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Potentiation of tumor necrosis factor-α expression by YC-1 in alveolar macrophages through a cyclic GMP-independent pathway

Tsong-Long Hwang[a,*], Chin-Chung Wu[b], Jih-Hwa Guh[c], Che-Ming Teng[d]

[a]*Graduate Institute of Natural Products, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan, ROC*
[b]*Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC*
[c]*School of Pharmacy, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC*
[d]*Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC*

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Abstract

Using cultured rat alveolar NR 8383 macrophages, this study investigated the effect of YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole], a soluble guanylyl cyclase (sGC) activator, on the production of tumor necrosis factor-α (TNFα). YC-1 enhanced lipopolysaccharide and interferon-γ (LPS/IFNγ)-induced TNFα formation in a concentration- and time-dependent fashion. YC-1 also caused an increasing effect on the TNFα mRNA level, suggesting that the transcriptional process was involved. However, further studies suggested that cyclic GMP did not mediate the potentiation of YC-1 on TNFα release, because (a) the sGC inhibitor and the protein kinase G inhibitor failed to block the effect; and (b) the cyclic GMP analogues, on the contrary, concentration-dependently diminished LPS/IFNγ-induced TNFα synthesis. In agreement with this finding, YC-1 produced changes in cell function but no changes in cyclic GMP and cyclic AMP levels or sGC activity. Pretreatment of the cells with cyclooxygenase inhibitors, a p38 mitogen-activated protein kinase inhibitor, a mitogen-activated protein kinase kinase (MEK) inhibitor, and a tyrosine kinase inhibitor did not attenuate the potentiation of TNFα release by YC-1. Cycloheximide prevented the YC-1-enhanced TNFα formation, implying that new protein synthesis was required. Interestingly, protein kinase C inhibitors enhanced the potentiation of YC-1 to a greater extent. Nevertheless, a protein kinase C activator, phorbol 12-myristate 13-acetate, failed to suppress the potentiation of TNFα production by YC-1. In summary, potentiation of TNFα release by YC-1 in LPS/IFNγ-activated alveolar macrophages is an additional mode of action of this compound that is independent of the elevation of cyclic GMP. Thus, caution needs to be used in attributing the YC-1-mediated response to the activation of sGC.

Keywords: Alveolar macrophage; Cyclic GMP; NR 8383; TNF; YC-1

1. Introduction

YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole], a chemically synthetic compound, has been identified as a direct activator of sGC. Initially, YC-1 was reported to inhibit platelet aggregation *in vitro* [1,2] and to attenuate platelet-rich thrombosis *in vivo* [3]. These effects were caused by an elevation of cyclic GMP levels in platelets. Cyclic GMP-increasing effects were also seen in vascular smooth muscle cells, and YC-1 caused relaxation of the endothelial-free aortic ring [4,5] and inhibition of neointima formation [6]. Subsequent studies with purified sGC demonstrated that YC-1 not only stimulates sGC directly, but also increases the responsiveness of this enzyme towards NO and CO [7–11]. In accordance with the results obtained with purified sGC, this synergistic response of YC-1 was shown on smooth muscle relaxation [4,12,13] and platelet aggregation [14]. Hence, YC-1 has been regarded as a useful tool to investigate the sGC/cyclic GMP pathway in various biological processes.

* Corresponding author. Tel.: +886-3-3274106; fax: +886-3-3274106.
E-mail address: htl@mail.cgu.edu.tw (T.-L. Hwang).

Abbreviations: cyclic AMP, cyclic adenosine 3′,5′-monophosphate; cyclic GMP, cyclic guanosine 3′,5′-monophosphate; COX, cyclooxygenase; ERK, extracellular signal-regulated protein kinase; IBMX, 3-isobutyl-1-methylxanthine; IFNγ, interferon-γ; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; NO, nitric oxide; PKC, protein kinase C; PKG, protein kinase G; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; TNFα, tumor necrosis factor-α.

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sGC catalyzes the conversion of GTP to cyclic GMP, a second messenger that modulates a variety of physiological functions, such as smooth muscle relaxation, platelet aggregation, and neurotransmitter release [15,16]. Recently, Ke *et al*. [17] showed that NO regulates actin reorganization in macrophages through the elevation of cyclic GMP. Cyclic GMP has also been reported to regulate TNFα synthesis in macrophages [18–20]. Alveolar macrophages play a significant role in the pathogenesis of several lung diseases including asthma and acute inflammation due to inhaled bacterial particles and dust. TNFα, an early cytokine produced by activated macrophages, plays an essential role in normal and pathological inflammatory reactions [21]. However, thus far, none of the studies has demonstrated the underlying mechanism of the actions of YC-1 on TNFα production in alveolar macrophages. Therefore, our experiments were performed to address the question of whether YC-1 could affect the production of TNFα in a rat alveolar macrophage cell line (NR 8383), and to elucidate the role of cyclic GMP in this function. However, the present data provided evidence that the potentiation of TNFα production by YC-1 was mediated by a cyclic GMP-independent pathway. Therefore, the cyclic GMP-independent action caused by YC-1 may mislead the interpretation of results drawn from studies of signal transduction, which use YC-1 specifically as an activator of sGC.

2. Materials and methods

2.1. Materials

YC-1 was chemically synthesized as described previously [22] and was dissolved in DMSO for a stock solution. Ham's F-12K medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and IFNγ (rat recombinant) were purchased from Gibco BRL. Ro 31-8220 [3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]]-3-(1-methyl-1*H*-indol-3-yl)maleimide; bisindolylmaleimide 1X, methanesulfonate], Go 6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-a)pyrrolo-(3,4-c)-carbazole], NS 398 [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide], PD 098059 (2′-amino-3′-methoxyflavone), and KT 5823 [(8*R**,9*S**,11*S**)-(−)-2-methyl-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo(a,g)cyclocta(cde)trinen-1-one] were obtained from Calbiochem. Cyclic AMP and cyclic GMP enzyme immunoassay kits were purchased from Amersham Pharmacia Biotech. TNFα ELISA kits and Quantikine rat TNFα mRNA kits were obtained from R&D. All other chemicals were purchased from Sigma. When drugs were dissolved in DMSO, the final concentration of DMSO in the culture medium did not exceed 0.2% (v/v) and did not affect the parameters measured.

2.2. Cell culture

The line of rat pulmonary alveolar macrophages (NR 8383) used throughout this study was obtained from the American Type Culture Collection (CRL-2192). NR 8383 cells were maintained in Ham's F-12K medium supplemented with 15% heat-inactivated FBS and antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin, 2.5 μg/mL of amphotericin B). The cells were grown in a humidified incubator at 37° and 5% CO_2. For further experiments, NR 8383 cells were seeded at a density of 5×10^5/mL in 24-well Costar plates and cultured in DMEM containing 2% heat-inactivated FBS.

2.3. TNFα production

TNFα production was induced by the addition of 100 ng/mL of LPS (phenol extracted *Escherichia coli* serotype 0128: B12; Sigma) and 10 U/mL of IFNγ. To assess the effects of several agents, drugs (such as YC-1, 8-bromo-cyclic GMP, dibutyryl-cyclic GMP, 8-bromo-cyclic AMP, dibutyryl-cyclic AMP, and forskolin) were added to the cells 15 min before the addition of LPS/IFNγ. At the designated time points, cell-free supernatants were harvested and kept frozen (−20°) until TNFα analysis. The amounts of TNFα were measured by using a commercially available ELISA kit. The assay was performed according to the instructions of the manufacturer.

In a separate series of experiments, we used several pharmacological inhibitors to examine the mechanism responsible for potentiation of TNFα production by YC-1. The cells were pretreated with inhibitors, such as ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; an sGC inhibitor), KT 5823 (a protein kinase G (PKG) inhibitor), PD 098059 (an MAP/ERK kinase (MEK) inhibitor), SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; a p38 mitogen-activated protein kinase (MAPK) inhibitor], genistein (a tyrosine kinase inhibitor), indomethacin (a non-selective cyclooxygenase (COX) inhibitor), NS 398 (a selective COX-2 inhibitor), Ro 31-8220 (a non-selective protein kinase C (PKC) inhibitor), and Go 6976 (a typical PKC inhibitor) for 30 min before YC-1 was added.

2.4. TNFα mRNA level

To investigate the process of YC-1-mediated TNFα production, TNFα mRNA was assayed. The mRNA level was estimated by using a colorimetric rat mRNA quantitation kit (R&D). Briefly, cells (5×10^5/500 μL in 24-well Costar plates) were stimulated with or without LPS/IFNγ for 3 hr in the presence or absence of YC-1. After centrifugation at 1200 *g* for 25 min at 4°, the pellets were suspended in 200 μL of lysis solution, and cell lysate samples were used to determine the concentration of mRNA. The assay was performed according to the instructions of the manufacturer.

2.5. Formation of cyclic nucleotides

The cyclic GMP and cyclic AMP contents were assayed using enzyme immunoassay kits. Cells were treated with LPS/IFNγ with or without pretreatment with YC-1 for 15 min, and the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 μM) was added 15 min before harvesting. Cyclic nucleotides were extracted at the indicated times by incubation of the cells with 0.5% dodecyltrimethylammonium bromide for 10 min at room temperature. The assay was performed according to the instructions of the manufacturer.

2.6. sGC activity

The NR 8383 cells were sonicated in ice-cold buffer containing 25 mM Tris–HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, 5 mM $MgCl_2$, 10 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride, and 10 μM pepstatin. Membrane fraction was removed by centrifugation at 100,000 *g* for 40 min at 4°. The supernatant was used as a source for the sGC sample. To assay the sGC activity, the reaction mixture contained 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM IBMX, 7.5 mM creatine phosphate, 3 U creatine phosphokinase, 1 mM GTP, and the sGC sample in a final volume of 0.2 mL. The reaction was carried out for 10 min at 37° and terminated by adding 0.8 mL of ice-cold 50 mM acetate buffer, pH 4.0, and boiling for 3 min [23]. The cyclic GMP contents were assayed using enzyme immunoassay kits.

2.7. Lactate dehydrogenase (LDH) release

The cytotoxic effects of the experimental conditions were determined by measuring LDH release into the cell culture medium using a commercially available method (Sigma). Cytotoxicity was expressed as percent LDH activity obtained from the cell-free medium compared to total LDH activity. Total LDH activity was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37°.

2.8. Statistics

Results are expressed as means ± SEM, and comparisons were made using Student's *t*-test. A probability of 0.05 or less was considered significant.

3. Results

3.1. Effect of YC-1 on TNFα production

NR 8383 cells were stimulated with LPS/IFNγ (100 ng/mL and 10 U/mL, respectively) to evoke TNFα production. Typically, a synergistic combination of stimuli is required for maximal induction of TNFα production in macrophages. Herein, LPS/IFNγ stimulation was used as a standard physiological activation protocol for TNFα formation. Activation of NR 8383 alveolar macrophages with LPS/IFNγ resulted in a time-dependent increase of TNFα formation, and the submaximal response at 8 hr (12.18 ± 1.07 ng/mL, N = 6) was conducted in this study (Fig. 1). YC-1 at concentrations of 10 and 30 μM, which caused only a minor effect by themselves, markedly enhanced TNFα production in a concentration- and time-dependent manner (Fig. 1).

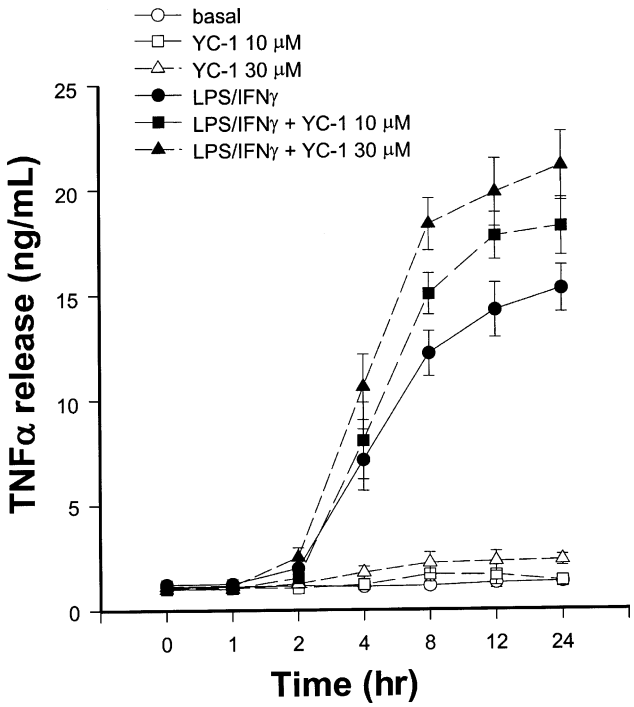

Fig. 1. Concentration- and time-dependent effects of YC-1 on TNFα production. Cells were pretreated with the indicated concentrations of YC-1 in the absence or presence of LPS/IFNγ. At the times indicated, culture supernatants were obtained and analyzed for TNFα formation, as described under "Section 2." Data represent the means ± SEM of four to seven experiments.

3.2. Role of cyclic nucleotide-elevating agents in the regulation of TNFα production

As shown in Fig. 2, pretreatment of cells with YC-1 (1–30 μM) resulted in a concentration-dependent increase of LPS/IFNγ-induced TNFα production up to 150% (N = 8; $P < 0.001$). The increase in TNFα formation induced by YC-1 was not reversed by ODQ (10 μM), a selective inhibitor of sGC, or KT 5823 (0.1 μM), a selective inhibitor of PKG (Fig. 3). Pretreatment of the cells with the cell-permeable analogues of cyclic GMP [8-bromo-cyclic GMP and dibutyryl-cyclic GMP (0.1–1000 μM)], the cell-permeable analogues of cyclic AMP [8-bromo-cyclic AMP (0.1–1000 μM) and dibutyryl-cyclic AMP (0.1–300 μM)], and an adenylyl cyclase activator [forskolin (0.01–10 μM)] concentration-dependently decreased LPS/IFNγ-induced TNFα production (Fig. 2). Cell viability was not affected

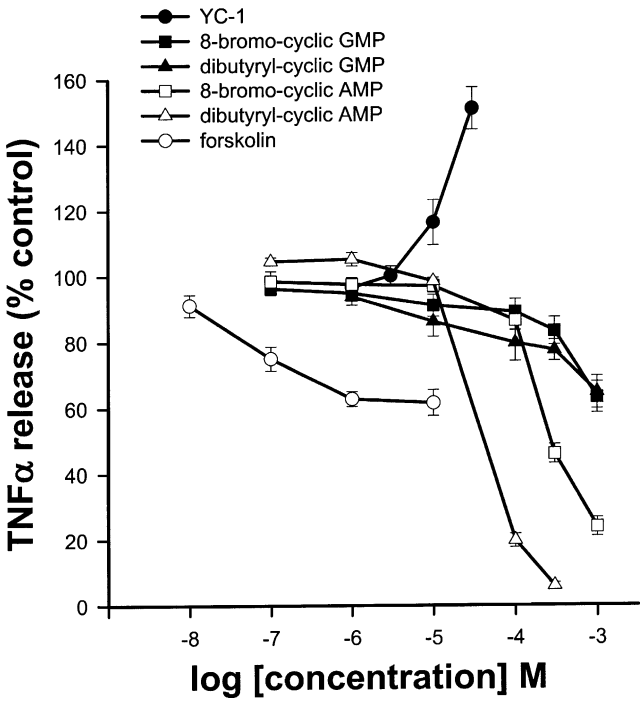

Fig. 2. Concentration-dependent effects of cyclic nucleotide-elevating agents on TNFα production. Cells were activated with LPS/IFNγ and pretreated with the indicated agents. After 8 hr, culture supernatants were obtained and analyzed for TNFα formation, as described under "Section 2." One hundred percent TNFα formation was 12.12 ± 0.49 ng/mL. Data represent the means ± SEM of six to eight experiments.

significantly when cultured with these agents, as assayed by LDH release. The maximal inhibition seen with the cyclic GMP analogues was relatively weaker than that produced by the cyclic AMP analogues. The rank order of potencies of these compounds in decreasing TNFα accumulation was dibutyryl-cyclic AMP > 8-bromo-cyclic AMP > dibutyryl-cyclic GMP ≥ 8-bromo-cyclic GMP. Moreover, sodium nitroprusside (SNP) (300 μM; an NO donor) and zaprinast (10 μM; a phosphodiesterase V inhibitor) did not alter LPS/IFNγ-induced TNFα production (data not shown). These observations suggested that the up-regulation of TNFα formation by YC-1 was not associated with cyclic GMP or cyclic AMP.

3.3. Effect of signaling transduction inhibitors on YC-1-induced TNFα production

To assess the signaling pathway of YC-1, we used several pharmacological inhibitors to examine the mechanism responsible for the potentiation of TNFα production by YC-1. Cycloheximide (1 μM) prevented the YC-1-enhanced TNFα release (data not shown), implying that new protein synthesis was required. Prostaglandin E_2 (PGE_2), a product of arachidonic acid metabolism *via* COX, is produced by macrophages in response to LPS stimulation and feeds back in an autocrine manner to inhibit TNFα production [24]. However, our data revealed that the non-selective COX inhibitor indomethacin (1 μM)

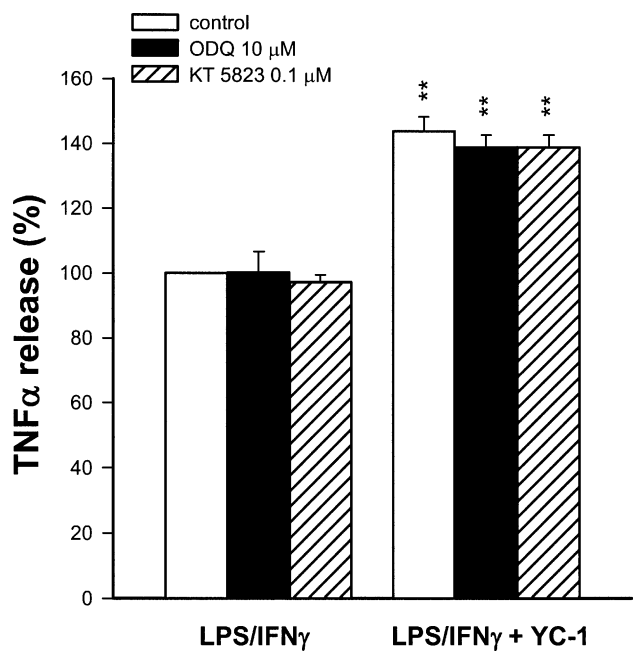

Fig. 3. Effect of sGC and PKG inhibitors on YC-1-potentiated TNFα production from activated macrophages. Cells were pretreated with ODQ (10 μM) or KT 5823 (0.1 μM) in the absence or presence of YC-1. After 8 hr, culture supernatants were collected, and TNFα release was determined by ELISA. One hundred percent TNFα formation was 12.44 ± 0.84 ng/mL. Data represent the means ± SEM of four experiments. Key: (**) $P < 0.001$ with respect to LPS/IFNγ.

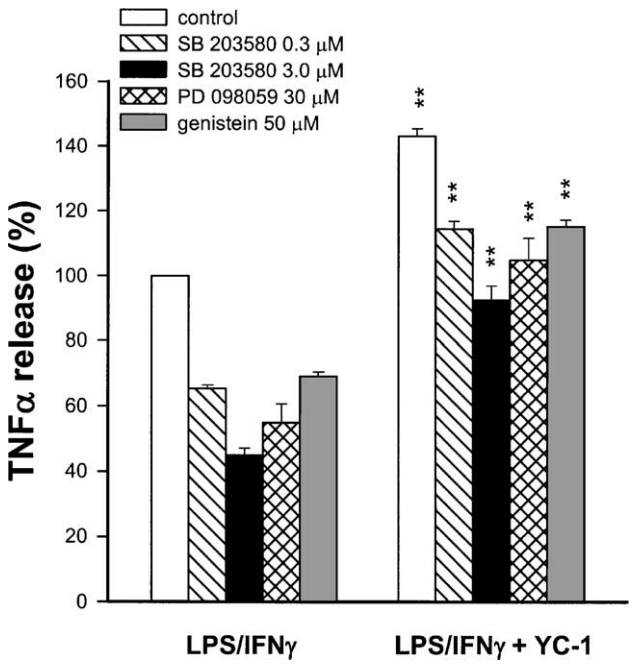

Fig. 4. Effect of MAPK and tyrosine kinase inhibitors on YC-1-potentiated TNFα production from activated macrophages. Cells were pretreated with SB 203580, PD 098059, or genistein in the absence or presence of YC-1 (30 μM). After 8 hr, culture supernatants were collected, and TNFα release was determined by ELISA. One hundred percent TNFα formation was 13.10 ± 0.57 ng/mL. Data represent the means ± SEM of eight experiments. Key: (**) $P < 0.001$ with respect to LPS/IFNγ.

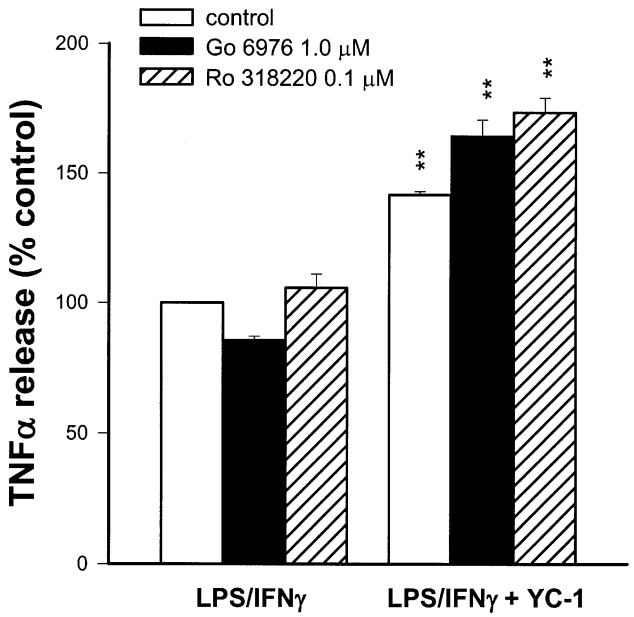

Fig. 5. Effect of PKC inhibitors on YC-1-potentiated TNFα production from activated macrophages. Cells were pretreated with Go 6976 (1.0 μM) or Ro 31-8220 (0.1 μM) in the absence or presence of YC-1 (30 μM). After 8 hr, culture supernatants were collected, and TNFα release was determined by ELISA. One hundred percent TNFα formation was 12.55 ± 0.72 ng/mL. Data represent the means ± SEM of six experiments. Key: (**) $P < 0.001$ with respect to LPS/IFNγ.

and the selective COX-2 inhibitor NS 398 (10 nM) did not alter either the LPS/IFNγ-induced TNFα production or the YC-1 potentiation (data not shown). Furthermore, application of PD 098059 (30 μM) and SB 203580 (0.3 and 3.0 μM) to inhibit MEK and p38 MAPK and of genistein (50 μM) to inhibit tyrosine kinases dramatically attenuated

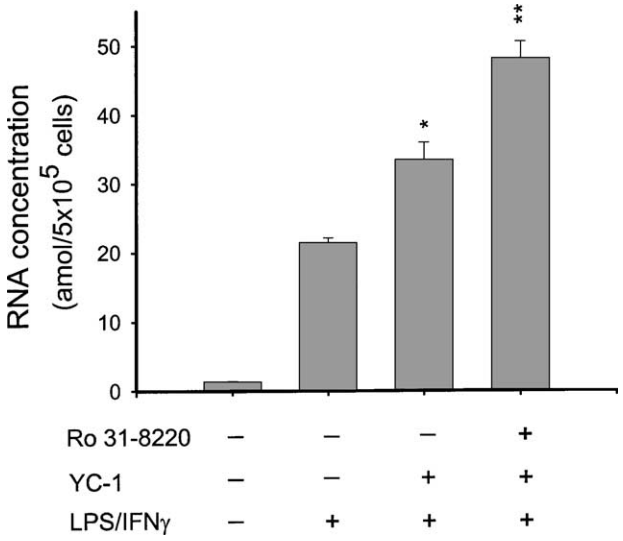

Fig. 6. Effect of YC-1 on TNFα mRNA level. Cells were stimulated with or without LPS/IFNγ for 3 hr in the presence or absence of YC-1 (30 μM), and then cell lysate samples were used to determine the concentration of TNFα mRNA as described under "Section 2." Ro 31-8220 (0.1 μM) was added before YC-1 for 30 min. Data represent the means ± SEM of four experiments. Key: (*) $P < 0.01$ and (**) $P < 0.001$ with respect to LPS/IFNγ alone.

the LPS/IFNγ-induced TNFα release, but did not alter the potentiating effect of YC-1 (Fig. 4). Interestingly, using two PKC inhibitors, Ro 31-8220 (0.1 μM) and Go 6976 (1.0 μM), which caused only a minor effect by themselves, enhanced the potentiation of YC-1 to a greater extent (Fig. 5). As shown in Fig. 5, 1.0 μM Go 6976 and 0.1 μM Ro 31-8220 enhanced the potentiation of YC-1 from 85.45 ± 1.56% to 164.21 ± 6.38% (N = 6; $P < 0.001$) and from 105.65 ± 5.43% to 173.45 ± 5.69% (N = 6; $P < 0.001$), respectively. The PKC activator phorbol 12-myristate 13-acetate (PMA; 0.1 μM) increased LPS/IFNγ-induced TNFα production (from 100 to 145.89 ± 3.93%, N = 4; $P < 0.001$). However, YC-1 still potentiated TNFα release in the pretreatment with PMA (from 147.83 ± 6.38 to 204.32 ± 10.31, N = 4; $P < 0.01$).

3.4. Increase of TNFα mRNA level by YC-1

Quantikine mRNA analysis was performed to determine whether YC-1 increases TNFα mRNA accumulation.

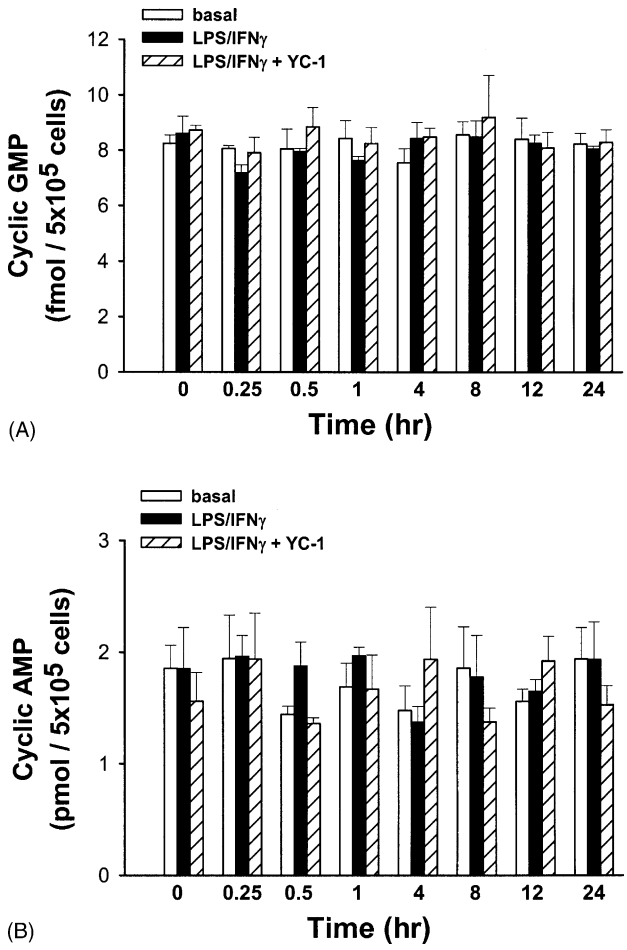

Fig. 7. Time-dependent effect of YC-1 on cyclic GMP and cyclic AMP concentrations. Cells were untreated (basal) or treated by LPS/IFNγ with or without preincubation with YC-1 (30 μM) for 15 min, and the non-selective phosphodiesterase inhibitor IBMX (100 μM) was added 15 min before harvesting. Cyclic GMP (A) and cyclic AMP (B) concentrations were determined by enzyme immunoassay kits at the indicated times as described under "Section 2." Data represent the means ± SEM of three experiments.

YC-1 (30 μM) caused a significant up-regulation of the LPS-induced TNFα mRNA level (Fig. 6). Ro 31-8220 (0.1 μM), which elicited a minor increase by itself, also potentiated the effect of YC-1 (Fig. 6).

3.5. Effects of YC-1 on cyclic GMP and cyclic AMP formation and the activity of sGC

The effects of YC-1 on cyclic GMP and cyclic AMP contents in LPS/IFNγ-activated macrophages and the sGC activity in macrophage cytosolic fractions were assayed using enzyme immunoassay kits. At the times indicated up to 24 hr, neither LPS/IFNγ nor LPS/IFNγ plus YC-1 treatment altered the levels of cyclic GMP or cyclic AMP when compared with the vehicle control (N = 3) (Fig. 7). YC-1 (up to 100 μM) and SNP (100 μM) failed to alter sGC activity (from 0.38 ± 0.11 pmol to 0.30 ± 0.46 pmol and 0.36 ± 0.24 pmol cyclic GMP/10 min per 2×10^6 cells, respectively, N = 4; both at $P > 0.05$). In contrast, YC-1 (10, 30, and 100 μM) activated sGC activity of human neutrophil cytosolic fractions in a concentration-dependent manner (data not shown), which is in agreement with the findings of Wang *et al*. [23].

4. Discussion

Besides NO and CO themselves, YC-1 represents the first activating pharmacopoeia of intracellular sGC in a biological milieu. YC-1 is a potent and direct activator of sGC [1,7] and mimics many of the known functions of NO, such as inhibition of platelet aggregation [2] and adhesion [25], as well as decrease of vascular smooth muscle proliferation [26] and contraction [4]. However, YC-1 seems to possess some additional effects that do not involve the activation of sGC. Indeed, it has been reported that YC-1 inhibits cyclic GMP-hydrolyzing phosphodiesterases in platelets [8,12] and smooth muscle cells [27]. In addition, YC-1 may cause an increase of the inotropic effect in ventricular myocardium [28], the blockage of calcium-activated and voltage-dependent potassium channels in pituitary GH_3 lactotrophs [29], and the stimulation of NO synthesis and release in endothelial cells [30], all of which are independent of the activation of sGC/cyclic GMP pathways. Recently, Wang *et al*. [31] demonstrated that inhibition of fMLP-stimulated intracellular calcium changes by YC-1 is mainly *via* the blockade of calcium entry through the inhibition of tyrosine kinase activity, but not *via* the sGC/cyclic GMP-related mechanism. Unfortunately, these additional actions of this compound still do not have a reliable explanation.

The present study demonstrates a potentiation by YC-1 on LPS/IFNγ-induced *TNFα* gene expression and TNFα production in NR 8383 macrophages. On the other hand, the cell-permeable cyclic GMP analogues 8-bromo-cyclic GMP and dibutyryl-cyclic GMP concentration-dependently decreased TNFα production in activated macrophages. In accordance with our findings, another paper reported an inhibition of TNFα production by cyclic GMP in murine bone marrow-derived macrophages [32]. However, cyclic GMP was also reported to stimulate TNFα synthesis in rat resident peritoneal macrophages [18] and in human pulmonary macrophages [19]. These data, together with our results, lead to the suggestion that the effect of cyclic GMP on TNFα production is highly dependent upon species and cell type. Clearly, additional work is required to define the action of cyclic GMP that diminishes TNFα secretion in NR 8383 cells. Our data provided a further two lines of evidence to suggest that cyclic GMP did not mediate the increasing effect of YC-1 on TNFα release. First, both the sGC inhibitor ODQ and the PKG inhibitor KT 5823 failed to block YC-1 potentiation. Second, YC-1 produced changes in cell function but not in the cyclic GMP level and sGC activity. Since large amounts of NO were released in activated macrophages, we hypothesized that YC-1 should markedly elevate cyclic GMP content through a synergistic response with NO. Our results demonstrated, however, that contrary to our hypothesis, YC-1 failed to alter the level of cyclic GMP. In view of the fact that YC-1 and SNP failed to affect the cytosolic sGC activity in NR 8383 cells, our data implied that the activity of sGC was minor in these cells. A similar result has been described by Kiemer and Vollmar [32], who indicated that no sGC has been detected in mouse macrophages. Obviously, additional work is required to define the action of YC-1 in cells bearing NO-sensitive sGC.

Next, because YC-1 inhibited cyclic AMP-hydrolysis phosphodiesterases [27], the action of cyclic AMP was also addressed. Our data indicated that the effect of YC-1 was also cyclic AMP-independent. Explanations for this result include the following. First, the cell-permeable cyclic AMP analogues 8-bromo-cyclic AMP and dibutyryl-cyclic AMP markedly decreased TNFα production in a concentration-dependent fashion. Second, the adenylyl cyclase activator forskolin also concentration-dependently reduced TNFα release. Third, YC-1 treatment did not change the levels of cyclic AMP.

The present study indicates that the effect of YC-1 appears to be more complex than originally described. To date, although more than one protein kinase has been proven to be responsible for the enhancement of TNFα production, the signaling mechanisms responsible for TNFα release are still not completely clear. Pretreatment with cycloheximide prevented the YC-1-enhanced TNFα release, implying that new protein synthesis was required. Treatment with PD 098059 and SB 203580, inhibitors of MEK and p38 MAPK, and with genistein, an inhibitor of tyrosine kinase, significantly decreased LPS/IFNγ-induced TNFα release, but did not affect the potentiation of YC-1. These data suggested that MEK, p38 MAPK, and tyrosine kinase are functionally important for the regulation of TNFα release in NR 8383 cells, but not requisite actions in the

regulation of TNFα production by YC-1. A previous report indicated that PGE_2 feeds back in an autocrine manner to inhibit TNFα production in LPS-stimulated rabbit alveolar macrophages [24]. However, our data revealed that indomethacin, a non-selective COX inhibitor and NS 398, a selective COX-2 inhibitor, do not alter either LPS/IFNγ-induced TNFα production or YC-1 potentiation. The controversial results may be due to the different cells used. PKC is a family of closely related serine/threonine kinases that appear to mediate various cellular functions [33]. In the present study, we showed that the LPS/IFNγ-induced TNFα production was not affected significantly by the PKC inhibitors Ro 31-8220 and Go 6976, indicating that PKC activation was not an obligatory event in the regulation of TNFα production by LPS/IFNγ. Interestingly, Ro 31-8220 and Go 6976 enhanced the potentiating effect of YC-1 to a greater extent. However, several reports have shown that Ro 31-8220 is not selective for PKC isoforms [34–36]. The regulatory role of PKC on the effect of YC-1 was examined by pretreatment with the PKC activator PMA. Although pretreatment of cells with PMA increased TNFα release, no effect was observed on the YC-1-enhanced TNFα production. Clearly, the underlying mechanisms responsible for this discrepancy will need to be investigated.

In summary, this is the first report demonstrating that YC-1 can increase *TNFα* gene expression and TNFα production in LPS/IFNγ-activated alveolar macrophages. The potentiation of TNFα release by YC-1 is an additional mode of action of this compound that is independent of the elevation of cyclic GMP. Therefore, investigators using YC-1 to examine the biological function of sGC may take the risk of obtaining results of a cyclic GMP-independent mechanism.

Acknowledgments

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