



A carbon monoxide-releasing molecule (CORM-3) attenuates lipopolysaccharide- and interferon- γ -induced inflammation in microglia

Mohamed G. Bani-Hani^{1,2}, David Greenstein², Brian E. Mann³,
Colin J. Green¹, Roberto Motterlini¹

¹Vascular Biology Unit, Department of Surgical Research, Northwick Park Institute for Medical Research, Harrow, United Kingdom

²Department of Vascular Surgery, North West London Hospitals NHS Trust, Northwick Park Hospital, Harrow, United Kingdom

³Department of Chemistry, University of Sheffield, Sheffield, United Kingdom

Correspondence: Roberto Motterlini, e-mail: r.motterlini@imperial.ac.uk

Abstract:

The development of carbon monoxide-releasing molecules (CO-RMs) in recent years helped to shed more light on the diverse range of anti-inflammatory and cytoprotective activities of CO gas. In this study, we examined the effect of a ruthenium-based water-soluble CO carrier (CORM-3) on lipopolysaccharide (LPS)- and interferon- γ (INF- γ)-induced inflammatory responses in BV-2 microglial cells and explored the possible mechanisms of action. BV-2 microglial cells were stimulated with either LPS or INF- γ in the presence of CORM-3 and the inflammatory response evaluated by assessing the effect on nitric oxide production (nitrite levels) and tumor necrosis factor- α (TNF- α) release. Similar experiments were also performed in the presence of inhibitors of guanylate cyclase (ODQ), NO synthase (L-NAME), heme oxygenase activity (tin protoporphyrin IX) or various mitogen-activated protein kinase (MAPK) inhibitors. CORM-3 significantly attenuated the inflammatory response to LPS and INF- γ as evidenced by a significant reduction ($p < 0.001$) in nitrite levels and TNF- α production ($P < 0.05$). Such effect was maintained in the presence of ODQ, L-NAME or tin protoporphyrin without showing any cytotoxicity. The use of an inactive form of CORM-3 that does not contain carbonyl groups (Ru(DMSO)₄Cl₂) failed to inhibit the increase in inflammatory markers suggesting that liberated CO mediates the observed effects. In addition, inhibition of phosphatidylinositol-3-phosphate kinase (PI3K) and extracellular signal-regulated kinase (ERK) pathways seemed to amplify the anti-inflammatory effect of CORM-3, particularly in cells stimulated with INF- γ . These results suggest that the anti-inflammatory action of CORM-3 could be exploited to mitigate microglia activation in neuro-inflammatory diseases.

Key words:

carbon monoxide-releasing molecules (CO-RMs), BV-2 microglia, LPS, interferon- γ , inflammation

Abbreviations: CO – carbon monoxide, CO-RMs – carbon monoxide-releasing molecules, HO-1 – heme oxygenase-1, iNOS – inducible nitric oxide synthase, L-NAME – L-nitroarginine methyl ester, LPS – lipopolysaccharide, LY294002 – 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride, ODQ – 1*H*-[1,2,4] Oxadiazolo[4,3-*a*]quinoxalin-1-one, PD98059 – 2'-amino-3'-methoxyflavone, SB203580 – 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole, SNPPIX – tin protoporphyrin IX, SP600125 – 1,9-Pyrazoloanthrone

Introduction

Carbon monoxide gas (CO) is now well recognized as a key modulator of the cellular response to inflammation and oxidative stress [6, 25, 38]. Under such pathological conditions, CO production is increased endogenously in conjunction with biliverdin and bilirubin following up-regulation of heme oxygenase-1 (HO-1), a stress inducible enzyme responsible for the degradation of heme [17]. The induction of the HO-1 system is regarded as a valuable intracellular defense against the generation of reactive oxygen species and the ensuing oxidant-mediated injury [15, 33]. Such protective effect appears to be ubiquitous in nature as it has been demonstrated in a variety of experimental models of diseases related to the cardiovascular and immune systems [46] as well as in several cell types including endothelial and cardiac cells [10, 14, 32], hepatocytes [54], and macrophages [51]. The expression of HO-1 has been also described in microglia, astrocytes and neurons following various pathological models of brain injury and neurodegeneration [5, 20, 39, 55]. As it is becoming increasingly evident that oxidative stress mediated by reactive oxygen and nitrogen species plays an important role in the pathogenesis of various kinds of inflammatory disorders [41, 43], it is then not surprising that the HO-1/CO pathway is emerging as an essential target for the resolution of inflammation [38]. Interestingly, CO gas has been shown to reduce the inflammatory response in a similar fashion to compounds that are known to possess anti-inflammatory properties and at the same time are potent HO-1 inducers [1, 48, 49]. Thus, an important functional link exists between up-regulation of HO-1 under stress conditions and the biological role of its products in counteracting cellular dysfunction.

In recent years, the intense investigation on the potential use of CO gas as a therapeutic agent lead to the

development of carbon monoxide-releasing molecules (CO-RMs), a novel class of compounds that have the ability to carry and release CO in physiological solutions [31]. To date, ruthenium-based transition metal carbonyls (CORM-2 and CORM-3) have been the most extensively tested CO carriers in biological systems [35] but other classes of chemicals are emerging as promising CO-releasing compounds [12, 36, 49]. Both CORM-2 and the water-soluble CORM-3 have been shown to exert a variety of physiological actions that are reminiscent of the effects of CO gas. These include vasorelaxation [16, 31], protection against ischemia-reperfusion injury [11, 47] and inhibition of platelet aggregation [9]. More recently, we have shown that CORM-3 is effective in reducing TNF- α and nitric oxide (NO) production in macrophages stimulated with lipopolysaccharide [48]. Similarly, we reported that CORM-3 markedly attenuated the inflammatory response to thrombin and interferon- γ (IFN- γ) in BV-2 microglia cells exposed to both normoxic and hypoxic conditions [4].

Microglia are the principal immune cells in the brain. They seem to play a dual role by amplifying the effects of inflammation and mediating cellular degeneration as well as protecting the nervous tissue [42]. Microglia cells activated by inflammation are seen in numerous pathologies that characterize the central nervous system; they can kill nearby neurons by releasing substantial amounts of cytotoxic mediators [29] such as NO, superoxide and peroxynitrite anions. They can also mediate the susceptibility of neurons to the toxic effects of glutamate [44]. In this study we examined the effect of the water soluble, ruthenium-based CORM-3 on LPS and IFN- γ induced neuroinflammation in BV-2 microglia to establish a potential biological role of CO in neuro-inflammation.

Materials and Methods

Cell culture

The BV-2 microglia cell line was a kind gift from Professor Rosario Donato (University of Perugia). The cell line was obtained and characterized as previously described [2]. Cells were cultured in 75 cm² flasks containing RPMI medium supplemented with 10%

FBS, 4 mM glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin and grown in a 5% CO₂ atmosphere at 37°C.

Reagents

Tricarbonylchloro(glycinato)ruthenium (CORM-3) was synthesized as previously described [11]. CORM-3 was freshly prepared as a 10 mM stock solution by dissolving the compound in pure distilled water. Since CORM-3 contains ruthenium as transition metal, a negative control (RuCl₂(DMSO)₄) soluble in water was used. Lipopolysaccharide (LPS), interferon-γ (INF-γ), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), L-nitroarginine methyl ester (L-NAME), tin protoporphyrin-IX (SnPPIX), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4pyridyl)-1H-imidazole (SB203580), 2'-amino-3'-methoxyflavone (PD98059), 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), 1,9-Pyrazoloanthrone (SP600125), and all other reagents were purchased from Sigma (USA) unless otherwise specified.

Experimental protocols

BV-2 microglia were treated with lipopolysaccharide (LPS) (1 µg/ml) or with interferon-γ (INF-γ) (10–15 ng/ml) in the presence or absence of either CORM-3 (75 µM), iCORM-3 (75 µM) or RuCl₂(DMSO)₄ (75 µM) for 24 h. To examine the potential anti-inflammatory action of CORM-3, NO generation (nitrite levels) and tumor necrosis factor-α (TNF-α) production were determined at the end of the incubation period. In a second set of experiments, which was designed to dissect the contribution of various enzymatic pathways, cells treated as above were challenged with specific inhibitors of soluble guanylate cyclase (ODQ, 10 µM), nitric oxide synthase (L-NAME, 50–100 µM) or heme oxygenase activity (SnPPIX, 10 µM). In a third set of experiments, which was designed to dissect the signal transduction mechanisms involved, cells were pre-incubated with various mitogen-activated protein kinases (MAPK) inhibitors for 1 h prior to the treatment with LPS or interferon-γ in the presence or absence of CORM-3, iCORM-3 or RuCl₂(DMSO)₄. Specifically, we used 10 µM SB203580 (P38 inhibitor), 25 µM LY294002 (PI3K inhibitor), 25 µM SP600125 (JNK inhibitor) and 25 µM PD98059 (ERK inhibitor). Cells were sub-

sequently incubated for 24 h and nitrite production and TNF-α levels were determined in the medium at the end of the incubation period. Cell viability (LDH release) was also assessed after the various treatments at the end of the experiments.

Determination of nitrite levels

Nitrite levels were determined using the Griess method as previously described by our group [4, 48]. Briefly, the medium from treated cells cultured in 24-well plates was removed and placed into a 96-well plate (50 µl per well). The Griess reagent was added to each well to begin the reaction, the plate was shaken for 10 min and the absorbance read at 550 nm on a Molecular Devices VERSAmax plate reader. The concentration of nitrite in each well was calculated from a standard curve generated with sodium nitrite (0–300 µM in cell culture medium).

Measurement of TNF-α production

The level of TNF-α present in each sample was determined using a commercially available kit from R&D Systems (Mouse TNF-α Quantikine immunoassay kit, Abingdon, UK) [4, 48]. The assay was performed according to the manufacturer's instructions. Briefly, cell culture supernatants were collected immediately after the treatment and spun at 13,000 × g for 2 min to remove any particulates. The medium was added to a 96-well plate precoated with affinity-purified polyclonal antibodies specific for the mouse TNF-α. An enzyme-linked polyclonal antibody specific for mouse TNF-α was added to the wells and left to react for 2 h followed by a final wash to remove any unbound antibody-enzyme reagent. The intensity of the color detected at 450 nm (correction wavelength 570 nm) was measured after addition of a substrate solution and was proportional to the amount of TNF-α produced.

Cell viability

Cell viability was determined using an assay for the release of lactate dehydrogenase (LDH) activity (Roche) using a cytotoxicity detection kit according to the manufacturer's instructions. Briefly, at the end of the incubation period, cell supernatants were collected and any cell residue was removed by centrifugation at 250 × g. The reaction mixture (which is

composed of the catalyst and the dye solution) was then added to the cell-free supernatant, incubated at room temperature for 15 min and the absorbance was measured at 490 and 690 nm. LDH activity was expressed as percentage of the maximal LDH release, which was obtained by lysis of the cells with Triton X-100 (1% in DMEM at 25°C).

Heme oxygenase activity assay

Microsomal fractions of BV-2 cells were frozen for 20 min then thawed for 5 min in a water bath and the process repeated three times. The protein concentration of each sample was then quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK). For the heme oxygenase activity measurement, a reaction mixture was added to a glass tube and consisted of: 300 μ l PBS (pH = 7.4) containing 2 mM MgCl₂; 3 mg rat liver cytosol fraction; 50 μ l glucose-6-phosphate (2 mM); 15 μ l glucose-6 phosphate dehydrogenase (0.2 U/l); 10 μ l liver microsomes (30 μ M in proteins); 25 μ l hemin (2 mM); 25 μ l NADPH (0.8 mM); 400 μ l sample. The samples derived from BV-2 cells scraped at the end of each treatment and the pellet resuspended in PBS (pH = 7.4) containing 2 mM MgCl₂. The tubes were vortexed for 2 min, sealed with Para-film (American National Cat, Greenwich, Connecticut, USA) and wrapped with aluminum foil to allow the reaction to take place in the dark. The tubes were left for 60 min in a water bath at 37°C and the reaction was terminated by adding 1 ml chloroform. The tubes were mixed vigorously for 3 min before and then were centrifuged at RCF 900 for 5 min. The resulting layers were then disturbed by vortex for 5 s and cells re-centrifuged again at RCF 1100 for 5 min. The extracted bilirubin (dissolved in the chloroform layer) was measured in the chloroform layer using a quartz cuvette (Sigma-Aldrich Chemicals, Poole, Dorset, UK) and the absorbance determined at 464 nm and 530 nm against a blank (chloroform) using a spectrophotometer (Uvikon 810p Spectrophotometer, Kontron Instruments Ltd, Watford, Hertfordshire, UK). Heme oxygenase activity was expressed as pmoles bilirubin/mg protein/h.

Statistical analysis

Differences among the groups were analyzed using one way ANOVA and Bonferroni test. Values were

expressed as mean \pm SEM and differences between groups were considered to be significant at $p < 0.05$.

Results

CORM-3 inhibits LPS- and interferon- γ -mediated increase in nitrite production in a dose-dependent manner

Similar to our previous findings observed with RAW macrophages [48], CORM-3 (10–75 μ M) markedly

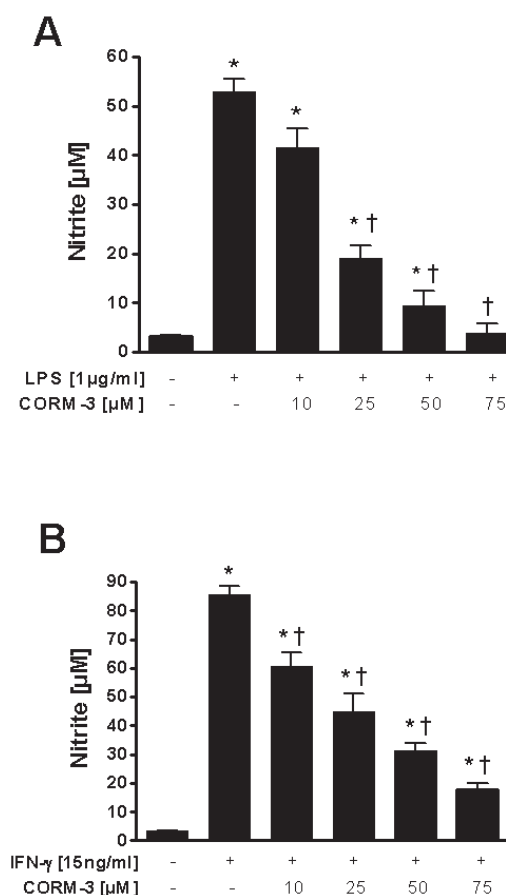


Fig. 1. CORM-3 attenuates nitrite production induced by lipopolysaccharide (LPS) or interferon- γ (INF- γ) in a dose-dependent manner in BV2 microglia cells. **(A)** Cells were stimulated with LPS (1 μ g/ml) in the presence of increasing concentrations of CORM-3 (10–75 μ M) for 24 h. At the end of the incubation period, nitrite levels were measured as an index of NO production using the Griess reagent assay as reported in Materials and Methods. **(B)** Cells were incubated with IFN- γ (15 ng/ml) in the presence of increasing concentrations of CORM-3 (10–75 μ M) for 24 h and nitrite levels determined at the end of the incubation period. Cells treated with medium alone represent the control group. Bars represent the mean \pm SEM, of 5–6 independent experiments. * $p < 0.05$ vs. control; † $p < 0.001$ vs. LPS or IFN- γ .

inhibited nitrite production in BV-2 microglia cells after exposure to LPS or INF- γ in a dose-dependent manner. A concentration of 75 μ M was sufficient to cause approximately a 90% inhibition in the increase in nitrite levels (Fig. 1 A, B). In addition, treatment with 75 μ M CORM-3 resulted in significant cytoprotection as evidenced by the reduction in the number of injured cells in the presence of either LPS or INF- γ (data not shown). Therefore, this concentration of CORM-3 was used in all subsequent experiments.

The inhibitory effect of CORM-3 on LPS-induced nitrite production in BV-2 microglia is not dependent on guanylate cyclase activation

Lipopolysaccharide (LPS) is the most common agent used to investigate the inflammatory responses in all types of tissues but it is known to elicit a massive inflammatory response particularly in microglia and macrophages. This inflammatory response is believed to be mediated by an increase in inducible nitric oxide

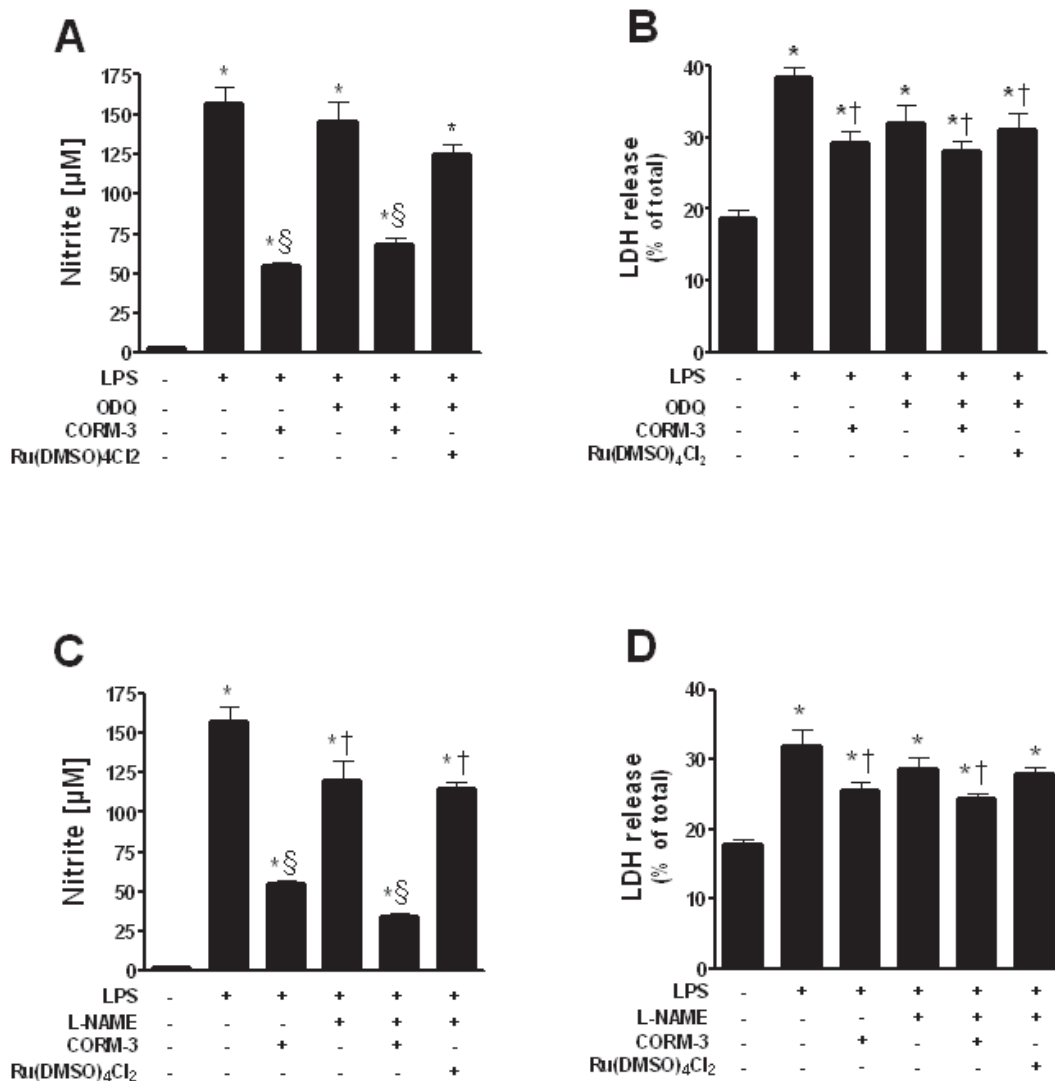


Fig. 2. The inhibitory effect of CORM-3 on LPS-induced nitrite production in BV2 cells is independent of soluble guanylate cyclase activation. (**A** and **B**) Cells were incubated with LPS (1 μ g/ml) in combination with ODQ (10 μ M) in the presence of 75 μ M of CORM-3 or Ru(DMSO) $_4$ Cl $_2$ (negative control) for 24 h. At the end of the incubation period, nitrite levels were measured as an index of NO production using the Griess reagent assay and cell viability determined by measuring LDH release as described in Materials and Methods. (**C** and **D**) Cells were incubated with LPS (1 μ g/ml) in combination with L-NAME (100 μ M) in the presence of 75 μ M of CORM-3 or Ru(DMSO) $_4$ Cl $_2$ for 24 h. Nitrite production and LDH release were determined at the end of the incubation period. Cells treated with medium alone represent the control group. Bars represent the mean \pm SEM of 6 independent experiments. * p < 0.05 vs. control; † p < 0.05 vs. LPS; § p < 0.01 vs. LPS

synthase (iNOS) expression resulting in the activation of signal transduction pathways through cGMP, which can be attenuated by the use of guanylate cyclase (ODQ) or NOS (L-NAME) inhibitors. In our experimental setting, we found that LPS markedly induced nitrite production to an extent that 10 μ M ODQ was only marginally cytoprotective with little effects on nitrite release (Fig. 2A, B, respectively). Under these conditions, 100 μ M L-NAME significantly reduced both nitrite release and cellular injury (Fig. 2C,

D). Interestingly, the effect of CORM-3 (75 μ M) was far more pronounced and markedly inhibited the increase in nitrite (Fig. 2 A, C) with an added cytoprotective effect evidenced by a significant reduction in LDH release (Fig. 2 B, D). An inactive form of CORM-3 that does not contain carbonyl groups (Ru(DMSO)₄Cl₂) was totally ineffective and did not show any change on nitrite levels and LDH release in the presence of ODQ or L-NAME suggesting that CO liberated from CORM-3 is responsible for the observed effects.

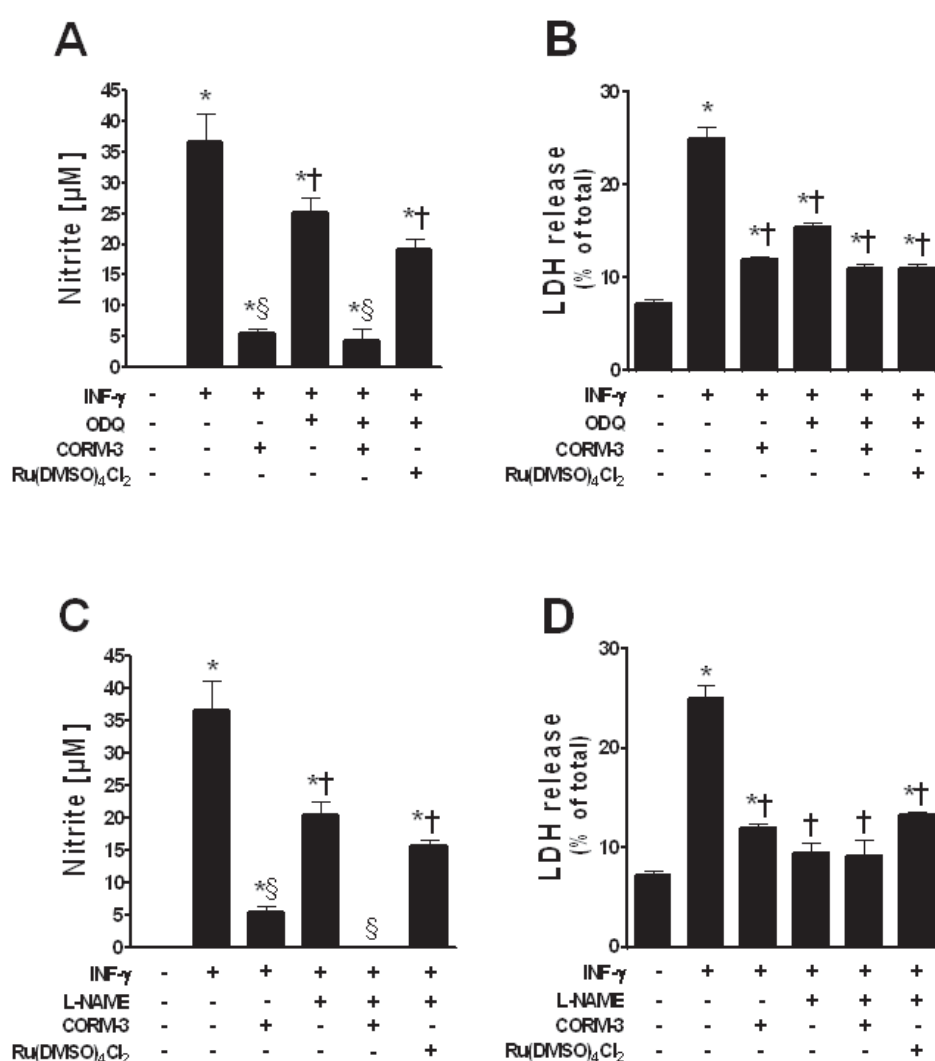


Fig. 3. The Inhibitory effect of CORM-3 on IFN- γ -induced nitrite production in BV2 cells is independent of guanylate cyclase activation and amplified by NOS inhibition. (**A** and **B**) Cells were incubated with IFN- γ (10 ng/ml) in combination with ODQ (10 μ M) in the presence of 75 μ M of CORM-3 or iCORM-3 (negative control) for 24 h. At the end of the incubation period, nitrite production was measured as an index of NO production using the Griess reagent assay and cell viability determined using the LDH assay as described in Methods. (**C** and **D**) Cells were incubated with IFN- γ (10 ng/ml) in combination with L-NAME (100 μ M) in the presence of 75 μ M of CORM-3 or iCORM-3 for 24 h. Nitrite production and LDH release were determined at the end of the incubation period as described in A and B. Cells treated with medium alone represent the control group. Bars represent the mean \pm SEM of 6 independent experiments per group. * $p < 0.05$ vs. control; † $p < 0.01$ vs. IFN- γ ; § $p < .001$ vs. IFN- γ

The inhibitory effect of CORM-3 on interferon- γ -induced nitrite production in BV-2 microglia is not affected by guanylate cyclase and is augmented by NOS inhibition

IFN- γ is a powerful microglia stimulant that has been used alone or in combination with other agents in models of neuro-inflammation [21, 23]. In our experimental setting, we found that IFN- γ markedly induced

nitrite production while ODQ and L-NAME significantly reduced this response in BV-2 microglia cells (Fig. 3A, C). However, CORM-3 (75 μ M) exerted a far more potent inhibition of nitrite release and maximized the total cytoprotective effect by significantly reducing cellular injury under these conditions (Fig. 3B, D). This effect appears to be due to CO as the inactive compound (Ru(DMSO)₄Cl₂) was totally ineffective.

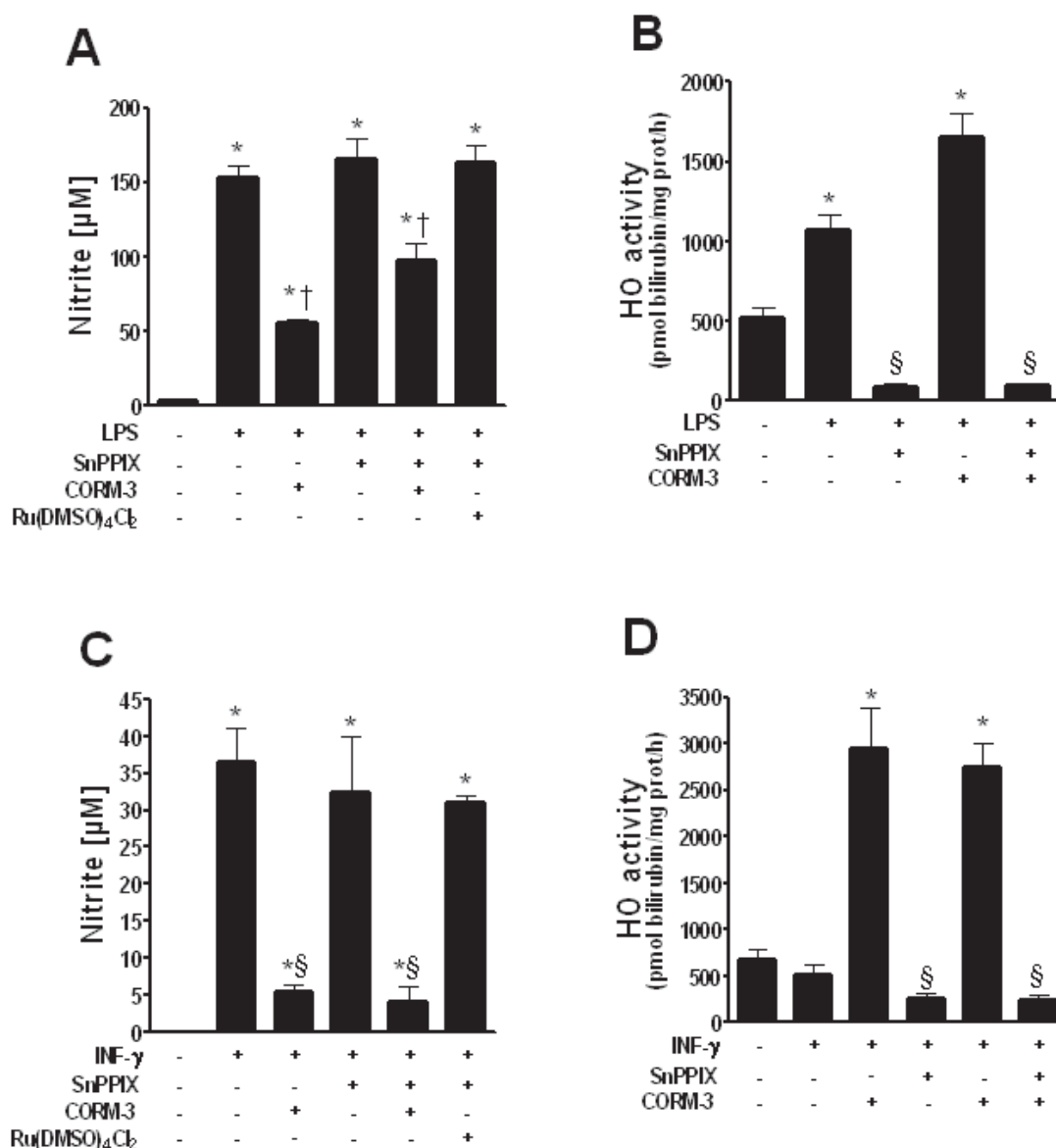


Fig. 4. The inhibitory effect of CORM-3 on LPS- or IFN- γ -induced nitrite production in BV2 cells is independent of endogenous heme oxygenase activity. (**A** and **C**) Cells were incubated with either LPS (1 μ g/ml) or IFN- γ (10 ng/ml) in combination with SNPPiX (10 μ M) in the presence of 75 μ M of CORM-3 or iCORM-3 (negative control) for 24 h. At the end of the incubation period, nitrite production was measured as an index of NO production using the Griess reagent assay. (**B** and **D**) Cells were incubated as in A and heme oxygenase activity determined at the end of 24 h incubation as described in Methods. Cells treated with medium alone represent the control group. Bars represent the mean \pm SEM. of 5 - 6 independent experiments per group. * p < 0.05 vs. control; † p < 0.05 vs. LPS or LPS plus CORM-3; § p < 0.001 vs. LPS or IFN- γ plus CORM-3

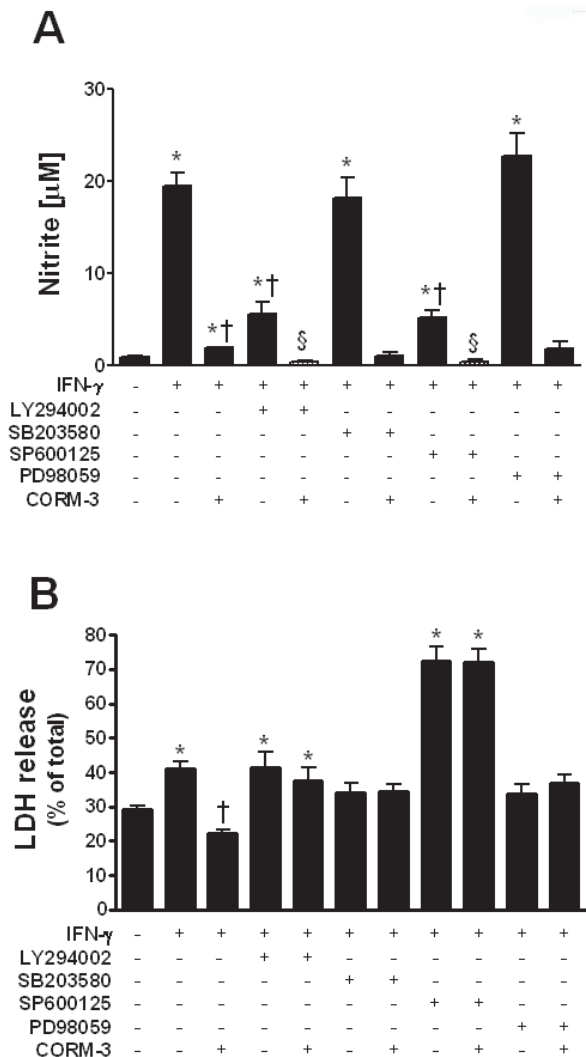


Fig. 5. CORM-3 amplifies the effects of MAPK inhibitors on IFN- γ -induced nitrite production. **(A)** Cells were treated with inhibitors of P38 (SB203580, 10 μ M), JNK (SP600125, 25 μ M), ERK (PD98059, 25 μ M) or PI3K (LY294002, 25 μ M) in serum-free medium for 1 h. Then cells were exposed for 24 h to complete medium (10% FBS) containing IFN- γ (10 ng/ml) and CORM-3 (75 μ M). At the end of the incubation period, nitrite production was measured as an index of NO production. **(B)** Cells were incubated as in A and cell viability determined after 24 h using the LDH release assay as described in Methods. Cells treated with medium alone represent the control group. Bars represent the mean \pm SEM of 5–6 independent experiments per group. * $p < 0.01$ vs control; † $p < 0.05$ vs. LPS; § $p < .001$ vs. LPS.

The anti-inflammatory effect of CORM-3 is independent of the endogenous activity of heme oxygenase

As previously demonstrated in RAW macrophages, CORM-3 has the ability to induce HO-1 activity by itself or in combination with other stimuli [48]. In BV-2

microglia cells we also found that CORM-3 significantly amplified heme oxygenase activity in the presence of either LPS (1 μ g/ml) or IFN- γ (10 ng/ml); these effects were totally abolished by tin protoporphyrin IX (SnPPIX, 10 μ M), an inhibitor of heme oxygenase activity (Fig. 4B, D). Interestingly, blockade of endogenous heme oxygenase activity increased the baseline production of nitrite in response to LPS (Fig. 4A). However, SnPPIX did not seem to affect

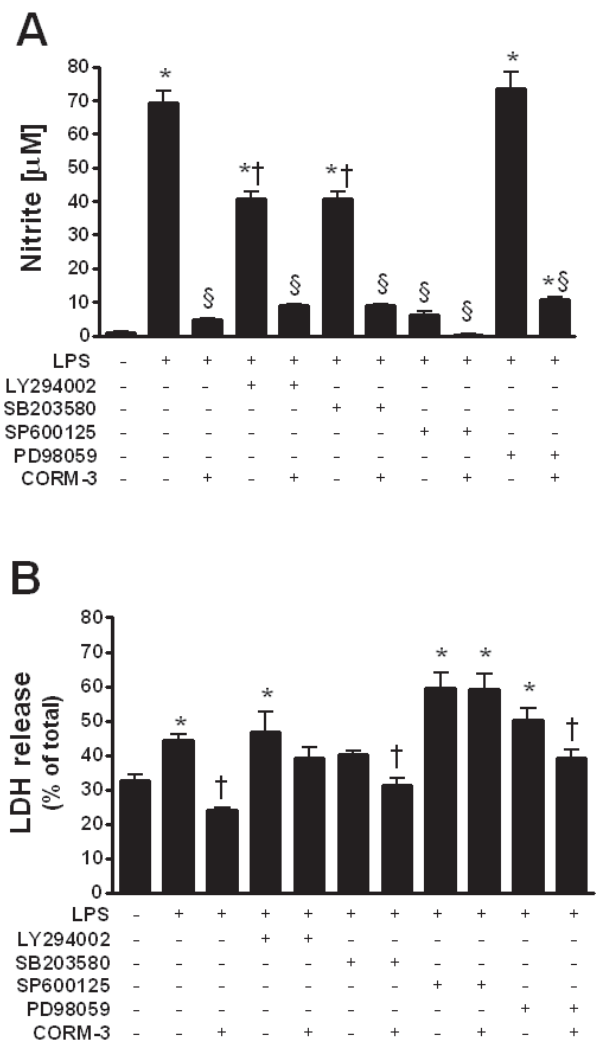


Fig. 6. CORM-3 augments the inhibitory effect of MAPK inhibitors on LPS-induced nitrite production. **(A)** Cells were treated with inhibitors of P38 (SB203580, 10 μ M), JNK (SP600125, 25 μ M), ERK (PD98059, 25 μ M) or PI3K (LY294002, 25 μ M) in serum-free medium for 1 h. Then cells were exposed for 24 h to complete medium (10% FBS) containing LPS (1 μ g/ml) and CORM-3 (75 μ M). At the end of the incubation period, nitrite production was measured as an index of NO production. **(B)** Cells were incubated as in A and cell viability determined after 24 h using the LDH release assay as described in Methods. Cells treated with medium alone represent the control group. Bars represent the mean \pm SEM of 5–6 independent experiments per group. * $p < 0.01$ vs. control; † $p < 0.01$ vs. LPS; § $p < 0.001$ vs. LPS.

the anti-inflammatory activity of CORM-3 and did not change the effect of the negative control (Ru(DMSO)₄Cl₂) further supporting the hypothesis that the liberated CO is responsible for the observed effects. Notably, there was a significant cytoprotective effect related to the use of SNPPIX which was not altered by the presence of CORM-3 or Ru(DMSO)₄Cl₂ (data not shown) and this can be attributed to a partial scavenging action of the protoporphyrin agent at the concentration used [18].

Blockade of the phosphatidylinositol-3-phosphate kinase (PI3K) pathway potentiates the inhibitory effect of CORM-3 on INF- γ -induced inflammation

As reported by previous reports, the PI3K pathway seems to play a more significant role than the p38 MAPK pathway in modulating INF- γ induced inflammation [23]. This was also observed in this study where a PI3K inhibitor (LY294002) potentiated the inhibitory effect of CORM-3 on nitrite production. In contrast, both P38 and ERK inhibitors (SB203580 and PD98059, respectively) did not affect NO production mediated by INF- γ (Fig. 5A). On the other hand, a JNK inhibitor (SP600125) significantly reduced the increase in nitrite release but markedly potentiated cellular damage (Fig. 5B). Notably, JNK inhibition totally abolished the cytoprotective effect of CORM-3 suggesting a role for this MAP kinase in CO-mediated cytoprotection.

The inhibitory effect of CORM-3 on LPS-induced nitrite production is not affected by MAPK inhibitors

In our setting, all MAPK inhibitors except PD98059 promoted, albeit to a different extent, a significant inhibition in nitrite increase after challenge with LPS; notably, only the JNK inhibitor, SP600125, potentiated the inhibitory effect of CORM-3 (Fig. 6A). However, the inhibition of JNK was associated with a significant increase in cellular damage. In addition, similar to our findings using INF- γ , inhibition of JNK in LPS-challenged cells abolished the cytoprotective effect of CORM-3 (Fig. 6B).

CORM-3 attenuates INF- γ -induced TNF- α production but only affects LPS-induced TNF- α release in the presence of P38 or ERK inhibitors

TNF- α is a pro-inflammatory cytokine that is up regulated after exposure to various stimuli. It is believed

to promote cellular injury and contributes to cellular apoptosis. In our setting, incubation of BV-2 microglia with INF- γ for 24 h resulted in a significant increase in TNF- α production (Fig. 7A). This response was markedly reduced by CORM-3 and not affected by the inactive form; this inhibitory effect was augmented by PI3K (LY294002) and ERK (PD98059) in-

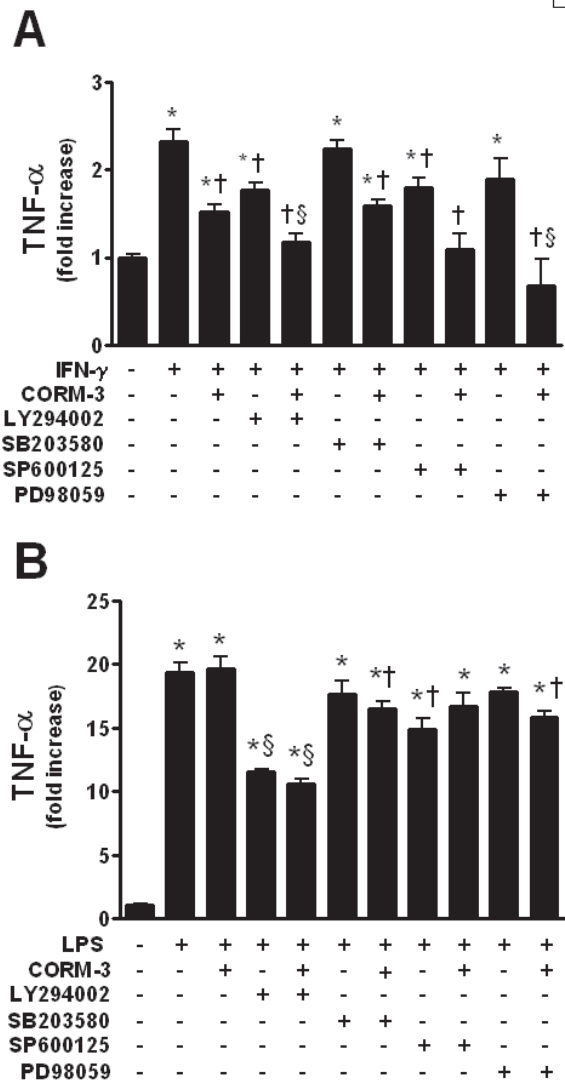


Fig. 7. CORM-3 attenuates INF- γ -induced TNF- α production but has no effect on the increase in TNF- α mediated by LPS. (**A** and **B**) Cells were treated with inhibitors of P38 (SB203580, 10 μ M), JNK (SP600125, 25 μ M), ERK (PD98059, 25 μ M) or PI3K (LY294002, 25 μ M) in serum-free medium for 1 h. Then cells were exposed for 24 h to complete medium (10% FBS) containing either LPS (1 μ g/ml) or INF- γ (10 ng/ml) in combination with CORM-3 (75 μ M) for 24 h. At the end of the incubation period TNF- α production was measured as described in the Methods. Cells treated with medium alone represent the control group. Bars represent the mean \pm SEM of 5 independent experiments per group. *p < 0.001 vs. control; † p < 0.05 vs. LPS or INF- γ ; § p < .001 vs. LPS or INF- γ

hibitors. Although LPS treatment also markedly increased TNF- α production, CORM-3 did not show any added benefit under these circumstances (Fig. 7B). However, the addition of CORM-3 to either P38 inhibitor (SB203580) or ERK inhibitor augmented their sub-optimal inhibition to reach the level of significance. Although LY294002 inhibited both nitrite and TNF- α by itself, it potentiated the effect mediated by CORM-3 only in the case of IFN- γ stimulation whereas it did not affect the activity of CORM-3 in LPS-stimulated BV-2 cells (Fig. 7A, B). On the other hand, ERK inhibition selectively amplified the effect of CORM-3 on TNF- α release with either IFN- γ or LPS stimulation without affecting NO production. Although JNK inhibition further increased the inhibitory effect of CORM-3 on nitrite production, it did not affect TNF- α release. However, JNK inhibition also showed an increased cellular injury; thus, the reduction in nitrite release may be attributed to reduced cellular mass rather than genuine inhibition of NO release. Notably, JNK inhibition totally abolished CORM-3-mediated cytoprotective effect. Collectively, our findings point to an involvement of the JNK pathway in CORM-3-mediated cytoprotective actions and confirm the complexity of CO interaction with different MAP kinases.

Discussion

Carbon monoxide-releasing molecules (CO-RMs) are emerging as a valuable tool to investigate the physiological function of CO because of their unique ability to transport and release this gas at precise and controlled amounts in cells and tissues [31, 34]. These CO carriers have been used successfully to modulate different cellular responses and functions in a variety of *in vitro*, *ex-vivo* and *in vivo* experimental models [11, 16, 19, 47]. Two major classes of CO-RMs have been identified and developed so far for their use in biological systems: 1) transition metal complexes, which contains either manganese (CORM-1), ruthenium (CORM-2 and CORM-3) or iron (CORM-F3) coordinated to carbonyl groups [11, 31, 35, 49]; and boranocarbonates (CORM-A1), which contain boron covalently bound to a carboxylic group that is converted to CO in the presence of hydrogen ions in aqueous solutions [34, 36]. The water-soluble CORM-3,

a ruthenium-based carbonyl complex that releases CO very rapidly ($t_{1/2} < 1$ min, at 37°C and pH = 7.4) has been the most extensively studied by our group and our collaborators; this compound showed to be cytoprotective in cardiac cells [11], act as an anti-proliferative agent in smooth muscle cells [53] and exert anti-inflammatory activities in macrophages [48]. More recently, we have reported that CORM-3 inhibits the inflammatory response *in vitro* by mitigating both NO and TNF- α production in BV-2 microglia cells challenged with thrombin [4]. In the present study we extend our findings by showing that CORM-3 can modulate neuroinflammation in microglia cells in response to LPS and INF- γ , two major players in the exacerbation of neuroinflammatory dysfunction.

Most neurological disorders involve activation and possibly dysregulation of microglia function [3]. This occurs as a consequence of the change in the redox state of microglia under intense oxidative and nitrosative stress that develop in the initial phases of many neuro-inflammatory conditions. Subsequently, neuronal inflammation leads to the release of cytokines such as TNF- α and IFN- γ , which finally leads to an exacerbation of tissue damage and cell death [26, 45]. Excessive production of reactive oxygen species (oxidative stress) and NO (nitrosative stress) is known to result in HO-1 induction in various cell types including endothelial cells and cardiomyocytes [13, 37]. Similarly, increased NO production in response to cytokines can cause autocrine and paracrine induction of HO-1 protein in microglia and astrocytes *in vitro* and *in vivo* [27]. Interestingly, cells with high HO content (e.g. cerebellar granule cells) are less affected by glial inflammatory reaction under normoxia and hypoxia [8]. More recent evidence has shown that astrocytes induced HO-1 in microglia can prevent excessive brain inflammation [30].

In the present study we found that stimulation of BV-2 microglia cells with LPS results in increased NO production (nitrite levels), an effect that was markedly attenuated in a concentration-dependent manner by addition of CORM-3. The fact that Ru(DMSO)₄Cl₂, a water-soluble inactive control that contains a ruthenium metal as in CORM-3 but does not release CO, did not cause any change in nitrite levels after stimulation with LPS emphasizes the direct involvement of CO in such effect. These data are in agreement with a previous report from our group showing that CORM-3 significantly decreased NO production as a consequence of induced iNOS expres-

sion following exposure of RAW macrophages to LPS [48]. In addition, two recent studies confirmed the inhibitory effect of CORM-2, another ruthenium-containing CO releaser, on LPS-induced NO production in microglia and macrophages [28, 52]. The pharmacological effects of CORM-3 were also manifested when INF- γ , a powerful pro-inflammatory cytokine, was used to induce neuroinflammation in BV-2 microglia. In fact, INF- γ alone had the ability to increase significantly both nitrite and TNF- α production, an effect that was markedly attenuated by CORM-3 but not the inactive compound. Furthermore, blockade of endogenous CO production by tin protoporphyrin-IX did not influence the ability of CORM-3 to reduce the inflammatory response suggesting a potential use of CO carriers as anti-inflammatory agents. These emerging properties of CO are in line with the ability of microglia to express the CO-producing enzyme HO-1 in response to oxidative stress [7, 27, 28] and are consistent with the protective role of the HO-1/CO pathway against neuronal injury in a model of permanent middle cerebral artery occlusion in mice [39]. The fact that CORM-3 was very effective in reducing nitrite production and attenuated LDH release in microglia in the presence of LPS or INF- γ is indicative of the possible therapeutic effect of CO in the context of neurodegeneration.

Mitogen activated protein kinases (MAPKs) are believed to play a significant role in the regulation of inflammatory response in the nervous system. However, the contribution of each individual pathway seems to vary according to the specific stimulus involved as stress-activated kinases (P38/JNK) seem to affect LPS-mediated inflammation [22] while extracellular signal-regulated kinase (ERK) seem to be more responsive to cytokine-mediated effects [40]. On the other hand, previous reports have shown that a phosphatidyl inositol 3 kinase (PI3K) inhibitor, LY294002, has a very potent effect on NO production and iNOS gene expression in the presence of INF- γ [23], while a P38 inhibitor (SB203580) has no effect by itself and needs the addition of ERK inhibitors (e.g. PD98059) to promote a significant effect in microglia [21, 50].

In this study we found that neither P38 nor JNK inhibition affect CORM-3-mediated anti-inflammatory action in INF- γ -induced inflammation. Nevertheless, blockade of the ERK pathway appears to selectively facilitate the inhibitory effect of CORM-3 on TNF- α release but not NO production; this could be attributed to the partial permissive role for ERK in cytokine production. Interestingly, PI3K inhibition amplified

the ability of CORM-3 to reduce both NO production and TNF- α release in microglia stimulated with INF- γ but seemed to act independently in the case of LPS-induced inflammation. A previous report in line with our findings showed that the expression of the main inflammatory mediators, iNOS and COX-2, involves activation of multiple signaling proteins, where PI3K activation appears to play a major role [24].

In conclusion, our results provide strong evidence that CO liberated from CORM-3 significantly reduces NO production in BV-2 microglia activated by LPS or INF- γ , an effect that is particularly relevant to neuro-inflammatory disorders. The effects mediated by CORM-3 appear to be independent of activation of HO-1 or guanylate cyclase and involves, at least in part, NOS inhibition. However, its effect on TNF- α release varies with the stimulus and signaling pathway involved. Similarly, the interaction of CORM-3 with MAPK pathways was rather selective and its cytoprotective activity seemed to be mediated by JNK pathway. Further research needs to be conducted to explore the molecular mechanism(s) by which CO transduces the anti-inflammatory response in tissues and how to exploit the full potential of CO-RMs in ameliorating glial responses in the context of neuroinflammatory and neurodegenerative disorders.

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