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Inflammation Research

The inhibitory effects of roflumilast on lipopolysaccharide-induced nitric oxide production in RAW264.7 cells are mediated by heme oxygenase-1 and its product carbon monoxide

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Abstract. *Objectives*: Heme oxygenase-1 (HO-1) is an enzyme that degrades heme into biliverdin, free iron, and carbon monoxide (CO). This enzyme is known to have cytoprotective and anti-inflammatory effects. In this study, we investigated whether roflumilast, a newly developed specific phosphodiesterase 4 (PDE4) inhibitor, mediates some of its anti-inflammatory effects by blocking nitric oxide (NO) and tumor necrosis factor alpha (TNF-α) via the induction of HO-1 expression in macrophages.
Methods: The expression of iNOS and HO-1 was analyzed by western blot analysis. The production of NO and TNF-α was assayed by Greiss and ELISA, respectively.
Results: Roflumilast markedly suppressed LPS-induced NO and TNF-α production and these phenomena were correlated with the induction of HO-1 protein levels. Moreover, the inhibitory effects of roflumilast on NO production were abrogated by a HO-1 inhibitor and a CO scavenger. Tricarbonyldichlrororuthenium(II) dimer, a CO releasing molecule significantly suppressed NO production.
Conclusions: These results suggested that roflumilast exerts its anti-inflammatory effects in macrophages through a novel mechanism that involves the action of HO-1 and its product, CO.

Key words: Heme oxygenase-1 – Carbon monoxide – Nitric oxide – Phosphodiesterase 4 inhibitor – Roflumilast – Tumor necrosis factor alpha

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Introduction

Heme oxygenases (HOs) are the rate-limiting enzymes in heme catabolism. They catalyse the oxidative degradation of heme into carbon monoxide (CO), free iron, and biliverdin, which subsequently is converted to bilirubin by bilirubin reductase [1, 2]. It is believed that these products derived from the catalysis of heme by HO may finally mediate the anti-oxidative, anti-proliferative and anti-inflammatory effects of HO-1 [3]. To date, three HO isozymes have been identified. Of these, HO-2 and HO-3 are constitutively expressed, whereas HO-1 is an inducible enzyme [4] and confers protective effects in the oxidative stress. HO-1 deficient mice show that HO-1 is important in the host's defense against oxidative stress that is accompanied by an increase of this enzyme in the inflammatory state [5]. Furthermore, the induction of HO-1 by chemical inducers, or selective overexpression, resulted in the cytoprotective effects both *in vitro* [6] and *in vivo* [7]. It has been reported that the induction of HO-1 activity and expression in macrophages inhibits a carragenin-induced pleural inflammation in rats [8]. The inhibition of HO-1 expression results in increased inflammatory responses, and pre-induction of HO-1 causes a decrease in inflammatory parameters [9, 10]. These results suggest that induction of HO-1 has beneficial effects that respond to a stressful state such as inflammation. However, the mechanisms of this biological phenomenon have not been clearly elucidated yet.

The pro-inflammatory cytokine TNF-α and reactive free radical, nitric oxide (NO) synthesized by inducible NO synthase (iNOS) are the major macrophage-derived inflammatory mediators. They have been reported to be involved in the development of inflammatory diseases [11]. The small amount of NO produced by constitutive NOS (cNOS) is an important regulator of physical homeostasis, whereas the large amount

of NO produced by iNOS has been correlated with the pathophysiology in inflammation. Thus, the inhibition of the excessive production of TNF-α and/or NO can be employed as a criteria to evaluate anti-inflammatory effects of drugs.

Phosphodiesterases (PDEs) are families of hydrolases that catalyze the hydrolysis of cyclic AMP (cAMP) and cyclic GMP (cGMP), and thus play key roles in the regulating signal responses to intracellular gradients of cAMP and cGMP [12, 13]. Among the cAMP-specific isozymes, PDE4 is the predominant family of PDEs expressed in inflammatory cells, including eosinophils [13–15], T lymphocytes [16], macrophages [17, 18], neutrophils [19] and dendritic cells [20]. Since PDE4 metabolizes cAMP, a signal molecule known to attenuate cell activation, PDE4 inhibitors cause an elevation of intracellular cAMP levels and subsequently decrease the production of numerous inflammatory mediators, including histamine and leukotrienes [21], and proinflammatory cytokines such as TNF-α and interleukin-1β (IL-1β) [22].

Roflumilast, the most potent and advanced PDE4 inhibitor [23, 24], has been demonstrated to be an effective anti-inflammatory agent in airway inflammatory diseases such as asthma [25]. Suppression of TNF-α production by roflumilast is well documented in LPS-induced inflammatory diseases [26]. Although roflumilast has beneficial biological effects, its mechanism in inflammatory cells has not yet been clearly demonstrated. It was recently reported that rolipram, a PDE4 specific inhibitor, and pentoxifylline, a general PDE inhibitor, suppressed NO production in islet cells [27] and macrophages [28].

In this study, we tested whether roflumilast suppresses pro-inflammatory mediators TNF-α and NO in macrophages and the mechanism involved, if any. Here, we report that roflumilast is a potent HO-1 inducer and inhibits NO production in LPS-stimulated RAW264.7 cells through the induction of HO-1. Our data also suggest that the protective effect of HO-1 on NO production is mainly mediated through its reaction product, CO.

Materials and methods

Materials

RPMI-1640, fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY). Roflumilast was synthesized by Korea Research Institute of Chemical Technology (KRICT) (Taejon, Korea). Murine TNF-α ELISA kit was purchased from BD bioscience (San Jose, CA). Cytotox 96 nonradioactive assay kit was obtained from Promega (Road Madison, WI). Polyclonal HO-1 antibody and monoclonal iNOS antibody were the products of Stressgen (Victoria, Canada) and Santa Cruz (Santa Cruz, CA), respectively. Zinc protoporphyrin IX (ZnPP) and Cobalt protoporphyrin IX (CoPP) were obtained from Porphyrin Products (Logan, UT). LPS, tricarbonyldichlororuthenium (II) dimmer (RuCO), deferoxamine mesylate (DFO) and hemoglobin (Hb) were purchased from Sigma Chemical (St. Louis, MO). All other reagents were the products of Sigma Chemical unless indicated otherwise.

Cell culture

The murine macrophage cell line RAW264.7 was obtained from the American Tissue Culture Collection (ATCC) (Manassas, VA). The cells were maintained at 5×10^5 cells/ml in complete RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicilline G (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Measurement of nitrite concentration

Accumulation of nitrite in the medium was measured by a colorimetric assay based on the Griess reaction [29]. Briefly, samples were reacted with 1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2.5% phosphoric acid at room temperature for 10 min, and nitrite concentration was determined by absorbance at 540 nm in comparison with sodium nitrite as a standard.

Cell cytotoxicity assay

Cellular toxicity was determined by lactate dehydrogenase (LDH) release in the medium. In brief, cells (5×10^5 cells/ml) were incubated with roflumilast or other agents. After 24 h incubation, LDH activity in the media was measured by using the assay kit (Cytotox 96 nonradioactive assay kit, Promega). Optical density (OD) at 490 nm was measured and quantified.

Western blot analysis

Protein samples (50–100 μg) were mixed with an equal volume of 2× SDS sample buffer, boiled for 5 min, and then separated through 8 or 10% PAGE-SDS gels. After electrophoresis, proteins were transferred to nylon membrane. The membranes were blocked in 5% dry milk (1 h), rinsed, and incubated with either HO-1 (1:1000) or iNOS (1:1000) antibodies in Tris-buffered saline (TBS) containing Tween 20 (0.1%) overnight at 4 °C. Primary antibody was removed by washing the membranes 4 times in TBS-T, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:1000). Following 4 times washes in TBS-T, bands were visualized by ECL and exposed to X-ray film.

TNF-α assay

The levels of TNF-α were measured in cell culture media using murine TNF-α ELISA kits (BD bioscience). Briefly, the cells (5×10^5/ml) were treated with LPS (100 ng/ml) in the presence of various concentrations of roflumilast (0.05–10 μM). Supernatants were removed and TNF-α production was quantified.

Statistical analysis

Data are represented as means ± SD of three separate experiments performed in triplicates. The significance of difference from the respective control for each experimental test condition was assayed by using Student's *t*-test for each paired experiments. A p value < 0.05, 0.01 or 0.001 was regarded as a significant difference.

Results

Roflumilast induces HO-1 protein in RAW264.7 cells

We examined the effects of different concentrations of roflumilast on HO-1 expression in RAW264.7 cells. As shown in Fig. 1A, the treatment of roflumilast for 12 h resulted in an increase in HO-1 protein levels in a concentration-dependent manner. Roflumilast caused a marked increase in HO-1 pro-

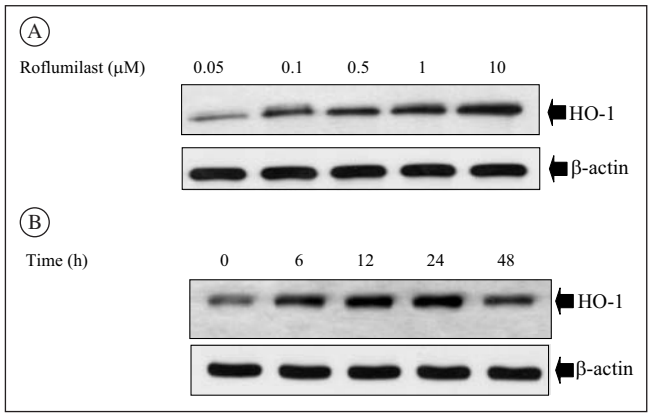

Fig. 1. Concentration- and time-dependent effects of roflumilast on HO-1 induction in RAW264.7 cells. (A) Cells (1×10^6) were cultured for 12 h with various concentrations of roflumilast (0.05–10 μM). HO-1 expression was detected by western blot analysis using a polyclonal antibody for rabbit HO-1. (B) Cells (1×10^6) were cultured for various times (0–48 h) with roflumilast (10 μM). At the indicated time points, the cells were harvested and HO-1 expression was detected as described in the Materials and methods.

tein level at the concentration of 10 μM (Fig. 1A). Thus, this concentration (10 μM) was chosen to determine the effect of roflumilast on HO-1 expression over time. Treatment with roflumilast resulted in a time-dependent increase in HO-1 protein expression, with maximal increases at 12–24 h (Fig. 1B). No cytotoxic effects of roflumilast were observed under this experimental condition (data not shown).

Roflumilast inhibits NO production and TNF-α secretion in LPS-stimulated RAW264. 7 cells

After 18 h incubation, unstimulated macrophages produced a background level of NO (about 2 μM) in the culture medium. When RAW264.7 cells were stimulated with LPS (100 ng/ml) for 18 h, the levels of nitrite, a stable oxidized product of NO, were markedly increased in the culture medium. However, in cells pre-treated with different concentrations of roflumilast for 12 h, LPS-induced NO production was inhibited in a concentration-dependent manner, complete inhibition being achieved at 10 μM (Fig. 2A). To investigate whether roflumilast inhibits NO production via the suppression of iNOS gene expression, we examined the induction of iNOS protein by western blot. The results showed that roflumilast decreased the levels of iNOS protein in LPS-stimulated cells in a concentration-dependent manner (Fig. 2B). When treated with LPS, cells increased TNF-α secretion, and this increase was inhibited in a concentration-dependent manner by co-treatment with roflumilast (Fig. 2C). No cytotoxic effects of roflumilast were observed under this experimental condition as measured by lactate dehydrogenase (data not shown).

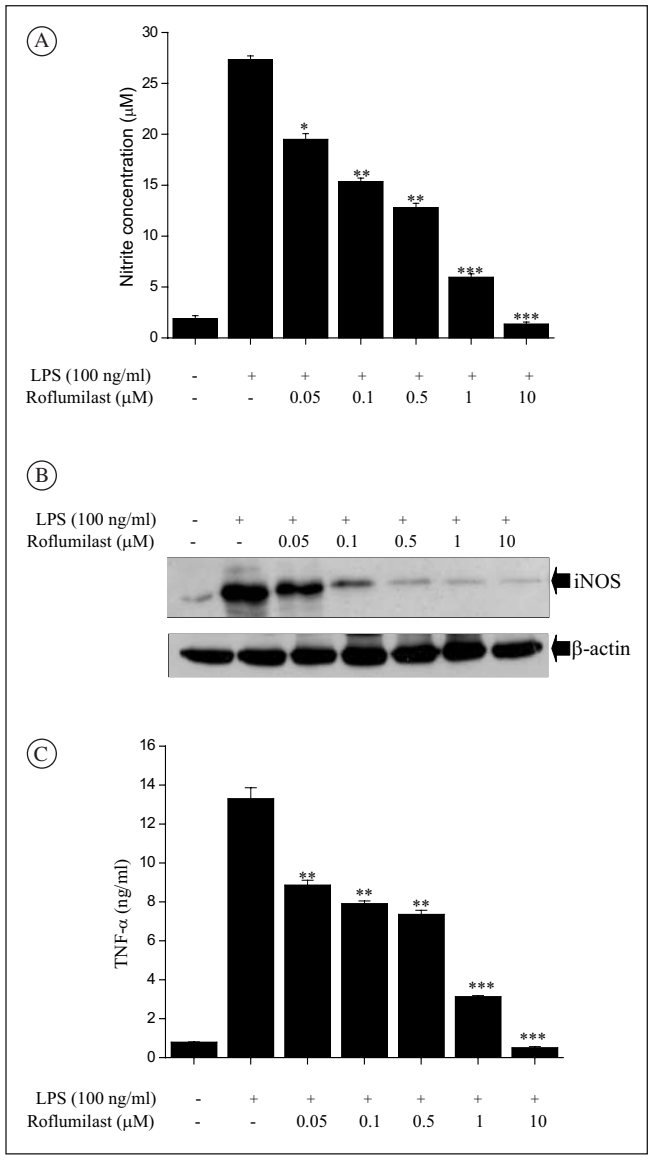

Fig. 2. Effects of roflumilast on NO production, iNOS expression and TNF-α secretion in RAW264.7 cells. RAW264.7 cells were pre-incubated with indicated concentrations of roflumilast (0.05–10 μM) for 12 h. Then, LPS (100 ng/ml) was given to the cells and incubation was continued for 18 h. (A) Nitrite levels were measured in the culture media of LPS-stimulated cells for 18 h by Griess reaction. (B) Levels of iNOS protein were measured in LPS-stimulated cells for 18 h by western blot using a monoclonal antibody for murine iNOS. (C) TNF-α secretion was measured in the 18 h culture media using a commercial ELISA kit. Data shown are means ± SD from three separate experiments done in triplicates. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus LPS alone.

Roflumilast-induced HO-1 mediates the inhibitory effects of NO production in LPS-stimulated RAW264.7 cells

Roflumilast suppressed NO production and iNOS induction, which was accompanied by the induction of HO-1 protein. Based on these results, we next examined if NO inhibition in roflumilast-treated cells is mediated through HO-1 induction by employing CoPP treatment, a well known HO-1

inducer [30], prior to LPS treatment. Preliminary experiments showed that CoPP induced a concentration-dependent induction of HO-1 with maximum effects achieved at 10 μM (data not shown). As shown in Fig. 3, pretreatment with CoPP (10 μM) for 12 h significantly inhibited LPS-induced NO production to a similar extent to roflumilast treatment, which was abrogated by ZnPP (10 μM), a competitive HO-1 inhibitor. Additionally, co-treatment with ZnPP (10 μM) significantly reversed roflumilast-induced suppression of NO production. The concentration of 10 μM of ZnPP was chosen based on preliminary experiments showing maximum effects without cytotoxicity. Roflumilast, CoPP and ZnPP alone had no effect on NO production under these conditions (data not shown). These results suggested that activation of HO-1 mediates inhibitory effects of roflumilast on LPS-induced NO production.

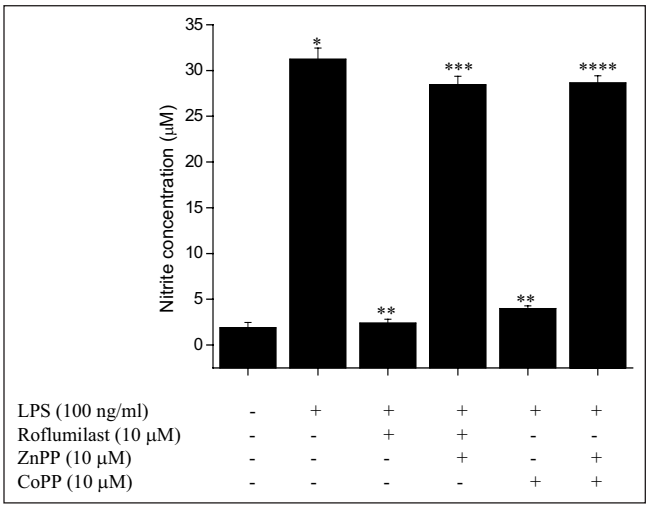

Fig. 3. Effects of CoPP and ZnPP on the LPS-induced NO production in RAW264.7 cells. Cells were pre-incubated with either 10 μM roflumilast or 10 μM CoPP for 12 h in the absence or presence of 10 μM ZnPP. LPS (100 ng/ml) was given to the cells and incubation was continued for 18 h. The nitrite concentration in culture medium was determined with Griess reaction. Data shown are means ± SD from three separate experiments done in triplicates. *$p < 0.001$ compared with untreated control, **$p < 0.001$ compared with LPS control, ***$p < 0.001$ compared with LPS+ roflumilast, and ****$p < 0.001$ compared with LPS+CoPP.

Inhibitory effect of HO-1 on NO production may be mediated via CO

As a possible mediator of HO-1 action induced by roflumilast against LPS-induced NO production, we hypothesized that CO, derived from heme degradation by HO-1 might exert potent anti-inflammatrory effects. Therefore, we carried out additional experiments to examine the effect of hemoglobin (Hb), a scavenger of CO [30], on NO production under the same experimental condition. RAW264. 7 cells were pre-treated for 12 h with roflumilast plus Hb and then treated with LPS. As shown in Fig. 4, Hb at 50 μM, the concentration that showed a maximum effect (data not shown), significantly reversed the inhibitory effect of roflumilast on LPS-induced NO production. Furthermore, treatment with RuCO (20 μM), a CO releasing compound, exhibited similar inhibitory effect on LPS-induced NO production in RAW264. 7 cells, whereas cotreatment with Hb and RuCO resulted in the reversal of the inhibitory effects of RuCO (Fig. 4). Similar experiments were performed to examine whether other HO-1 by-products or free iron might also affect the inhibitory effect on NO production. We used deferoxamine (DFO, 100 μM), an iron chelator, in combination with roflumilast in LPS-treated cells and measured NO production. As shown in Fig. 4, DFO had little effect on NO production, suggesting that the inhibitory effect of roflumilast on the NO production was little affected by the presence of free iron (Fig. 4). Hb, RuCO and DFO alone had no significant effects on cell viability and NO production under these conditions (data not shown). These results suggest that CO released by HO-1 is a major player of roflumilast action in the inhibition of LPS-induced NO production in the RAW264.7 cells.

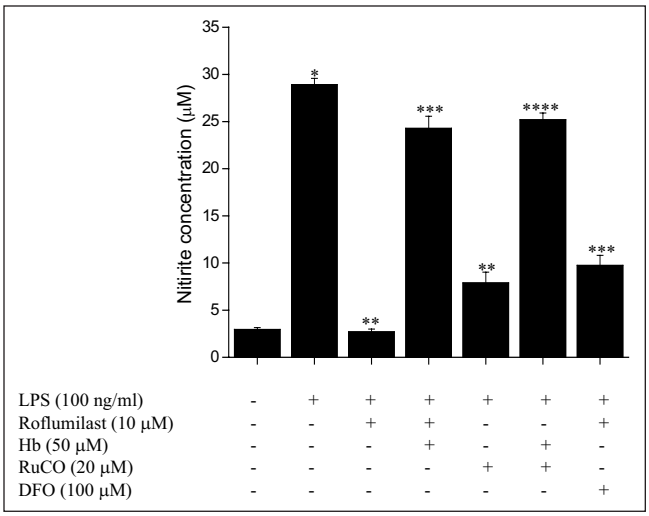

Fig. 4. Effects of Hb, RuCO and DFO on the LPS-induced NO production in RAW264.7 cells. Cells were pre-incubated with 10 μM roflumilast either in the absence or presence of 50 μM Hb or in the absence or presence of 10 μM DFO for 12 h. Cells were also pre-incubated with 20 μM RuCO in the absence or presence of 50 μM Hb for 12 h. Then, LPS (100 ng/ml) was given to the cells and incubation was continued for 18 h. The nitrite concentration in culture medium was determined with Griess reaction. Data shown are means ± SD from three separate experiments done in triplicates. *$p < 0.001$ compared with untreated control, **$p < 0.001$ compared with LPS control, ***$p < 0.001$ compared with LPS+ roflumilast, and ****$p < 0.01$ compared with LPS+ RuCO.

Discussion

Roflumilast is a novel potent PDE4-selective inhibitor and has been demonstrated to be an effective anti-inflammatory agent against airway inflammation [23–25]. Its potential usefulness in human asthma has been demonstrated in clinical studies [26, 31]. However, the mechanism of anti-inflammatory action was not fully understood. In this study, we mainly demonstrated that roflumilast was able to inhibit

LPS-induced NO production in RAW264. 7 cells and this effect was correlated with HO-1 induction.

NO and HO-1 are interesting products in physiologic and pathophysiologic systems that have stimulated much interest. In particular, the interplay between these systems has been subjected to many debates because of the possibilities of using them as therapeutic targets. The recent discovery of the possibility for inflammation control by PDE4 inhibitors, modulating iNOS gene expression and NO production, has offered another possibility to the anti-inflammatory drugs currently being developed. It was previously reported that rolipram, a specific PDE4 inhibitor, and pentoxifylline, a general PDE inhibitor, inhibited NO production in islet cells [27] and macrophages [28]. Initially, we investigated the role of roflumilast on LPS-induced NO production in RAW264.7 cells, which showed a concentration-dependent inhibition of NO production by roflumilast.

Colville-Nash et al. [32] previously reported that control of HO-1 activity affected the outcome of inflammation, and a PPAR-γ agonist inhibited NO production in conjunction with HO-1 expression. Accordingly, induction of HO-1 may play a role in the suppression of NO production. Therefore, in this study, we investigated whether the inhibition of NO production by roflumilast was related to its ability to induce HO-1 expression in macrophages. Interestingly, roflumilast is a strong HO-1 inducer in the range of 0.05 μM–10 μM. Pre-incubation of RAW264.7 cells with either roflumilast or a HO-1 inducer, CoPP, suppressed LPS-induced NO production. These data suggest that inhibition of NO by roflumilast in LPS-stimulated cells parallels the induction of HO-1 expression. ZnPP is a common HO-1 inhibitor and the inhibition of HO-1 activity by ZnPP has been reported in several studies [33–35]. In the present study, ZnPP attenuated the inhibitory activities of roflumilast on LPS-induced NO production. In conclusion, these findings demonstrated that NO and HO-1 pathways exhibited a certain degree of interdependency. Consistent with our observations, a recent study also supported the role of HO-1 in the inhibitory effects of flavonoid on LPS-induced NO production [36]. The relationship between HO-1 induction and NO inhibition by the PDE4 inhibitor, roflumilast, was first suggested in this study.

HO-1 expression may have further effects on inflammation via the production of heme breakdown products. These include CO, which may inhibit iNOS activity by binding to and inactivating the heme moiety on iNOS enzyme; biliverdin and bilirubin, which may scavenge oxidative products by direct antioxidant effects; and iron, which affects cellular redox potentials [37, 38]. It has been reported that CO exerts anti-inflammatory effects through directly modulating iNOS activity. However, the detailed mechanism is not clear yet. We demonstrated in the present study that CO released from heme degradation by HO-1 was responsible for suppressive properties of roflumilast on NO production. Hb, a CO scavenger, reversed the inhibitory effects of roflumilast on LPS-induced NO production while RuCO, a CO releasing compound, effectively suppressed the NO production. On the contrary, iron chelator DFO did not fully reverse the effect of roflumilast on NO production. Therefore, we suggest that HO-1 expression and the ensuing formation of the HO metabolite, CO, by HO-1 may be a novel pathway by which roflumilast could inhibit LPS-induced NO production.

In summary, our results suggest that induction of HO-1 by roflumilast may lead to decrease of NO production induced by LPS through enhanced CO accumulation. Our data together with the previous reports suggest that, roflumilast, a specific PDE4 inhibitor, may be useful for the control of a wide variety of inflammatory states and other pathologies related to the overproduction of NO.

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