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Modulation of thrombin-induced neuroinflammation in BV-2 microglia by a carbon monoxide-releasing molecule (CORM-3)

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Abbreviations: HO-1**,** heme oxygenase-1; CO, carbon monoxide; CO-RMs, carbon monoxide-releasing molecules.

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Abstract

Carbon monoxide-releasing molecules (CO-RMs) are emerging as a new class of pharmacological agents that regulate important cellular function by liberating CO in biological systems. Here, we examined the role of CORM-3 in modulating neuro-inflammatory responses in BV-2 microglial cells, considering its practical application as a novel therapeutic alternative in the treatment of stroke. BV-2 microglia cells were incubated for 24 h in normoxic conditions with thrombin alone or in combination with interferon-γ to simulate the inflammatory response. Cells were also subjected to 12 h hypoxia and reoxygenated for 24 h in the presence of thrombin and IFN-γ. In both set of experiments, the anti-inflammatory action of CORM-3 was evaluated by assessing its effect on nitric oxide production (nitrite levels) and TNF-α release. CORM-3 (75 μM) did not show any cytotoxicity and markedly attenuated the inflammatory response to thrombin and interferon-γ in normoxia and to a lesser extent in hypoxia as evidenced by a reduction in nitrite levels and TNF-α production. Inactive CORM-3, which does not liberate CO and is used as a negative control, failed to prevent the increase in inflammatory mediators. Blockade of endogenous CO production by tin protoporphyrin-IX did not change the anti-inflammatory activity of CORM-3 suggesting that CO liberated from the compound is responsible for the observed effects. In addition, inhibition of the mitogen-activated protein kinases PI3K and ERK amplified the anti-inflammatory effect of CORM-3. These results suggest that the anti-inflammatory activity of CORM-3 could be exploited to mitigate microglia activity in stroke and other neuro-inflammatory diseases.

Introduction

Stroke is the major cause of disabilities in adults leaving more than half of the survivors dependent on others for everyday activities (Wolfe et al., 2000). It is also a major cause of dementia, depression, epilepsy, and falls (Rothwell et al., 2004). This pathological event is characterized by blockade of blood supply to the brain and consequently oxygen and glucose deprivation, which lead to necrotic cell death and tissue infarct. Rescuing the surrounding partially ischemic penumbra depends on the severity of brain edema, subsequent neuro-inflammation and production of free radicals.

Microglia are the main inflammatory reacting cells in the brain after ischemia (Suk, 2004). They act through redox-sensitive inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and NAD(P)H oxidase which produce NO, superoxide, peroxynitrite and other reactive oxygen species (ROS) that mediate their phagocytic ability. Free radicals generated during the inflammatory process can directly damage neurons by interacting with their lipid-rich membranes, or by increasing AMPA/Kainate receptor susceptibility to the toxic effects of glutamate (Zhao et al., 2004).

Another important element in the cascade of events leading to the progression and exacerbation of stroke is thrombin. Apart from its basic role in forming and propagating the obstructing clot, thrombin contributes to stroke-related brain injury at more than one level. It is now well recognized that thrombin contributes to the formation of brain edema (Guan et al., 2004), neuro-inflammation (Hanisch et al., 2004), and neuronal damage (Smirnova et al., 1998). These effects are further augmented by decreased levels of oxygen (hypoxia) and the consequent formation of ROS and pro-inflammatory cytokines.

Since the late 90s, carbon monoxide gas (CO) emerged from the shadows of being a catabolic byproduct with no physiological significance into full recognition as a ubiquitous key modulator of cellular response, particularly in its role as signaling mediator and contributor to cell survival during conditions of oxidative stress (Motterlini et al., 2005a). CO is produced by the enzyme heme oxygenase (HO) through heme degradation. HO exists as constitutive (HO-2) and inducible (HO-1) isoforms, the latter being of particular importance in stressful conditions because of its ability to attenuate oxidative stress-mediated injury in response to a wide range of stimuli including ischemia reperfusion injury (Clark et al., 2000). In fact, HO-1 induction has been demonstrated to protect against such injuries in almost all cell types including cardiomyocytes (Clark et al., 2003), hepatocytes (Amersi et al., 1999), lung tissue (Minamino et al., 2001) and neuronal tissue (Panahian et al., 1999). Many of the protective effects of HO are now believed to be mediated by CO (Otterbein et al., 2000), which has been shown to reduce the inflammatory response in a similar fashion to compounds that are known to potently induce HO-1 (Sawle et al., 2005).

In recent years more emphasis on the possible therapeutic role of CO incited researchers towards the development of new methods to facilitate its use in biological systems; this approach lead eventually to the development of carbon monoxide-releasing molecules (CO-RMs) (Clark et al., 2003; Motterlini et al., 2002; Motterlini et al., 2005b; Motterlini et al., 2005a). These compounds are biologically compatible CO carriers that can liberate CO in physiological solutions. Different chemical classes of CO-RMs are currently being developed but the most extensively studied so far are transition metal carbonyls, which contain ruthenium or iron as core metal and can release CO with different rates in aqueous solutions (Clark et

al., 2003; Motterlini et al., 2002; Fairlamb et al., 2005; Sandouka et al., 2006). Ruthenium-based CO-RMs (CORM-2 and CORM-3) have been tested in different models of disease showing promising beneficial effects and are ideal to use because they are easy to synthesize, they provide a predictable pattern of CO release and have no toxic effects at low micromolar concentrations (Clark et al., 2003; Motterlini et al., 2002; Sawle et al., 2005; Motterlini et al., 2005a; Sandouka et al., 2006). In this study we examined the effect of CORM-3 on thrombin induced neuro-inflammation in BV2 microglia in normoxic and hypoxic conditions to establish a potential biological role of CO in stroke and neuro-inflammation.

MATERIALS AND METHODS

Reagents: Tricarbonylchloro(glycinato)ruthenium (II) ([Ru(CO)3Cl(glycinate)] or CORM-3) was synthesized as previously described (Clark et al., 2003; Foresti et al., 2004). CORM-3 was freshly prepared as a 10 mM stock solution in pure distilled water. An inactive form, which is incapable of releasing CO, was used as a negative control. CORM-3 was 'inactivated' (iCORM-3) by adding the compound to cell culture medium and leaving it for 18 h at 37 °C in a 5% CO_2 humidified atmosphere to liberate CO. The iCORM-3 solution was finally bubbled with nitrogen to remove the residual CO present in the solution. Since CORM-3 contains ruthenium as transition metal, $RuCl_2(DMSO)_4$ was also used as an additional negative control. Thrombin, Interferon-γ, tin protoporphyrin-IX, SB203580, SP600125, PD98059, LY294002 and all other reagents were purchased from Sigma unless otherwise specified.

Cell culture: The mouse BV2 microglia cell line was a kind gift from Professor Rosario Donato (University of Perugia). The cell line was obtained and characterized as previously described (Adami et al., 2001). Cells were initially grown to confluence in 75 cm^2 flasks using RPMI containing 10% FBS, 4 mM glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin in H_2O-saturated 5% CO_2 atmosphere at 37 °C. Cells were subsequently sub-cultured (1:3 ratio) and grown to 90% confluence in 24-well plates prior to testing.

Experimental protocol: BV-2 microglia were treated with thrombin (10 U/ml) alone or in combination with interferon-γ (10 ng/ml) in the presence or absence of CORM-3

(75 µM) or iCORM-3 (75 µM). Cells were then incubated under normoxic conditions for 24 h. To examine the potential anti-inflammatory action of CORM-3, nitrite levels and TNF-α production were determined at the end of the incubation period.

In a similar set of experiments, cells were treated with thrombin (10 U/ml) alone or in combination with interferon-γ (10 ng/ml) in the presence or absence of CO-RMs under hypoxic conditions (95% N_2/5% CO_2) using an established protocol previously described by our group (Motterlini et al., 2000). Cells were exposed to hypoxia for 12 h and then reoxygenated in a normoxic environment for an additional 24 h. CORM-3 or iCORM-3 was applied to cells prior to placing them in the hypoxic chamber and no addition was made during the reoxygenation period. Nitrite production and TNF-α levels were also determined from medium samples at the end of the incubation period. In addition, cell viability was assessed by different methods at the end of the experiments.

Determination of nitrite levels: Nitrite levels were determined using the Griess method as previously described by our group (Sawle et al., 2005). 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 nitrite level in each sample 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 tumor necrosis factor-α (TNF- α) present in each sample was determined using a commercially available ELISA kit from R&D Systems (Mouse TNF-α Quantikine immunoassay kit, Abingdon, U.K.) (Sawle et al., 2005). The assay was performed according to manufacturers' instructions. Briefly, cell culture supernatants (n=5 per each group) 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 Alamar Blue assay kit (Serotec, U.K.) and carried out according to the manufacturers' instructions as previously reported by us (Sawle et al., 2005). The assay is based on the detection of metabolic activity of living cells using a redox indicator, which changes from an oxidized (blue) form to a reduced (red) form. The intensity of the red color is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 and 600 nm and expressed as a percentage of control. An assay for the release of lactate dehydrogenase (LDH) activity was also performed using a cytotoxicity detection kit according to manufacturers' instructions (Roche). Briefly, at the end of the incubation period, cell supernatants (n=6 per each group) were collected and any cell residue was removed by centrifugation at 250 x 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 after which the absorbance was measured at 490 and 690 nm. LDH activity was expressed as percentage of maximal LDH release, which was obtained by lysis of cells with Triton X-100 (1% in DMEM at 25 °C).

Statistical analysis: Differences among the groups were analyzed using one way ANOVA and Bonferroni tests. Values were expressed as mean ± S.E.M. and differences between groups were considered to be significant at $p < 0.05$.

RESULTS

CORM-3 attenuates thrombin-induced nitrite release in BV-2 microglia under normoxic and hypoxic conditions. Thrombin is a well known inducer of NO release from microglia under normoxic conditions (Ryu et al., 2000), a phenomena responsible for its devastating effect on neurons. We examined the effects of ruthenium-based CO-RMs on this response. Preliminary experiments were conducted in order to determine the most effective concentration of CORM-3 to reduce thrombin (or INF-γ)-mediated increase in nitrite production. We found that CORM-3 (10-100 μM) significantly reduced nitrite increase in a concentration-dependent manner (data not shown). Since the inhibition of nitrite production by 75 μM CORM-3 was not markedly different from the one elicited by 100 μM (82.6±0.7 vs. 90.2±0.8%, respectively) and because at 75 μM we did not observe any change in cell viability on microglia, neurons and astrocytes, this ideal concentration was used in all future experiments. As shown in Figure 1A, exposure to thrombin for 24 h markedly induced nitrite release in BV-2 cells. This response was significantly reduced by CORM-3 but not by the inactive compound (iCORM-3) indicating that such effect can be attributed to the liberated CO. It is important to note that cell viability was preserved in all the treatments performed (Figure 1B) suggesting that the effect of CORM-3 and the consequent release of CO are not toxic to the cells. The increase in NO production (nitrite levels) induced by thrombin was further augmented following exposure of BV2 cells to hypoxia/reoxygenation (Figure 2A). Hypoxia/reoxygenation also caused a significant cellular damage which was exacerbated by the presence of thrombin (Figure 2B). Interestingly, CORM-3 (but not iCORM-3) promoted a significant reduction in nitrite production induced by

thrombin under hypoxic conditions and significantly reduced the consequent reoxygenation-mediated cellular damage (Figure 2A and 2B).

CORM-3 attenuates thrombin and interferon-γ induced nitrite release in BV-2 microglia under normoxic and hypoxic conditions. Interferon-γ (IFN-γ) is a strong microglial stimulant produced mainly by the lymphocytes and increases the microglial ability to induce and propagate inflammation (Blasko et al., 2001). It is commonly used alone or in combination with other stimuli (e.g. LPS) to simulate and investigate neuro-inflammation. We examined the effect of IFN-γ on BV-2 cells in the presence of thrombin. Predictably, the combination of thrombin with IFN-γ amplified nitrite production (Figure 3A) and CORM-3 once again significantly attenuated this effect. Although iCORM-3 partially affected nitrite levels, the effect of CORM-3 was far more pronounced ($p<0.01$, CORM-3 vs. iCORM-3) and no significant cellular damage was detected by all the treatments (Figure 3B). Similarly, but to a different extent, CORM-3 was able to partially reduce nitrite increase by the combination of thrombin and IFN-γ following hypoxia/reoxygenation (Figure 4A). Under these conditions, a marked increase in cell injury (LDH release) was observed and both CORM-3 and iCORM-3 partially reduced the toxic effect (Figure 4B).

CORM-3 attenuates thrombin and interferon-γ induced TNF-α production in BV-2 microglia under normoxic and hypoxic conditions. TNF-α is a key immuno-modulatory and pro-inflammatory cytokine that is up regulated after stroke. It is believed to promote cellular injury during and after the stressful events (Wang et al.,

2004a), and contributes to cellular apoptosis. In our setting, incubation of BV-2 microglia with thrombin plus IFN-γ for 24 h resulted in a significant increase in TNF-α production (Figure 5A). This response was markedly reduced by CORM-3 and not affected by the inactive form. Treatment of cells for 12 h under hypoxic conditions followed by 24 h of reoxygenation in the presence of thrombin and IFN-γ amplified TNF-α production (Figure 5B). Addition of CORM-3 prior to hypoxia-reoxygenation caused a significant decrease in TNF-α production mediated by the combination of thrombin and IFN-γ, an effect that was not reproduced by the iCORM-3.

The anti-inflammatory effect of CORM-3 is independent of the endogenous activity of heme oxygenase. To test whether endogenous CO may contribute to the anti-inflammatory action of CORM-3, we used the heme oxygenase inhibitor tin protoporphyrin-IX (SnPPIX, 10 μM). Blockade of heme oxygenase activity significantly increased the baseline production of nitrite in response to the combination of thrombin and IFN-γ (Fig. 6A), an effect also seen in the presence of CORM-3 alone (data not shown). However, SnPPIX did not seem to affect the anti-inflammatory activity of CORM-3 and did not change the effect of the CO-depleted compound (iCORM-3) further supporting the hypothesis that CO liberated by CORM-3 is responsible for the observed effects. Notably, there was no cytotoxicity related to the use of CORM-3, iCORM-3, or tin protoporphyrin-IX (Fig. 6B). It has to be noted that the presence of SnPPIX reduced LDH release promoted by the combination of thrombin and IFN-γ.

Contribution of mitogen-activated protein kinases (MAPKs) on the anti-inflammatory activity mediated by CORM-3. In our model of thrombin-Interferon induced

inflammation we found that the anti-inflammatory effect of CORM-3 was potentiated by blockade of PI3-K and partially by ERK. Interestingly, ERK inhibitor PD98059 seemed to selectively augment CORM's inhibitory effect on TNF-α release (Fig 7B) without affecting NO production (Fig. 7A). On the other hand, PI3K inhibitor LY294002 potentiated the anti-inflammatory activity of CORM-3 in reducing both NO production and TNF-α release (Fig. 7A and 7B). We did not observe any contribution by other classical MAPK pathways as P38 (SB203580) and JNK (SP600125) inhibitors did not alter the CORM-3-mediated attenuation of NO production and TNF-α release in response to thrombin and INF-γ (Fig. 7A and 7B).

DISCUSSION

Carbon monoxide (CO) is a pivotal signaling mediator that regulates a variety of cellular functions in mammals. Due to its versatility and pleiotropic properties, it is becoming apparent that this gas produced endogenously by the enzyme heme oxygenase could be exploited for treating different disorders and pathological states. The importance of CO as a potential therapeutic agent is exemplified by the recent development of CO-releasing molecules (CO-RMs), a new class of chemicals that have been specifically designed to carry and deliver small amounts of CO into biological environments (Motterlini et al., 2003; Motterlini et al., 2005b; Motterlini et al., 2005a). Previous studies using water-soluble CO-RMs have demonstrated the ability of these compounds to exert vasodilatory and hypotensive effects (Motterlini et al., 2005a), mitigate ischemia-reperfusion injury (Clark et al., 2003; Motterlini et al., 2002), prevent organ graft rejection (Clark et al., 2003) and attenuate the inflammatory response in stimulated macrophages (Sawle et al., 2005). Here, we extend our previous investigations by showing that CORM-3, a transition metal carbonyl that is fully soluble in aqueous solutions and rapidly liberates CO, modulates neuroinflammation in microglia cells.

It is known that cerebral ischemia triggers a strong inflammatory response that involves early recruitment of microglia, which proceeds and predominates over macrophage infiltration in brain tissue (Schilling et al., 2003). The brain is particularly sensitive to oxidative stress due to its high rate of oxygen consumption, elevated lipid content and increased rate of reactive oxygen species (ROS) generation. Although the initial insult to neurons may not be caused directly by ROS, the resulting events mediated by increased free radicals production can cause

secondary damage that is far greater and is pathologically relevant to neurodegeneration (Crack and Taylor, 2005). As a result of breaching the blood brain barrier, serum factors such as thrombin leak into the brain to come into direct contact with neurons and astroglia. The presence of thrombin in direct contact with brain tissue induces inflammatory infiltration, brain edema, and reactive gliosis (Nishino et al., 1993). Thrombin has a direct toxic effect on neurons by inducing apoptosis (Smirnova et al., 1998) and an indirect damaging effect through activating microglia (Carreno-Muller et al., 2003) leading to stimulation of NO and ROS production (Ryu et al., 2000; Hanisch et al., 2004; Kim et al., 2002). As a consequence of the change in the redox state of these cells, cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon-γ (IFN-γ) are released and this further contributes to the exacerbation of tissue damage (Kim et al., 2002; Rothwell, 1997).

In the present study we found that stimulating BV2 microglia cells with thrombin results in increased NO production (nitrite levels) and this effect is markedly attenuated by addition of CORM-3. The fact that the inactive control (iCORM-3), which does not release CO, was far less effective in reducing nitrite levels after stimulation with thrombin emphasizes the direct involvement of CO in the observed effect. It needs to be pointed out that the partial beneficial effect of iCORM-3 observed in some experiments, particularly on cell viability, might be due either to the compound itself and/or to the residual carbonyl groups still present in the molecule (Clark et al., 2003; Motterlini et al., 2005a). These data are in agreement with a previous report showing that CORM-3 significantly decreased NO production as a consequence of induced iNOS expression following exposure to endotoxin (Sawle et al., 2005). Interestingly, we also found that exposure of BV2 cells to

hypoxia-reoxygenation in the presence of thrombin caused a much higher production of nitrite compared to thrombin alone and CO liberated from CORM-3 was still very effective in reducing this inflammatory response. It is known that NO generated from iNOS exacerbates ischemia-reperfusion injury to the brain and that NO and other cytokines are highly increased in microglia in response to hypoxia (Park et al., 2002). The fact that CORM-3 was very effective in reducing nitrite production and attenuated LDH release in microglia in the presence of thrombin under hypoxic conditions is indicative of the potential therapeutic effect of CO in the context of brain injury mediated by ischemic events. The beneficial effects of CORM-3 were also manifested when INF-γ, one of the most potent stimulants of microglia, was introduced in our experimental protocol to simulate the synergistic effect of pro-inflammatory cytokines in maximizing cellular damage and driving multiple destructive cascades. Indeed, we found that the combination of thrombin and INF-γ synergized NO production in BV2 cells under normoxic conditions, an effect that was markedly attenuated by CORM-3 but not the inactive compound. The protective effect of CORM-3 in reducing nitrite levels by the combination of thrombin and INF-γ was only partially observed under hypoxia; this might be explained by the fact that the combination of cytokines and low oxygen tension is too extreme to BV2 microglia as it caused severe cellular damage (> 90% LDH release), which is likely to be irreversible. However, the salutary effects of CORM-3 were clearly evident when TNF-α levels were measured in microglia exposed to thrombin plus INF-γ either under normoxia or hypoxia-reoxygenation. In both conditions, we found that CORM-3 significantly reduced TNF-α in response to the pro-inflammatory cytokines; this effect can be attributed to CO as iCORM-3 was far less effective. These data are in agreement with and supported by a previous study from our group showing

that CO-RMs can effectively mitigate the TNF-α release from murine macrophages stimulated *in vitro* with lipopolysaccharide (Sawle et al., 2005). Furthermore, blockade of endogenous heme oxygenase-derived CO, bilirubin and biliverdin by tin protoporphyrin-IX (10 μM) did not affect CORM-3 induced anti-inflammatory or cytoprotective activity. The bioactive properties of CO emerging from the present investigation are in line with the ability of microglia to express the CO-producing enzyme heme oxygenase (HO-1) (Kitamura et al., 1998) and are sustained by the neuroprotective role of HO-1 against cerebral ischemia, an effect that is likely mediated by CO (Panahian et al., 1999). It has to be noted that the presence of SnPPIX reduced LDH release promoted by the combination of thrombin and IFN-γ. Although this could be counterintuitive, we have previously shown that SnPPIX, apart from acting as a competitive inhibitor of heme oxygenase activity, also possesses some scavenging properties against free radicals and this could explain SnPPIX's favorable effect on cell viability obtained in this study (Foresti et al., 1999).

Mitogen activated protein kinases (MAPKs) are believed to play a significant role in the regulation of inflammatory response in cerebral ischemia (Wang et al., 2004b). However, the contribution of each individual pathway is rather difficult to define because of the complex interactions between all the involved pathways that can directly or indirectly affect the final outcome. MAPK families can be classified as stress activated (P38/JNK) or extra-cellular signal-regulated kinase (ERK). In addition, phosphatidyl inositol 3 kinase (PI3K) is now proven to play a crucial role in cell survival signals and modulation of iNOS expression (Hwang et al., 2004). Recent studies have shown the involvement of P38, JNK, ERK and PI3K pathways in the brain tissue response to ischemia (Barone et al., 2001; Takagi et al., 2000; Ferrer et al., 2003; Crack and Taylor, 2005).

The importance of each kinase varies with the type of stimulus**.** In ischemia reperfusion (I/R) induced inflammation P38 seems to play a major role while JNK 's role is rather permissive or modulatory related more to cytokine regulation. In addition, although ERK is not stress-induced it appears to participate, at least partly, in the production of inflammatory cytokines (Toledo-Pereyra et al., 2004), whereas thrombin-induced NO release is inhibited by p38 (SB203580) and ERK (PD98059) inhibitors (Ryu et al., 2000). However, SB203580 had no effect by itself on IFN-γ-mediated NO release and needed the addition of PD98059 to reach a significant inhibitory effect in microglia (Han et al., 2002; Shen et al., 2005). On the other hand, PI3K had a very potent effect on IFN-γ-induced NO production and iNOS gene in microglia, an effect that is likely mediated by STAT1 and partly by NF-kB (Hwang et al., 2004).

In our settings P38 inhibition had a minimal effect on NO production and TNF-α release (the apparent increase in TNF-α level is due to decreased cell viability in the presence of SB203580, data not shown). JNK inhibition reduced both inflammatory markers (the increase in TNF-α was also associated with an increased cell injury, data not shown). However, neither P38 nor JNK inhibition seemed to affect CORM-3-mediated anti-inflammatory action. Although ERK inhibition had a relatively small inhibitory effect on both NO and TNF-α release, its effect on CORM-3 activity was rather selective facilitating CORM-3 inhibitory effect on TNF-α release but not NO production, which can be attributed to the partial permissive role for ERK in cytokine production. On the other hand, PI3K inhibition had a significant inhibitory effect by itself and potentiated the effect of CORM-3 in reducing both NO production and TNF-α release. More mechanistic studies are required to better understand the interaction between the signal transduction pathways involved; interestingly, 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 appears to play a major role (Jang et al., 2005).

In conclusion, our results provide strong evidence that CO liberated from CO-RMs significantly reduces the inflammatory response in BV-2 microglia stimulated by thrombin, cytokines and hypoxia-reoxygenation, an effect that is particularly relevant to stroke-related neuro-inflammatory response and cellular damage. CORM-3 seems to affect a spectrum of pathways in the cellular inflammatory response that extends from direct enzymatic inhibition to interaction with MAPK pathways. Further research needs to be conducted to explore the exact mechanism(s) by which CO transduces the signal into an anti-inflammatory response and how CO-RMs can be best exploited for maximizing the neuromodulatory activity of CO in the context of stroke and protection of brain tissue against ischemia-reperfusion injury.

References

Adami C, Sorci G, Blasi E, Agneletti AL, Bistoni F, and Donato R (2001) S100B expression in and effects on microglia. *Glia* **33**:131-142.

Amersi F, Buelow R, Kato H, Ke B, Coito AJ, Shen XD, Zhao D, Zaky J, Melinek J, Lassman CR, Kolls JK, Alam J, Ritter T, Volk HD, Farmer DG, Ghobrial RM, Busuttil RW, and Kupiec-Weglinski JW (1999) Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. *J Clin Invest* **104**:1631-1639.

Barone FC, Irving EA, Ray AM, Lee JC, Kassis S, Kumar S, Badger AM, White RF, McVey MJ, Legos JJ, Erhardt JA, Nelson AH, Ohlstein EH, Hunter AJ, Ward K, Smith BR, Adams JL, and Parsons AA (2001) SB 239063, a second-generation p38 mitogen-activated protein kinase inhibitor, reduces brain injury and neurological deficits in cerebral focal ischemia. *J Pharmacol Exp Ther* **296**:312-321.

Blasko I, Ransmayr G, Veerhuis R, Eikelenboom P, and Grubeck-Loebenstein B (2001) Does IFNgamma play a role in neurodegeneration? *J Neuroimmunol* **116**:1-4.

Carreno-Muller E, Herrera AJ, de Pablos RM, Tomas-Camardiel M, Venero JL, Cano J, and Machado A (2003) Thrombin induces in vivo degeneration of nigral dopaminergic neurones along with the activation of microglia. *J Neurochem* **84**:1201-1214.

Clark JE, Foresti R, Sarathchandra P, Kaur H, Green CJ, and Motterlini R (2000) Heme oxygenase-1-derived bilirubin ameliorates post-ischemic myocardial dysfunction. *Am J Physiol Heart Circ Physiol* **278**:H643-H651.

Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R, and Motterlini R (2003) Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. *Circ Res* **93**:e2-e8.

Crack PJ and Taylor JM (2005) Reactive oxygen species and the modulation of stroke. *Free Radic Biol Med* **38**:1433-1444.

Fairlamb IJS, Duhme-Klair AK, Lynam JM, Moulton BE, O'Brien CT, Whitwood AC, Sawle P, Hammad J, and Motterlini R (2005) *η4*-Pyrone metal carbonyl complexes as effective CO-releasing molecules (CO-RMs). *Bioorg Med Chem Lett* **16**:995-998.

Ferrer I, Friguls B, Dalfo E, and Planas AM (2003) Early modifications in the expression of mitogen-activated protein kinase (MAPK/ERK), stress-activated kinases SAPK/JNK and p38, and their phosphorylated substrates following focal cerebral ischemia. *Acta Neuropathol (Berl)* **105**:425-437.

Foresti R, Green CJ, and Motterlini R (2004) Generation of bile pigments by haem oxygenase: a refined cellular strategy in response to stressful insults. *Biochem Soc Symp* **71***:*177-192.

Foresti R, Sarathchandra P, Clark JE, Green CJ, and Motterlini R (1999) Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis. *Biochem J* **339**:729-736.

Guan JX, Sun SG, Cao XB, Chen ZB, and Tong ET (2004) Effect of thrombin on blood brain barrier permeability and its mechanism. *Chin Med J (Engl)* **117**:1677-1681.

Han IO, Kim KW, Ryu JH, and Kim WK (2002) p38 mitogen-activated protein kinase mediates lipopolysaccharide, not interferon-gamma, -induced inducible nitric oxide synthase expression in mouse BV2 microglial cells. *Neurosci Lett* **325**:9-12.

Hanisch UK, Van Rossum D, Xie Y, Gast K, Misselwitz R, Ariola S, Goldsteins G, Koistinaho J, Kettenmann H, and Moller T (2004) The microglia-activating potential of thrombin: The protease is not involved in the induction of proinflammatory cytokines and chemokines. *J Biol Chem*. **279**:51580-51587.

Hwang SY, Jung JS, Lim SJ, Kim JY, Kim TH, Cho KH, and Han IO (2004) LY294002 inhibits interferon-gamma-stimulated inducible nitric oxide synthase expression in BV2 microglial cells. *Biochem Biophys Res Commun* **318**:691-697.

Jang BC, Paik JH, Kim SP, Shin DH, Song DK, Park JG, Suh MH, Park JW, and Suh SI (2005) Catalase induced expression of inflammatory mediators via activation of NF-kappaB, PI3K/AKT, p70S6K, and JNKs in BV2 microglia. *Cell Signal* **17**:625-633.

Kim KY, Kim MY, Choi HS, Jin BK, Kim SU, and Lee YB (2002) Thrombin induces IL-10 production in microglia as a negative feedback regulator of TNF-alpha release. *Neuroreport* **13**:849-852.

Kitamura Y, Matsuoka Y, Nomura Y, and Taniguchi T (1998) Induction of inducible nitric oxide synthase and heme oxygenase-1 in rat glial cells. *Life Sci* **62**:1717-1721.

Minamino T, Christou H, Hsieh CM, Liu Y, Dhawan V, Abraham NG, Perrella MA, Mitsialis SA, and Kourembanas S (2001) Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. *Proc Natl Acad Sci U S A* **98**:8798-8803.

Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, and Green CJ (2002) Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res* **90**:E17-E24.

Motterlini R, Foresti R, Bassi R, Calabrese V, Clark JE, and Green CJ (2000) Endothelial heme oxygenase-1 induction by hypoxia. Modulation by inducible nitric-oxide synthase and S-nitrosothiols. *J Biol Chem* **275**:13613-13620.

Motterlini R, Mann BE, and Foresti R (2005a) Therapeutic applications of carbon monoxide-releasing molecules (CO-RMs). *Expert Opin Investig Drugs* **14**:1305-1318.

Motterlini R, Mann BE, Johnson TR, Clark JE, Foresti R, and Green CJ (2003) Bioactivity and pharmacological actions of carbon monoxide-releasing molecules. *Curr Pharm Des* **9**:2525-2539.

Motterlini R, Sawle P, Bains S, Hammad J, Alberto R, Foresti R, and Green CJ (2005b) CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. *FASEB J* **19**:284-286.

Nishino A, Suzuki M, Ohtani H, Motohashi O, Umezawa K, Nagura H, and Yoshimoto T (1993) Thrombin may contribute to the pathophysiology of central nervous system injury. *J Neurotrauma* **10**:167-179.

Otterbein LE, Bach FH, Alam J, Soares M, Tao LH, Wysk M, Davis RJ, Flavell RA, and Choi AM (2000) Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* **6**:422-428.

Panahian N, Yoshiura M, and Maines MD (1999) Overexpression of heme oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. *J Neurochem* **72**:1187-1203.

Park SY, Lee H, Hur J, Kim SY, Kim H, Park JH, Cha S, Kang SS, Cho GJ, Choi WS, and Suk K (2002) Hypoxia induces nitric oxide production in mouse microglia via p38 mitogen-activated protein kinase pathway. *Brain Res Mol Brain Res* **107**:9-16.

Rothwell NJ (1997) Cytokines and acute neurodegeneration. *Mol Psychiatry* **2**:120-121.

Rothwell PM, Coull AJ, Giles MF, Howard SC, Silver LE, Bull LM, Gutnikov SA, Edwards P, Mant D, Sackley CM, Farmer A, Sandercock PA, Dennis MS, Warlow CP, Bamford JM, and Anslow P (2004) Change in stroke incidence, mortality, case-fatality, severity, and risk factors in Oxfordshire, UK from 1981 to 2004 (Oxford Vascular Study). *Lancet* **363**:1925-1933.

Ryu J, Pyo H, Jou I, and Joe E (2000) Thrombin induces NO release from cultured rat microglia via protein kinase C, mitogen-activated protein kinase, and NF-kappa B. *J Biol Chem* **275**:29955-29959.

Sandouka A, Fuller BJ, Mann BE, Green CJ, Foresti R, and Motterlini R (2006) Treatment with carbon monoxide-releasing molecules (CO-RMs) during cold storage improves renal function at reperfusion. *Kidney Int* **69**:239-247.

Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ, and Motterlini R (2005) Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Br J Pharmacol* **145**:800-810.

Schilling M, Besselmann M, Leonhard C, Mueller M, Ringelstein EB, and Kiefer R (2003) Microglial activation precedes and predominates over macrophage infiltration in transient focal cerebral ischemia: a study in green fluorescent protein transgenic bone marrow chimeric mice. *Exp Neurol* **183**:25-33.

Shen S, Yu S, Binek J, Chalimoniuk M, Zhang X, Lo SC, Hannink M, Wu J, Fritsche K, Donato R, and Sun GY (2005) Distinct signaling pathways for induction of type II NOS by IFNgamma and LPS in BV-2 microglial cells. *Neurochem Int* **47**:298-307.

Smirnova IV, Zhang SX, Citron BA, Arnold PM, and Festoff BW (1998) Thrombin is an extracellular signal that activates intracellular death protease pathways inducing apoptosis in model motor neurons. *J Neurobiol* **36**:64-80.

Suk K (2004) Minocycline suppresses hypoxic activation of rodent microglia in culture. *Neurosci Lett* **366**:167-171.

Takagi Y, Nozaki K, Sugino T, Hattori I, and Hashimoto N (2000) Phosphorylation of c-Jun NH(2)-terminal kinase and p38 mitogen-activated protein kinase after transient forebrain ischemia in mice. *Neurosci Lett* **294**:117-120.

Toledo-Pereyra LH, Toledo AH, Walsh J, and Lopez-Neblina F (2004) Molecular signaling pathways in ischemia/reperfusion. *Exp Clin Transplant* **2**:174-177.

Wang X, Feuerstein GZ, Xu L, Wang H, Schumacher WA, Ogletree ML, Taub R, Duan JJ, Decicco CP, and Liu RQ (2004a) Inhibition of tumor necrosis factor-alpha-converting enzyme by a selective antagonist protects brain from focal ischemic injury in rats. *Mol Pharmacol* **65**:890-896.

Wang ZQ, Wu DC, Huang FP, and Yang GY (2004b) Inhibition of MEK/ERK 1/2 pathway reduces pro-inflammatory cytokine interleukin-1 expression in focal cerebral ischemia. *Brain Res* **996**:55-66.

Wolfe CD, Tilling K, and Rudd AG (2000) The effectiveness of community-based rehabilitation for stroke patients who remain at home: a pilot randomized trial. *Clin Rehabil* **14**:563-569.

Zhao W, Xie W, Le W, Beers DR, He Y, Henkel JS, Simpson EP, Yen AA, Xiao Q, and Appel SH (2004) Activated microglia initiate motor neuron injury by a nitric oxide and glutamate-mediated mechanism. *J Neuropathol Exp Neurol* **63**:964-977.

FOOTNOTES

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FIGURE LEGENDS

Figure 1. CORM-3 attenuates thrombin-induced nitrite production in BV2 microglia cells. (*A*) Cells were incubated with thrombin (10 U/ml) alone or in combination with 75 μM 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*) Cells were incubated as reported in A and cell viability determined after 24 h using the Alamar Blue assay as described in Methods. Cells treated with medium alone represent the control group. Bars represent the mean ± S.E.M. of 5-6 independent experiments per group. *$p<0.05$ vs. control; † $p<0.05$ vs. thrombin (Thr).

Figure 2. CORM-3 attenuates thrombin-induced nitrite production in BV2 microglia cells under hypoxic conditions. (*A*) Cells were incubated with thrombin (10 U/ml) alone or in combination with 75 μM CORM-3 or iCORM-3 (negative control) for 12 h using a hypoxia chamber (95% N_2, 5%CO_2) and then reoxygenated in normal atmosphere (21% O_2, 5% CO_2) 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*) Cells were incubated as in A and cell viability determined after 36 h using the LDH assay as described in Methods. Cells treated with medium alone represent the control group. Bars represent the mean ± S.E.M. of 5-6 independent experiments per group. *$p<0.001$ vs. control; † $p<0.001$ vs. thrombin (Thr) plus hypoxia-reoxygenation (H/R).

Figure 3. CORM-3 attenuates the combined effect of thrombin and IFN-γ on nitrite production in BV2 microglia. (*A*) Cells were incubated with thrombin (10 U/ml) plus

IFN-γ (10 ng/ml) alone or in the presence of either CORM-3 (75 μM) or iCORM-3 (75 μM, 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) Cells were incubated as in A and cell viability determined after 24 h using the LDH assay as described in Methods. Cells treated with medium alone represent the control group. Bars represent the mean ± S.E.M. of 5-6 independent experiments per group. *p<0.001 vs. control; † p<0.001 vs. thrombin (Thr) and Interferon-γ (IFN-γ).

Figure 4. CORM-3 attenuates the combined effect of thrombin and IFN-γ on nitrite production under hypoxic conditions. (*A*) Cells were incubated with the combination of thrombin (10 U/ml) and IFN-γ (10 ng/ml) alone or in the presence of either CORM-3 (75 μM) or iCORM-3 (75 μM, negative control) for 12 h in hypoxia chamber (95% N_2, 5%CO2) and then reoxygenated in normal atmosphere (21% O_2, 5% CO_2) 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) Cells were incubated as in A and cell viability determined after 36 h using the LDH assay as described in Methods. Cells treated with medium alone represent the control group. Bars represent the mean ± S.E.M. of 5-6 independent experiments per group. *p<0.001 vs. control; †p<0.001 vs. thrombin (Thr) plus interferon-γ (IFN-γ) plus hypoxia-reoxygenation (H/R).

Figure 5. CORM-3 attenuates the combined effect of thrombin and IFN-γ on TNF-α production under normoxic and hypoxic conditions. (*A*) Cells were incubated with the combination of thrombin (10 U/ml) plus IFN-γ (10 ng/ml) alone or in the

presence of either CORM-3 (75 μM) or iCORM-3 (75 μM, negative control) for 24 h. At the end of the incubation period TNF-α production was measured as described in the Methods. (B) Cells treated as in A but incubated for 12 hrs under hypoxia and then reoxygenated in normal atmosphere for 24 hrs. At the end of the incubation period TNF-α production was measured. Cells treated with medium alone represent the control group. Bars represent the mean ± S.E.M. of 5-6 independent experiments per group. *$p<0.001$ vs. control; † $p<0.001$ vs. thrombin (Thr) plus interferon-γ (IFN-γ) or Thr+INF-γ+H/R.

Figure 6. The anti-inflammatory effect of CORM-3 is independent of endogenous HO activity. (*A*) Cells were treated with a combination of thrombin (Thr, 10 U/ml) and IFN-γ (10 ng/ml) alone or in the presence of tin protoporphyrin IX (SnPPIX, 10 μM). Simultaneously, CORM-3 (75 μM) or iCORM-3 (75 μM, negative control) were added to the solution and all incubated for 24 h. 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 ± S.E.M. of 5-6 independent experiments per group. *$p<0.001$ vs. control; $^{\ddagger}p<0.01$ vs. Thr+IFN-γ; $^{\dagger}p<0.05$ vs. Thr+IFN-γ+SnPPIX.

Figure 7. The anti-inflammatory effect of CORM-3 is amplified by PI3K inhibition. (*A*) Cells were treated with P38 MAPK inhibitor SB203580 (10 μM), JNK MAPK inhibitor SP600125 (25 μM), ERK MAPK inhibitor PD98059 (25 μM) and PI3K

inhibitor LY294002 (25 μM) in serum free medium for 1h. Then cells were exposed for 24 h to complete medium (10% FBS) containing thrombin (Thr, 10 U/ml)-IFN-γ (10 ng/ml) alone or in combination with either CORM-3 (75 μM) or iCORM-3 (75 μM, negative control). 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 TNF-α production determined after 24 h by an ELISA kit as described in Methods. Cells treated with medium alone represent the control group. Bars represent the mean ± S.E.M. of 5-6 independent experiments per group. * $p<0.001$ vs. control; $^{\dagger}p<0.001$ vs. Thr+IFN-γ; $^{\ddagger}p<0.05$ vs. Thr+IFN-γ; § $p<0.05$ vs. Thr+IFN-γ+CORM-3.

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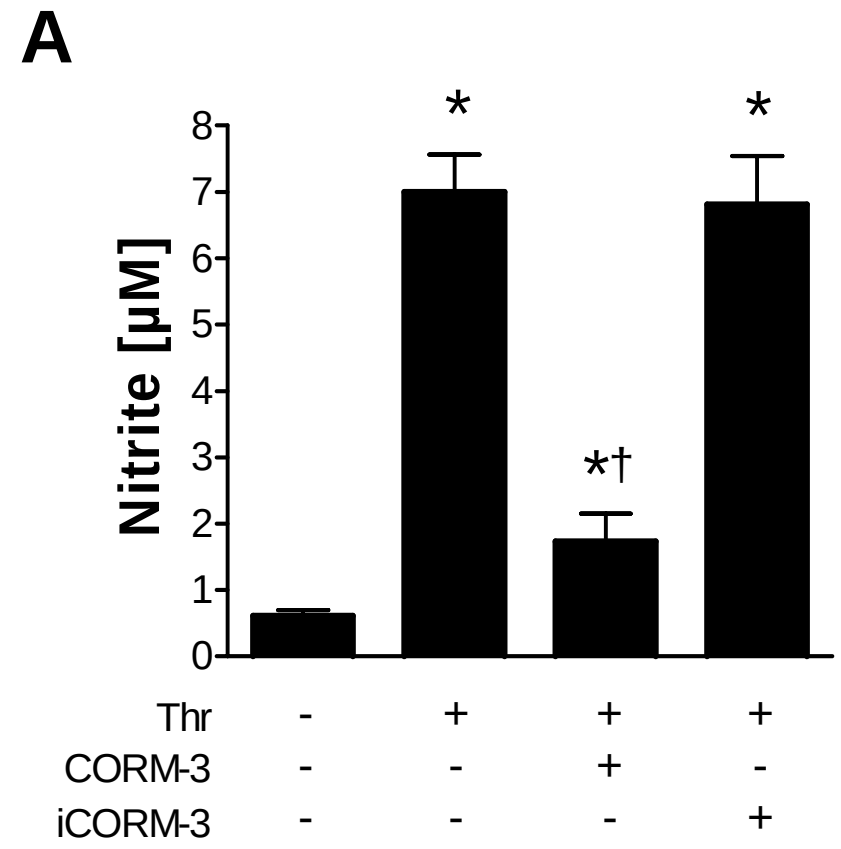
Figure 1



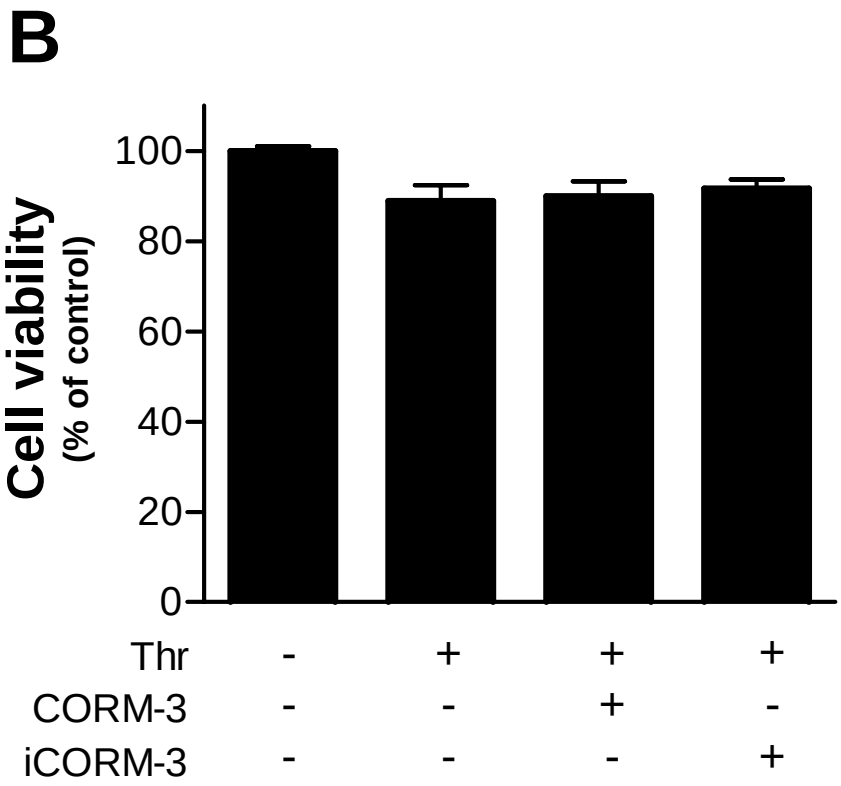


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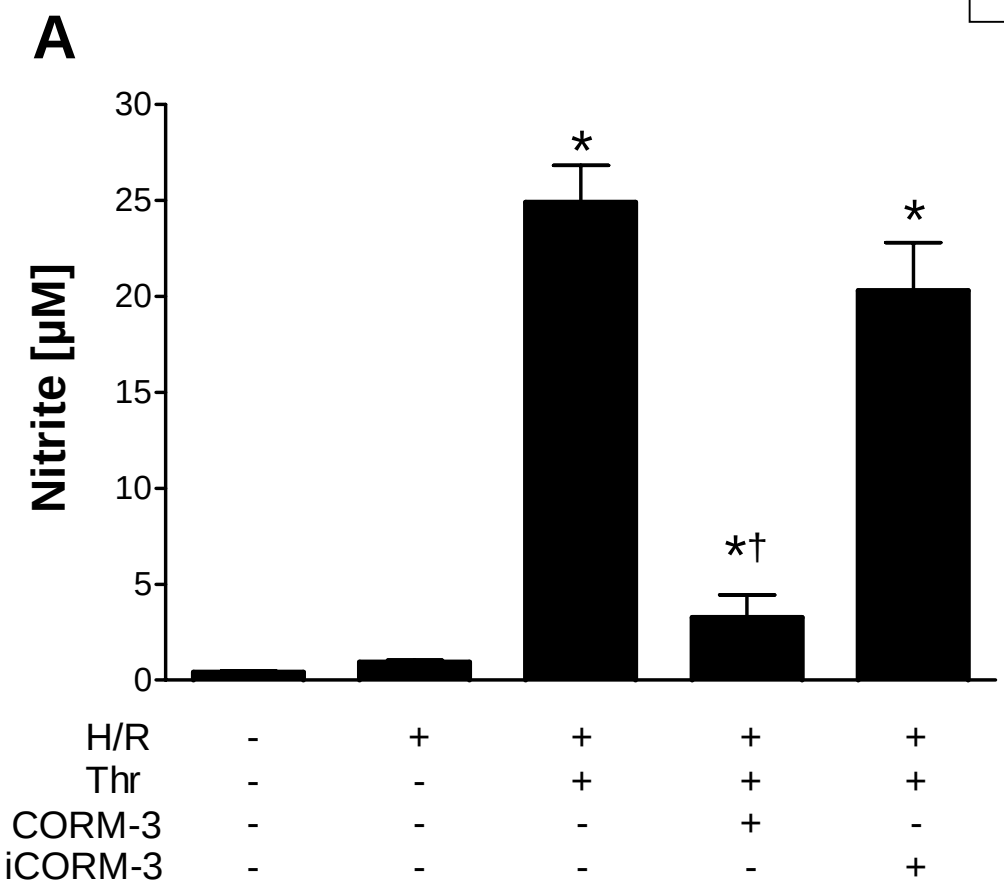
Figure 2



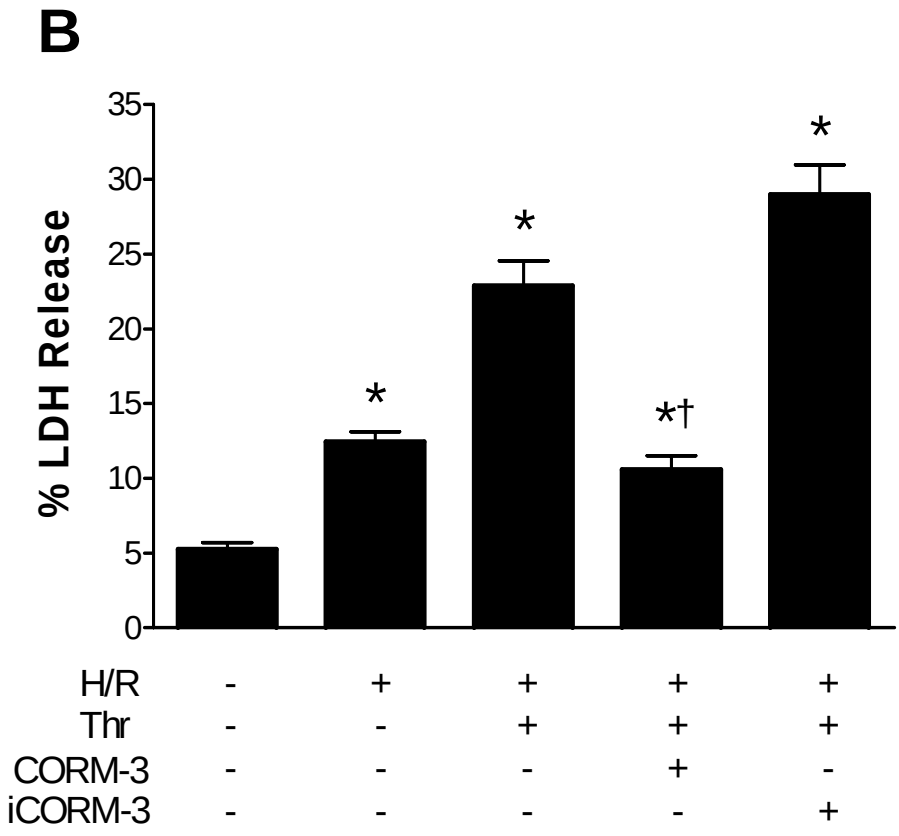


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Figure 3

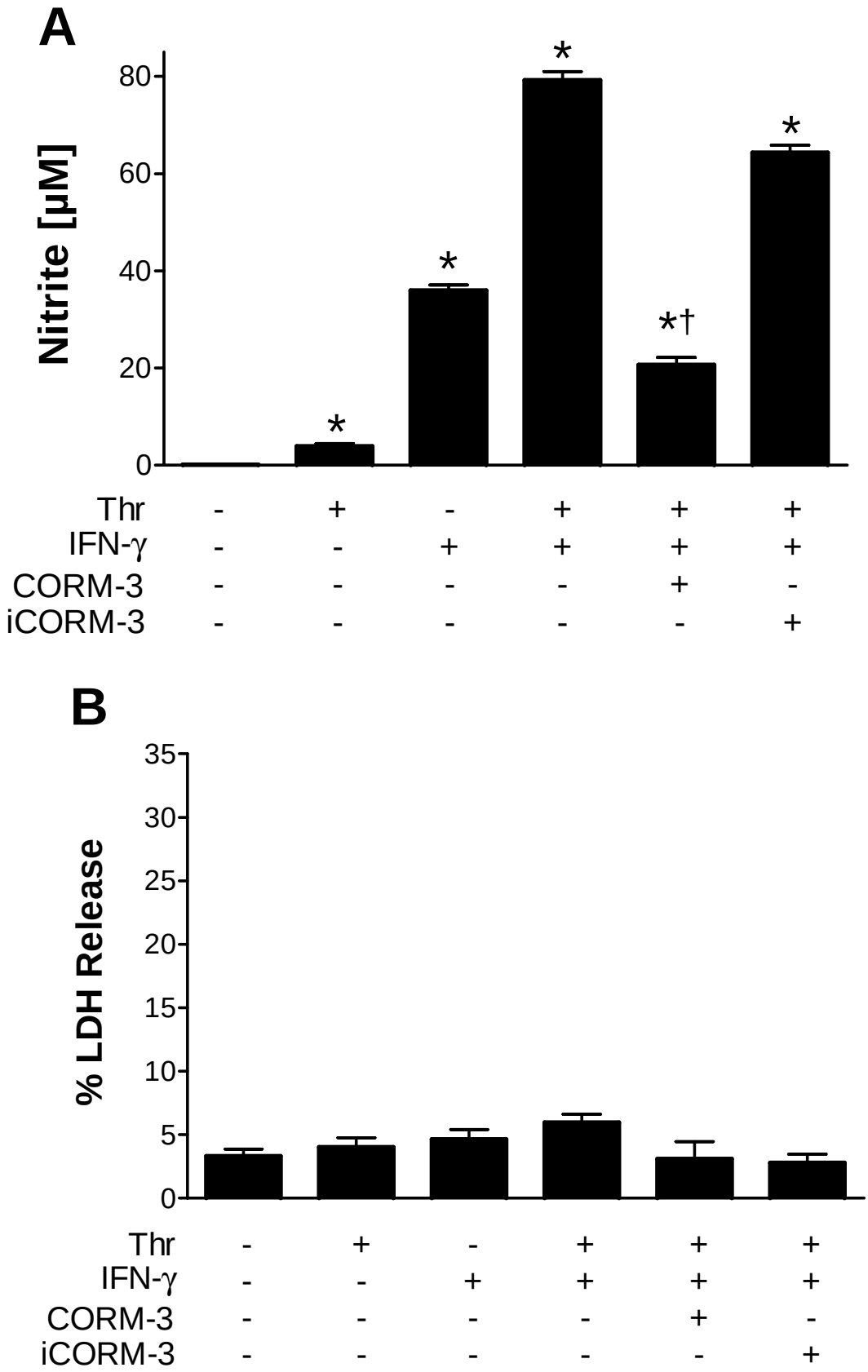

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Figure 4

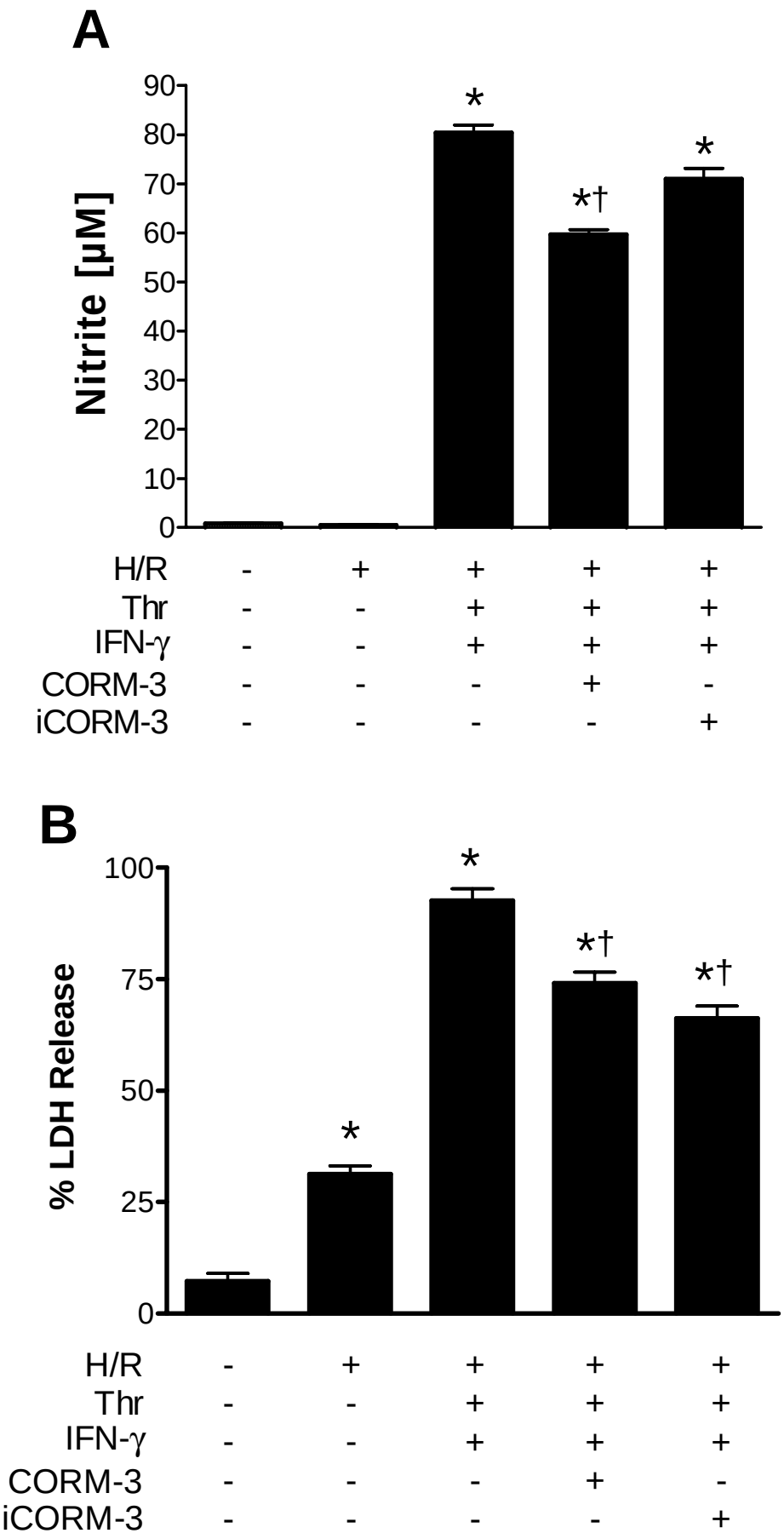

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Figure 5

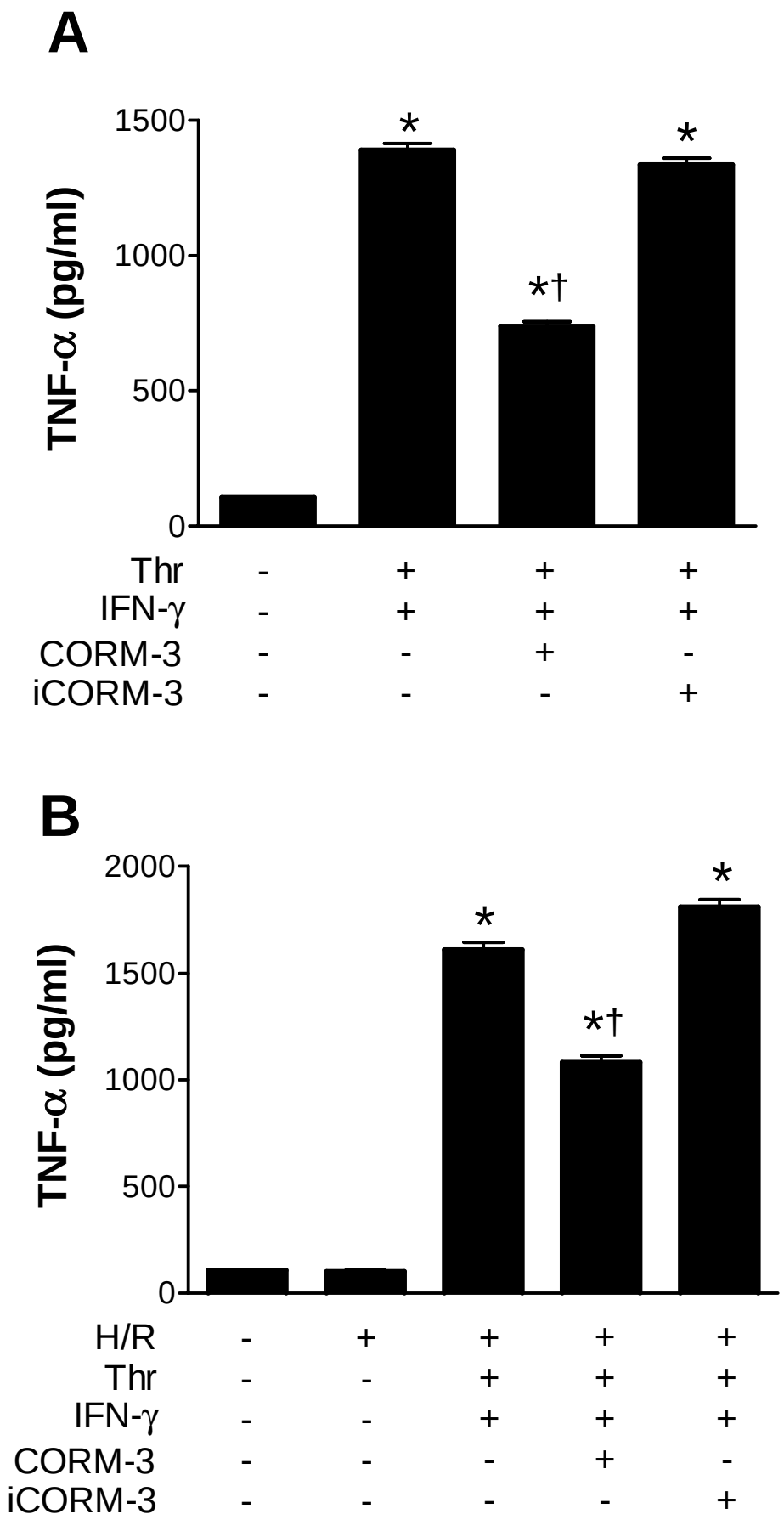

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Figure 6

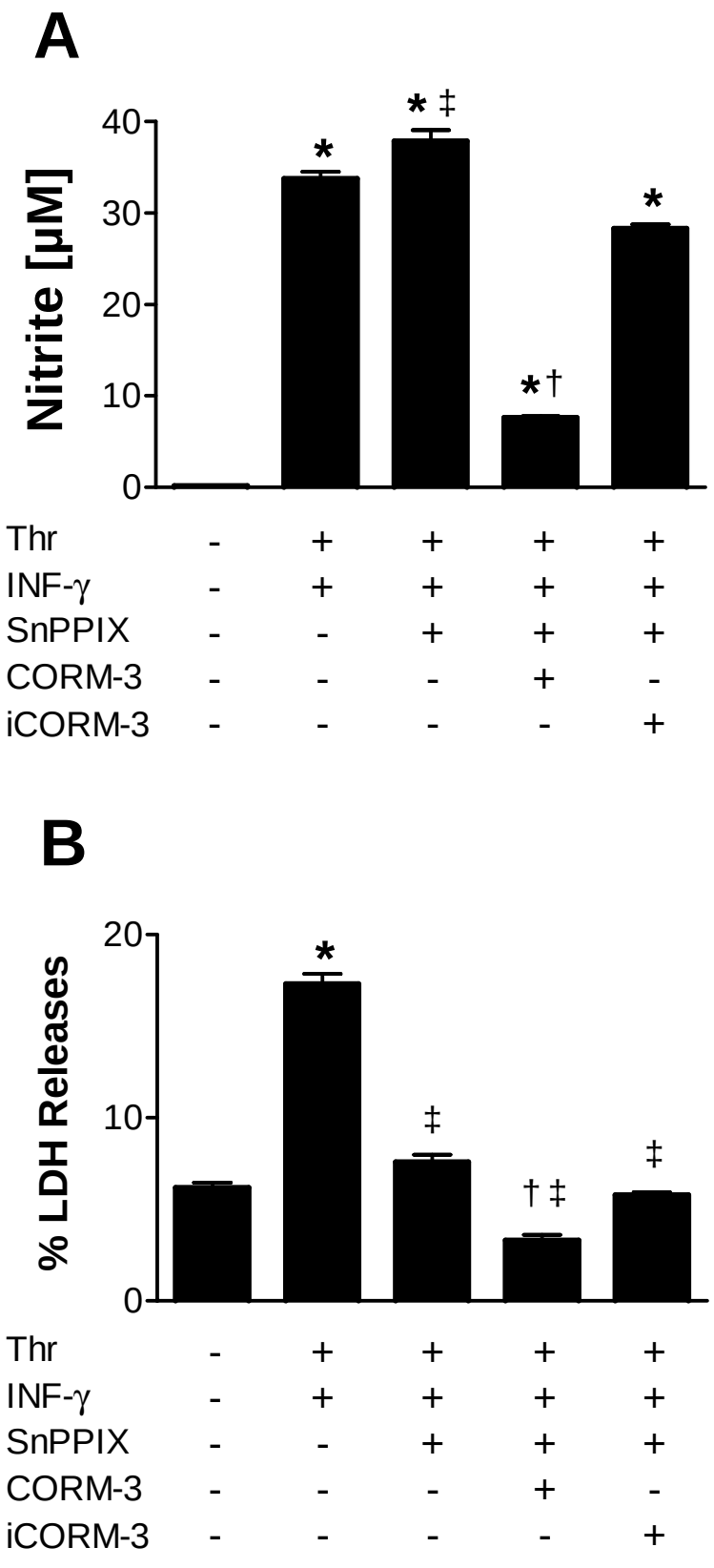

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Figure 7