γ [4]. In many recent studies, it has been found that the expression and activity of iNOS are increased in chronic diseases, such as inflammation or cancer [5,6]. Furthermore, excessive NO has been observed in tissue damages associated with type I diabetes, arthritis, or nephritis [7], suggesting that deregulation of iNOS expression is linked to many diseases.

The molecular regulation of iNOS expression is complex and occurs at multiple levels [8,9]. iNOS expression is primarily regulated at the level of transcription. It is well known that iNOS transcription is induced by the activation of transcription factors, including NF-κB, AP-1, IRF, and NF-IL6, which individually act on NF-κB, AP-1, IRF, and NF-IL6 cis-acting elements within the iNOS promoter [10–12]. iNOS expression is also regulated at the levels of post-transcription, such as iNOS mRNA stability [13] or translation [14,15]. Furthermore, it is well documented that iNOS expression is linked to activation of intracellular signaling proteins, including PI3K, PKCs, JAK-2, or mitogen-activated protein kinases, such as ERKs and p38s [16–19], suggesting that signaling proteins are additional regulatory points in iNOS expression.

ROS are produced in cells or tissues under physiological and pathological conditions. Excessive ROS have been implicated in diseases, such as inflammation or cancer [20]. The fundamental defense of the organism against ROS is accomplished by the action of cellular ROS scavenging proteins, such as catalase, superoxide dismutase, or glutathione peroxidase and/or by the intracellular levels of antioxidant compounds, including glutathione, albumin, or transferin. Recent studies have shown that in vivo or in vitro treatments of antioxidant enzymes, such as catalase or superoxide dismutase and antioxidant compounds, such as *N*-acetylcysteine are effective in reducing inflammation and/or cancer [21,22] probably by their ROS scavenging ability. From the accumulating evidence, it is believed that antioxidant enzymes may play a negative role in inflammation and/or cancer. However, though the mechanism is unclear, it has been previously reported that macrophages produce substantial amounts of nitrite and nitrate after the addition of catalase, an antioxidant enzyme scavenging H_2O_2 [23]. Considering that nitrite and nitrate are derived from NO, which is primarily made by NOS enzymes, we hypothesized that the production of nitrite and nitrate in macrophages as a resultant of catalase treatment might be correlated with induction of iNOS expression, which produces NO, a source of nitrite and nitrate.

In this study, we investigated the effect of catalase on iNOS expression in Raw 264.7 macrophages, and the molecular and signaling mechanisms involved.

2. Materials and methods

2.1. Materials

Catalase (C-40), aprotinin, leupeptin, and AT were purchased from Sigma. PD98059 (an inhibitor of ERKs), SB203580 (a p38 inhibitor), LY294002 (an inhibitor of PI3K), SP600125 (an inhibitor of JNKs), Z-Leu-Leu-Leu-H (MG132, a proteosomal and NF-κB inhibitor), rapamycin (an inhibitor of mammalian target of rapamycin/S6 kinase (mTOR/S6K)), and PTX (a G_i inhibitor) were purchased from Biomol. Bradford reagent was from Biorad. An anti-iNOS polyclonal antibody was purchased from Upstate Biotech. An anti-IκB-α antibody was from Santa Cruz. Antibodies against anti-rabbit or mouse secondary horseradish peroxidase and ECL Western detection reagents were bought from Amersham Biosciences. RPMI 1640, Dulbecco's Modified Eagle's Medium (DMEM), penicillin, and streptomycin were purchased from GIBCO-BRL. BAY 11–7085 was purchased from Calbiochem-Nova-biochem Corp.

2.2. Cell culture and preparation of whole cell lysates

Raw 264.7 murine macrophages were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin. BV2 murine microglial macrophage-like cells, HT-29 (colon), and A549 (lung) human carcinoma cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. For preparation of whole cell lysates, cells were washed with ice-cold phosphate buffered saline, and lysed in a buffer [50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate, 1% Nonidet P-40, 1 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl]. After centrifugation at 12,000 rpm for 20 min at 4°, the supernatant was collected, and the protein concentration was determined.

2.3. Western blot analysis

Equal amounts of protein (40 μg/lane) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane (Millipore). The membrane was then washed with Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl), containing 0.05% Tween 20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with antibodies of iNOS (1:2000), IκB-α (1:2000), or actin (1:10,000). The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the ECL Western detection reagents.

2.4. Measurement of NO

After treatment of catalase or LPS, Raw cell culture medium was saved for the measurement of nitrite, an indicator of NO production. Briefly, culture medium (100 μL) was incubated with 100 μL of Griess reagent (0.1% napthalethylenediamine dihydrochloride, 1% sulfanilamine, and 2.5% H_3PO_4, Promega). Absorbance of the mixture was then measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

2.5. Determination of catalase activity

Catalase activity was measured as described previously [24,25] with a slight modification. In principle, H_2O_2 oxidizes ferrous to ferric ion that forms a blue–purple complex at optical density (OD) of 560 nm (no oxidation of ferrous by low or absence of H_2O_2 develops yellow color). Briefly, catalase (500 U) was incubated without or with different concentrations of AT, an inhibitor of catalase activity [26], at room temperature for 1 h in 100 μL 50 mM potassium phosphate buffer (pH 7.5). One-tenth of the reaction was then incubated with H_2O_2 at room temperature for 10 min. The reaction was finally incubated in 1 mL of a solution, containing 200 μM xylenol orange and 500 μM ferrous ammonium sulfate (1:1). The color development was measured at OD 560 nm. The final concentration of H_2O_2 used in the assay was about 2 mM.

2.6. iNOS promoter/luciferase assay

Briefly, 1 μg of murine iNOS promoter/luciferase DNA along with 20 ng of control pRL-TK DNA (Promega) was transiently transfected into 1.5×10^6 Raw 264.7 cells/well in 6-well plates, using LipoFectamine/Plus reagents (Invitrogen) according to the instructions provided by the manufacturer. After 24 h post-transfection, cells were treated with catalase, and incubated for an additional 4 h. Cells were then washed, lysed, followed measurement of luciferase activity, using a luciferase assay kit (Promega). The luciferase activity was normalized with the expression of control pRL-TK.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with the RNAzol-B (Tel-Test) according to the instructions provided by the manufacturer. Three micrograms of total RNA were reverse transcribed, using M-MLV reverse transcriptase (Promega) following the manufacturer's manual instruction. Single stranded cDNA was then amplified by PCR with specific primers of iNOS and GAPDH: iNOS sense; 5′-GACAAGCTGCATGTGACATC-3′; iNOS anti-sense; 5′-GCTGGTAGGTTCCTGTTGTT-3′; GAPDH sense; 5′-GGTGAAGGTCGGTGTGAACG-3′; GAPDH anti-sense; 5′-GGTAGGAACACGGAAGGCCA-3′. The following PCR conditions were applied: iNOS, 25 cycles of denaturation at 94° for 30 s, annealing at 52° for 30 s, and extension at 72° for 30 s; GAPDH, 18 cycles of denaturation at 94° for 30 s, annealing at 57° for 30 s, and extension at 72° for 30 s. GAPDH was used as an internal control to evaluate relative expression of iNOS.

3. Results

3.1. Induction of iNOS mRNA and protein expression in Raw cells by catalase

We initially measured the effects of different concentrations of catalase on iNOS protein and mRNA expression in Raw 264.7 macrophages by Western and RT-PCR experiments, respectively. As shown in Fig. 1A, catalase dose-dependently induced iNOS protein expression. We next determined whether catalase-induced iNOS protein expression was due to increased iNOS mRNA levels. Similar to the pattern of iNOS protein expression by catalase, there was a dose-dependent iNOS mRNA expression by catalase (Fig. 1A). The kinetic of iNOS expression by catalase was next determined. As shown in Fig. 1B, iNOS protein was weakly induced at 4 h catalase treatment, followed drastic increase of iNOS protein thereafter. iNOS mRNA was visible at 2 h catalase treatment, followed big increase in iNOS mRNA levels at 4 h catalase treatment. High level of iNOS mRNA was maintained up to 24 h after the addition of catalase. Control actin protein or GAPDH mRNA was not changed by treatment with catalase in different doses and times (Fig. 1A and Fig. 1 B). The efficiency of catalase in inducing iNOS expression was next evaluated by the extent of iNOS expression by LPS, a well-known iNOS inducer. As shown in Fig. 1C, the data demonstrated that iNOS protein expression induced by catalase was almost comparable to that induced by LPS in Raw cells, suggesting the efficiency of catalase to induce iNOS expression. In addition, like treatment of LPS increased high amounts of NO, catalase treatment also led to increased production of NO in Raw cells, suggesting that catalase induces the expression of a functionally active iNOS. We next investigated whether other antioxidant enzyme, such as superoxide dismutase mimics iNOS expression as catalase does. As anticipated, treatment of catalase (500 U/mL) induced high iNOS expression, whereas treatment of superoxide dismutase (500 or even 2000 U/mL) failed to induce iNOS expression in Raw cells (Fig. 1D), suggesting the specificity of catalase to induce iNOS expression.

3.2. Catalase-induced iNOS expression in Raw 264.7 macrophages is required for the catalase enzymatic activity

We next determined whether catalase-induced iNOS expression was truly mediated by the action of catalase,

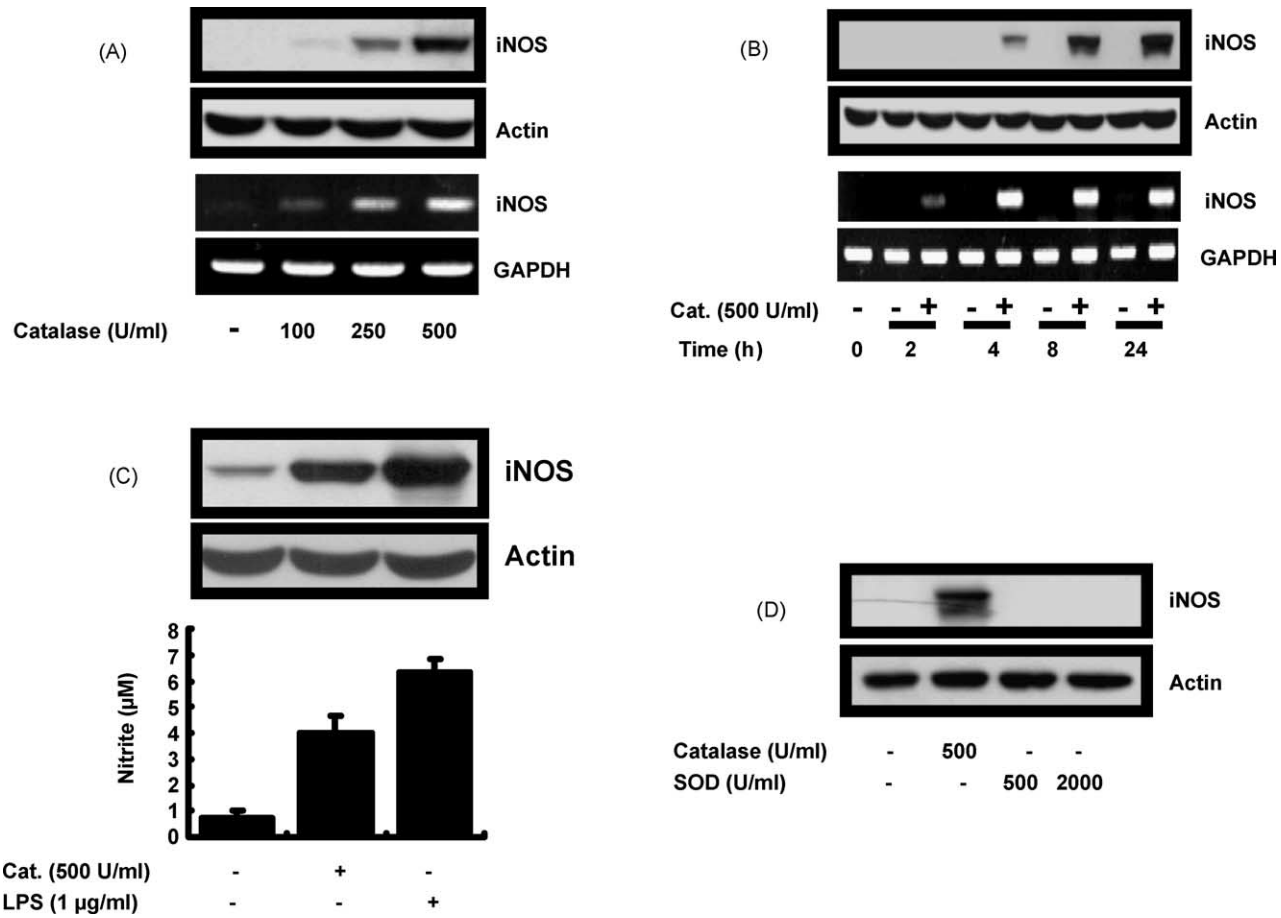

Fig. 1. The effects of catalase on iNOS expression in Raw cells. (A) Raw cells were treated with catalase (100, 250, or 500 U/mL) for 8 h. Whole cell lysates and total RNA were prepared, and analyzed for iNOS or actin Western and iNOS or GAPDH RT-PCR, respectively. (B) Raw cells were treated with catalase for the indicated times (2, 4, 8, or 24 h). At each time point, whole cell lysates and total RNA were prepared, and used for iNOS or actin Western and iNOS or GAPDH RT-PCR, respectively. (C) Raw cells were treated for 8 h with catalase or LPS, a well-known iNOS inducer. Whole cell lysates and cell culture medium from catalase- or LPS-treated cells were then prepared and saved, respectively, and assayed for determining iNOS protein expression and accumulated nitrite, an indicator of NO production, respectively. (D) Raw cells were treated for 8 h with the indicated concentrations of catalase or superoxide dismutase (SOD), another antioxidant enzyme. Whole cell lysates were prepared, and used for determining iNOS protein expression from catalase- or SOD-treated cells.

using proteinase K, a protein-degrading enzyme or AT, an inhibitor of catalase activity [26]. As shown in Fig. 2A, catalase that was pretreated for 1 h with proteinase K before addition to cells failed to induce iNOS expression in Raw cells. For testing the effectiveness of AT as a catalase activity inhibitor, catalase was incubated without or with different concentrations of AT in a cell-free system. Catalase was then exposed to H_2O_2, the genuine catalase substrate, and measured its ability to degrade H_2O_2. As shown in Fig. 2B, AT dose-dependently inhibited the activity of catalase, confirming that AT is a catalase activity inhibitor. Raw cells were then exposed for 8 h to catalase that was pretreated for 1 h without or with different concentrations of AT before addition to cells. iNOS expression in these cells was then measured by iNOS Western blot. As shown in Fig. 2C, catalase that was not pretreated with AT induced iNOS expression, while catalase that was preinactivated by AT (particularly at 30 mM) greatly lost its iNOS inducing ability. The data of AT inhibition of catalase-induced iNOS expression led us to consider that the basal level of H_2O_2 in Raw cell culture might cause a tonic inhibition of iNOS expression. We therefore measured the basal level of H_2O_2 in the supernatant of Raw cell culture. Unfortunately, we were unable to measure the basal level of H_2O_2 in the supernatant, which might be due to that our H_2O_2 measurement system was not so sensitive to detect the basal level of H_2O_2 or that there might be no or extremely low basal level of H_2O_2 in the supernatant. Alternatively, we tested whether exogenous addition of H_2O_2 affects iNOS expression by catalase. As shown in Fig. 2D, there was no effect of exogenous addition of H_2O_2 at 0.5 or 2 mM on basal iNOS expression (in the absence of catalase) or induced iNOS expression by treatment of catalase in Raw cells. Collectively, these data demonstrate that the catalase activity is required for iNOS expression in Raw cells.

3.3. Catalase increased iNOS transcription in Raw cells

iNOS expression is primarily regulated at the level of transcription. To molecularly understand the mechanism of

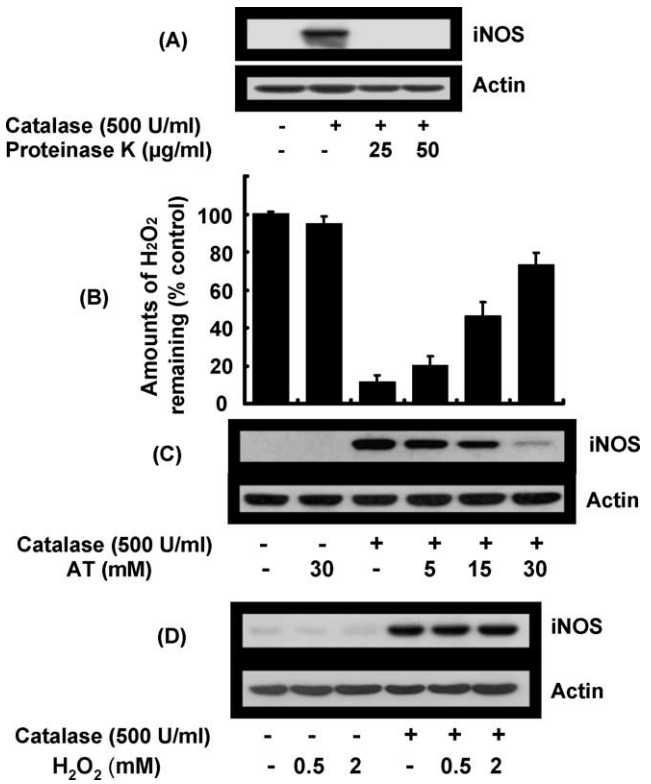

Fig. 2. The effects of proteinase K, AT, or exogenous addition of H_2O_2 on basal or catalase-induced iNOS expression in Raw cells. (A) Catalase was preincubated with different concentrations of proteinase K for 30 min before addition to cells. The proteinase K-treated catalase was then exposed to Raw cells for 8 h. Whole cell lysates were made, and used for iNOS or actin Western. (B) Catalase activity was measured as in detail described in Materials and methods. Briefly, catalase was preincubated for 1 h without or with different concentrations of AT, a catalase activity inhibitor in cell-free systems. The conditioned catalase was then exposed to H_2O_2. Catalase activity was evaluated by the degree of H_2O_2 degradation, which was measurable by a color development at OD 560 nm. (C) Raw cells were treated for 8 h with catalase that was pretreated for 1 h without or with AT in different concentrations before addition to cells. Whole cell lysates were made, and used for iNOS or actin Western. (D) Raw cells were treated for 8 h with catalase in the presence of H_2O_2 with indicated concentrations. Whole cell lysates were made, and used for iNOS or actin Western.

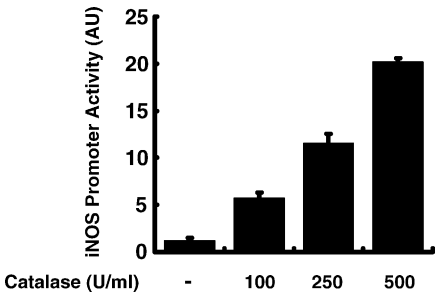

Fig. 3. The effects of catalase on iNOS transcription in Raw cells. Raw cells were co-transfected with 1 μg of the murine iNOS promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 24 h. Transfected cells were then treated with the indicated concentrations of catalase for additional 4 h. Cell lysates were prepared, and used for reporter gene activity. AU indicates arbitrary unit. Data are mean ± S.E. of three independent experiments.

catalase-induced iNOS expression, we next measured the effects of catalase on iNOS transcription in Raw cells. For this, Raw cells were transiently transfected with a luciferase construct, containing the murine iNOS promoter for 24 h, and the transfected cells were then treated with different concentrations of catalase for additional 4 h. As shown in Fig. 3, catalase dose-dependently increased the expression of iNOS promoter-driven luciferase, suggesting that catalase increases iNOS transcription in Raw cells.

3.4. NF-κB dependence of catalase-induced iNOS expression in Raw cells

Transcription factors, including NF-κB, IRF, AP-1, or NF-IL6 have been identified as important regulators in iNOS transcription [10–12]. Recently, we have demonstrated that NF-κB transcription factor is rapidly (within 30 min) activated following the exposure of catalase in Raw cells [27]. Therefore, we determined the role of NF-κB in catalase-induced iNOS expression in Raw cells, using MG132, a NF-κB inhibitor. The effectiveness of MG132 as a NF-κB inhibitor was assessed in this study by measuring the degree of blockage of the degradation of IκB-α, an inhibitory protein of NF-κB. Raw cells were pretreated for 1 h with different concentrations of MG132, and then cells were exposed to catalase for 0.5 or 8 h, following the measurement of IκB-α or iNOS protein expression levels in these cells. Pretreatment with MG132 particularly at 6 μM that effectively blocked catalase-induced IκB-α degradation in Raw cells (Fig. 4A) also resulted in partial inhibition of iNOS expression induced by catalase in Raw cells (Fig. 4B). We next ensured whether MG132 inhibition of catalase-induced iNOS expression is through inhibition of NF-κB activity by directly measuring catalase-induced NF-κB promoter activity in the presence of MG132. Apparently, pretreatment of MG132 at 6 μM effectively suppressed NF-κB promoter-driven luciferase expression in response to catalase (Fig. 4C). Similarly, there was concentration-dependent inhibition of catalase-induced iNOS protein expression (Fig. 4D) and NF-κB promoter-driven luciferase expression (Fig. 4E) by pretreatment of BAY 11-7085, another NF-κB inhibitor that inhibits IKK. These results strongly suggest that NF-κB in part mediates catalase-induced iNOS expression in Raw cells.

3.5. PI3K regulates catalase-induced iNOS expression in Raw cells

It has been demonstrated that iNOS expression is linked to activation of intracellular signaling proteins, including ERKs, p38s, PKCs, or JAK-2 [16–19]. Recently, we have reported that ERKs, p38s, JNKs,

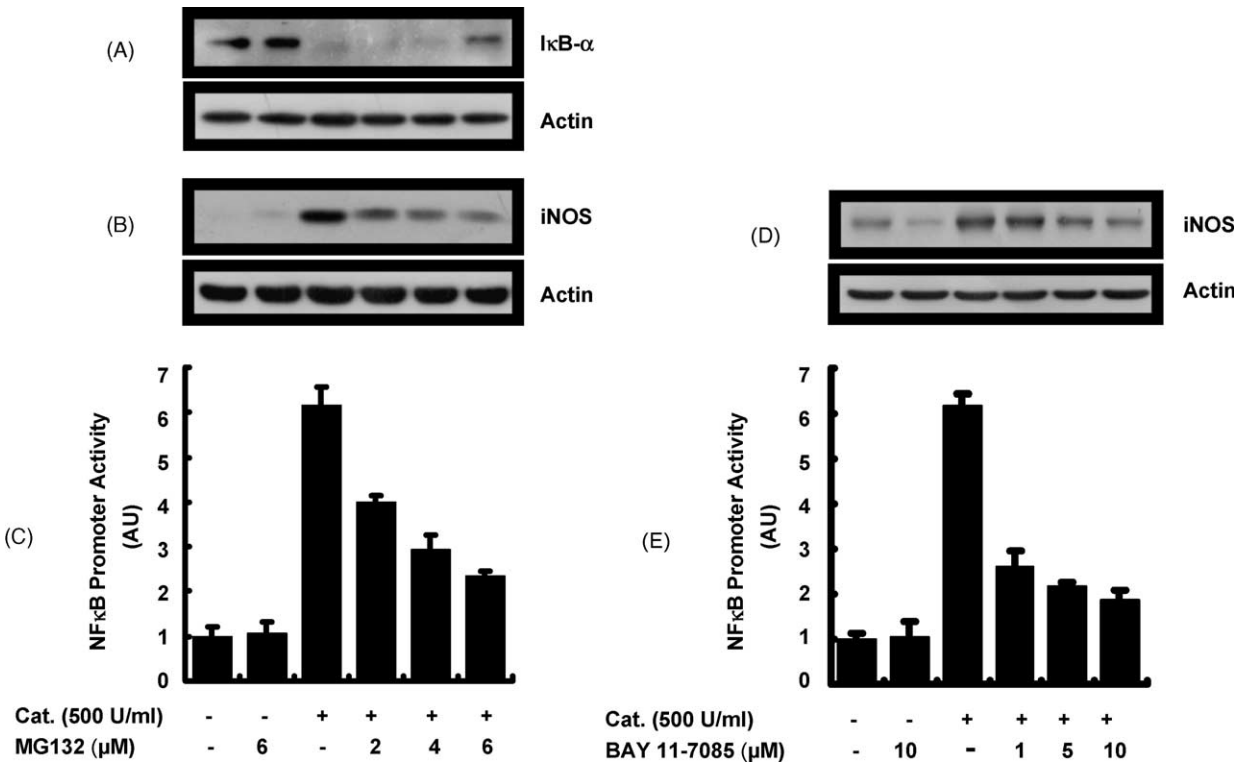

Fig. 4. The effects of MG132 or BAY 11-7085, inhibitors of NF-κB, on catalase-induced iNOS expression, IκB-α degradation, or NF-κB promoter activity in Raw cells. (A) Raw cells were pretreated for 1 h with the indicated doses of MG132, a NF-κB inhibitor and then treated with catalase for additional 30 min. Cell lysates were prepared, and analyzed for IκB-α or actin Western. (B) Raw cells were pretreated for 1 h with the indicated concentrations of MG132. Cells were then treated with catalase for additional 8 h. Cell lysates were made, and used for iNOS or actin Western. (C) Raw cells were co-transfected with NF-κB promoter/luciferase DNA along with control pRL-TK DNA for 24 h, following treatment with the indicated concentrations of catalase for 4 h in the presence of MG132 with the indicated concentrations. Cell lysates were prepared, and assayed for reporter gene activity. AU indicates arbitrary unit. Data are mean ± S.E. of three independent experiments. (D) Raw cells were pretreated for 1 h with the indicated concentrations of BAY 11-7085. Cells were then treated with catalase for additional 8 h. Cell lysates were made, and used for iNOS or actin Western. (E) Raw cells were co-transfected with NF-κB promoter/luciferase DNA along with control pRL-TK DNA for 24 h, following treatment with the indicated concentrations of catalase for 4 h in the presence of BAY 11-7085 with the indicated concentrations. Cell lysates were prepared, and assayed for reporter gene activity. AU indicates arbitrary unit. Data are mean ± S.E. of three independent experiments.

and PI3K are activated following treatment with catalase in Raw cells [27]. This led us to test the role of ERKs, p38s, JNKs, or PI3K in catalase-induced iNOS expression in Raw cells. As shown in Fig. 5A, catalase-induced iNOS protein expression in Raw cells was strongly abrogated by pretreatments of LY294002 or wortmanin (inhibitors of PI3K), while pretreatments with PD98059 (an ERKs inhibitor), SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), rapamycin (an inhibitor of mTOR/S6K), or PTX (a G_i inhibitor) had no or little effect on catalase-induced iNOS protein expression in Raw cells. These results strongly suggest that PI3K specifically mediates catalase-induced iNOS protein expression in Raw cells. To additionally understand the role of PI3K pathway in catalase-induced iNOS expression, Raw cells were pretreated for 1 h with LY294002 in different concentrations, and then treated with catalase for additional 8 h. The steady-state iNOS mRNA levels, iNOS mRNA stability, and iNOS transcription in these cells were then measured by RT-PCR, actinomycin D chase, and luciferase experiments, respectively. As shown in Fig. 5B, LY294002 dose-dependently reduced the steady-state iNOS mRNA levels induced by catalase. Furthermore, LY294002 increased the turnover of iNOS mRNA in the presence of actinomycin D; the half-life of iNOS mRNA decreased from 6 to 4 h (Fig. 5C). Moreover, LY294002 suppressed the expression of iNOS promoter-driven luciferase induced by catalase in Raw cells (Fig. 5D). SP600125 that did not change catalase-induced iNOS expression (Fig. 5A) had no effect catalase-induced iNOS transcription in Raw cells (Fig. 5D). These results strongly suggest that catalase-induced iNOS expression in Raw cells is largely dependent on the activation of PI3K pathway, which appears to regulate iNOS expression by increasing transcription, steady-state transcripts levels, and mRNA stability.

3.6. Partial PI3K regulation of catalase-induced NF-κB-α activation in Raw cells

It is reported that PI3K regulates iNOS expression via activation of NF-κB pathway [28,29]. Moreover, cross-

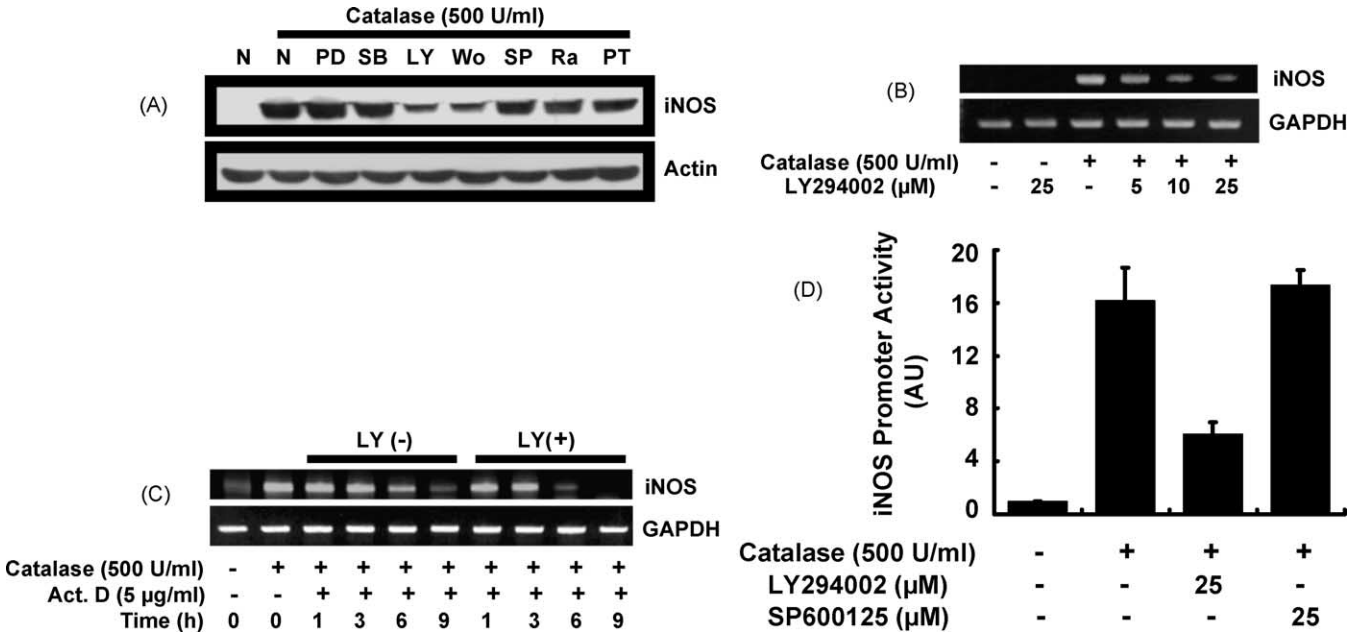

Fig. 5. The effects of LY294002 on iNOS transcription, steady-state transcripts levels, and mRNA stability induced by catalase in Raw cells. (A) Raw cells were pretreated for 1 h without or with PD98059 (50 μM, an inhibitor of ERKs), SB203580 (25 μM, an inhibitor of p38s), LY294002 (25 μM, an inhibitor of PI3K), wortmanin (300 nM, an inhibitor of PI3K), SP600125 (25 μM, an inhibitor of JNKs), rapamycin (10 nM, an inhibitor of mTOR/p70S6K), or PTX (500 ng/ml, an inhibitor of G_i). Raw cells were then treated with catalase for additional 8 h. Whole cell lysates were made, and used for iNOS or actin Western. N, no inhibitor; PD, PD98059; SB, SB203580; LY, LY294002; Wo, wortmanin; SP, SP600125; Ra, rapamycin; PT, PTX. (B) Raw cells were pretreated for 1 h with different concentrations of LY294002, and then treated with catalase for additional 8 h. Total RNA was isolated and used for iNOS or GAPDH RT-PCR. (C) Raw cells were initially incubated without or with catalase for 8 h to highly induce iNOS transcripts. The conditioned media were then removed, and cells were further treated with catalase for the indicated times in the presence of actinomycin D (5 μg/mL) along with or without LY294002 (25 μM). At each time, total RNA was prepared, and used for iNOS or GAPDH RT-PCR. (D) Raw cells were co-transfected with 1-μg of the murine iNOS promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 24 h. Transfected cells were then treated with catalase for additional 4 h in the absence or presence of LY294002 (25 μM) or SP600125 (25 μM). Cell lysates were prepared, and used for reporter gene activity. AU indicates arbitrary unit. Data are mean ± S.E. of three independent experiments.

signaling between PI3K and NF-κB has been postulated [30,31]. This led us to test whether PI3K lies upstream of NF-κB in catalase-elicited signaling pathway in Raw cells. Interestingly, inactivation of PI3K by LY294002 caused inhibition of catalase-induced NF-κB promoter driven-luciferase expression in a concentration-dependent manner in Raw cells (Fig. 6), suggesting that PI3K may act as an upstream regulator of NF-κB activation by catalase in Raw cells. Considering that LY294002 inhibits catalase-induced iNOS transcription in Raw cells as shown in Fig. 5D, these results may also suggest that PI3K may involve in catalase-induced iNOS transcription by up-regulating NF-κB in Raw cells.

3.7. iNOS expression by catalase seems to be macrophage cell type-specific

We next tested whether catalase induction of iNOS expression was unique to Raw cells. Catalase was exposed for 8 h to Raw cells, BV2 microglial macrophage-like cells, HT29 (colon), or A549 (lung) cancerous epithelial-like cells. The response of iNOS expression by catalase in these cells was then analyzed by iNOS Western blot. As shown in Fig. 7, catalase induced the expression of iNOS in BV2 cells, whereas HT29 and A549 cells did not express iNOS in response to catalase. These results suggest that catalase induction of iNOS expression may be macrophage lineage cell-type specific.

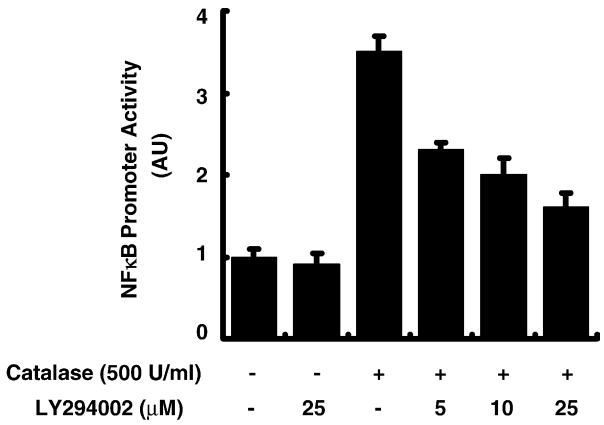

Fig. 6. PI3K seems be an upstream regulator of NF-κB activation in response to catalase in Raw cells. Raw cells were co-transfected with NF-κB promoter/luciferase DNA along with control pRL-TK DNA for 24 h, following treatment with the indicated concentrations of catalase for 4 h in the presence of LY294002 with the indicated concentrations. Cell lysates were prepared, and assayed for reporter gene activity. AU indicates arbitrary unit. Data are mean ± S.E. of three independent experiments.

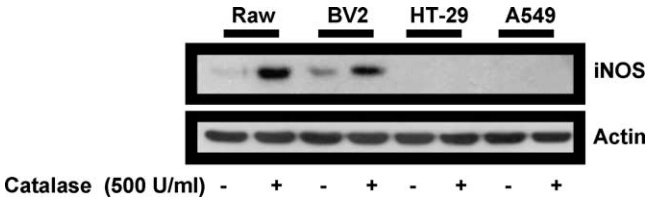

Fig. 7. Effects of catalase on iNOS expression in other types of cells. In addition to Raw cells, catalase (500 U/mL) was also exposed to BV2 microglial macrophage-like cells, HT-29 (colon), or A549 (lung) cancer epithelial-like cells for 8 h. Whole cell lysates were then made, and used for iNOS or actin Western.

4. Discussion

In present study, we have demonstrated for the first time that catalase induces the expression of iNOS in Raw cells by increasing iNOS transcription and mRNA stability. Our data have indicated that the induction is required for the activity of catalase, based on the facts that catalase which lost its activity by AT concomitantly lost its iNOS inducing ability. Importantly, our data have addressed that catalase-induced iNOS expression in Raw cells is correlated with the activation of NF-κB transcription factor and PI3K signaling pathway.

The antioxidant enzyme catalase mainly functions to degrade H_2O_2 into water and oxygen. However, it has been previously reported that macrophages produce high amounts of nitrite and nitrate in response to catalase [23]. Given that nitrite and nitrate are derived from NO, we hypothesized that the molecular event associated with the production of nitrite and nitrate in macrophages after the addition of catalase might be through the induction of iNOS, an enzyme producing NO. In present study, we have found that catalase dose- and dose-dependently induces the expression of iNOS in Raw cells. Importantly, by carrying out some biochemical experiments with AT, a catalase activity inhibitor [26] or proteinase K, a protein degradation enzyme, we have determined that iNOS expression in Raw cells is truly mediated by catalase. To our knowledge, it is the first report demonstrating induction of iNOS expression in macrophages after the addition to catalase.

It has been reported that iNOS expression is regulated at multiple levels, including transcription, post-transcription, and translation [8,9]. In present study, we have found that catalase induces the expression of iNOS promoter-driven luciferase in a concentration-dependent manner in Raw cells, suggesting that the induction is due to iNOS transcriptional activation. As previously reported, iNOS transcription is largely dependent on the activities of transcription factors, such as NF-κB, AP-1, IRF, or NF-IL6, which, respectively, bind to NF-κB, AP-1, IRF, and NF-IL6 consensus sequences within the iNOS promoter for the induction of iNOS [10-12]. In this study, we have found that blockage of NF-κB pathway by MG132, a NF-κB inhibitor, results in partial suppression of catalase-induced iNOS transcription (data not shown) and iNOS protein expression in Raw cells, which is associated with inhibition of catalase-induced degradation of IκB-α and NF-κB promoter activity after pretreatment of MG132 in particular at 6 μM. Moreover, the efficiency of MG132 inhibition of catalase-induced iNOS expression via inhibition of NF-κB promoter activity is further confirmed by use of BAY 11-7085, another inhibitor of NF-κB that inhibits IKK, which effectively reduces catalase-induced NF-κB promoter activity and iNOS expression in Raw cells. Therefore, these results suggest that NF-κB in part mediates catalase-induced iNOS expression by iNOS transcriptional up-regulation. Since, inactivation of NF-κB by MG132 only partially suppresses iNOS expression in response to catalase, it is also likely that additional transcriptional factors other than NF-κB regulate catalase-induced iNOS expression in Raw cells. It will be interesting to see what additional transcriptional factors are involved in the process of iNOS expression in response to catalase in these cells.

Catalase normally exists in a tetrameric form with four subunits (each has about 60 kDa), and is impermeable into cell plasma membrane [28]. In agreement with this, it was previously demonstrated by a flow cytometric analysis that catalase was bound to the surface of macrophages [23]. From the evidence, we hypothesized that catalase might exert its iNOS inducing activity through outside of cells. Our recent data demonstrating that catalase induces rapid activation of ERKs, p38s, JNKs, and PI3K in Raw cells [27] may further strengthen this hypothesis. Activation of ERKs, p38s, PKCs, JAK-2, or PI3K pathway has been implicated in iNOS expression [16–19,28,29]. Specifically, we have found that catalase-induced iNOS protein expression in Raw cells is greatly blocked by pretreatment with LY294002 or wortmannin, but not by pretreatment with PD98059, SB203580, or SP600125. Moreover, the data of RT-PCR and iNOS promoter analyses have shown that inactivation of PI3K pathway by LY294002 results in suppression of catalase-induced iNOS mRNA expression and iNOS promoter activity. These results strongly suggest that PI3K regulates catalase-induced iNOS expression by increasing steady-state iNOS transcripts levels and iNOS transcription. It has been reported that PI3K regulates iNOS expression through activation of NF-κB pathway [28,29]. Furthermore, cross-signaling between PI3K and NF-κB has been postulated [30,31]. Based on these previous reports and our present data showing involvement of PI3K and NF-κB in catalase-induced iNOS expression, we thought that PI3K might regulate catalase-induced iNOS expression via NF-κB. In our experiments, we have observed that inactivation of PI3K pathway by LY294002 which strongly inhibits catalase-induced phosphorylation of p70S6K, a well-known downstream effector of PI3K, and degradation of IκB-α (data not shown) also reduces catalase-induced NF-κB promoter-driven luciferase expression. These results suggest that PI3K regulation of iNOS expression in response to catalase is through

NF-κB activation and that PI3K is upstream of NF-κB activation induced by catalase in Raw cells.

No data has been at present available about the role of PI3K in the regulation of post-transcriptional iNOS mRNA stability. In present study, we have shown that iNOS mRNA induced by catalase is stabilized for several hours in the presence of actinomycin D, a transcription inhibitor, suggesting that catalase induces post-transcriptional iNOS mRNA stabilization in Raw cells. Interestingly, we have found that LY294002 increases the turnover of iNOS mRNA; the half-life of iNOS mRNA decreases from 6 to 4 h. Therefore, these results suggest that PI3K is involved in post-transcriptional iNOS mRNA stabilization in response to catalase in Raw cells. To our knowledge, it is the first report addressing a role of PI3K pathway in post-transcriptional iNOS mRNA stabilization. It is well understood that PI3K activation is often followed by receptor stimulation or activation of receptor associated proteins upon treatment with extracellular stimuli [32–34]. In present study, we have observed that PTX, an inhibitor of G_i protein has no or little effect on catalase-induced iNOS expression in Raw cells, therefore, suggesting that iNOS expression by catalase may be not through activation of a receptor coupled PTX-sensitive G_i class of heterotrimeric G proteins. It will be interesting to see which particular receptor or upstream events are activated following the addition of catalase and involved in iNOS expression and/or PI3K activation in Raw cells.

Given that catalase-induced iNOS expression in Raw cells is inhibited by AT, a catalase activity inhibitor, as shown in the present study, we thought that that catalase-induced iNOS expression might be through removal of H_2O_2, and the basal level of H_2O_2 might cause a tonic inhibition of iNOS expression in Raw cells. Unfortunately, we have not detected the basal level of H_2O_2 in the supernatant of Raw cell culture. It may be explained by that our H_2O_2 measurement system used here may be not so sensitive to detect it or that there may be no or extremely low basal level of H_2O_2 in the supernatant. Alternatively, we have tested the effect of exogenous addition of H_2O_2 on basal or catalase-induced iNOS expression in Raw cells. However, our present data showing that exogenous addition of H_2O_2 has no effect on basal and catalase-induced iNOS expression in Raw cells indicate that though catalase-induced iNOS expression is necessary for catalase activity, iNOS expression by catalase appears to be not through removal of H_2O_2, suggesting presence of other mechanism. It is noteworthy to mention the previous findings by Han et al. [35] in which catalase treatment rather inhibits iNOS expression and NF-κB activation induced by LPS in Raw 264.7 cells that may be due to scavenging LPS-induced H_2O_2, which is known to involve in up-regulation of iNOS expression [35].

Additionally, we have shown that catalase can induce the expression of iNOS in BV2 microglial macrophage-like cells, but not in HT-29 (colon) and A549 (lung) cancer epithelial-like cells. These results may indicate that iNOS expression in response to catalase is likely to be macrophage lineage type-specific.

In conclusion, we have shown that catalase induces iNOS expression in Raw 264.7 macrophages. Our data suggest that the induction is at least mediated via two molecular and signaling pathways. One may be through the increase of iNOS transcription that is in part related with the activation of NF-κB transcription factor and PI3K pathway. The other may be through the increase of post-transcriptional iNOS mRNA stability that seems to be dependent on PI3K pathway. The findings and data presented here may suggest a hitherto unknown function of catalase that up-regulates iNOS.

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