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Differential Modulation by Exogenous Carbon Monoxide of TNF-α Stimulated Mitogen-Activated Protein Kinases in Rat Pulmonary Artery Endothelial Cells

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ABSTRACT

Heme oxygenase-1 (HO-1) is an enzyme that is highly inducible by various cellular stressors, especially oxidant injury. Our laboratory and others have demonstrated that induction of HO-1 exerts an antiinflammatory effect both *in vitro* and *in vivo*. We hypothesized that carbon monoxide (CO), a major catalytic byproduct of heme catalysis by HO-1, may mediate this antiinflammatory effect by modulating signal transduction pathways, in particular the mitogen-activated protein (MAP) kinase pathway. Confluent primary cultures of rat pulmonary artery endothelial cells (RPAEC) were treated with tumor necrosis factor-α (TNF-α; 50 ng/ml), and whole-cell extracts were assayed for phosphorylated ERK1/2, JNK1/2, and p38 MAP kinases. These three major MAP kinase pathways were activated by TNF-α in a time-dependent manner. RPAEC treated with TNF-α in the presence of a low concentration of CO (1%) exhibited marked attenuation of the phosphorylation of ERK1/2 MAP kinase when compared with cells treated with TNF-α alone. A similar effect was seen on the upstream MEK 1/2 kinase. Interestingly, CO (1%) accentuated TNF-α-induced phosphorylated p38 MAP kinase. These effects of exogenous CO on the ERK1/2 and p38 systems could be replicated by overexpression of HO-1 in RPAEC, using an adenoviral vector. As these MAP kinases are implicated in the regulation of various inflammatory molecules and adhesion molecules, our data provide a potential mechanism by which HO-1, acting via CO, may modulate the inflammatory response by differential activation of the MAP kinase pathway. Antioxid. Redox Signal. 4, 241–248.

INTRODUCTION

HEME OXYGENASE (HO) is the rate-limiting enzyme that catalyzes the conversion of heme to biliverdin, liberating free iron and carbon monoxide (CO) as by-products of the catabolic process (13). This enzyme, which exists in three isoforms in the body, HO-1, 2, and 3, is highly conserved through evolution, and deletions of the gene encoding the enzyme are highly lethal *in vivo*. Whereas HO-2 and HO-3 are constitutively expressed, HO-1 is inducible. Apart from physiological induction by its substrate heme, HO-1 is highly inducible by a variety of inflammatory insults, especially oxidative stressors involved in the inflammatory response (5). Recent evidence that mice (9) and humans (14) with a HO-1-deficient genotype exhibit increased inflammatory indices has confirmed earlier reports that induction of HO-1 has potent cytoprotective effects in several animal models of inflammation (4, 6). The exact mechanism by which HO-1 exerts this beneficial effect is not known, but bilirubin (11), ferritin (1), and CO (8) have all been implicated in mediating HO-1-induced cytoprotection.

The mitogen-activated protein (MAP) kinase system is a canonical phosphorylation pathway for the transduction of signals from cell membrane, through the cytoplasm to the nucleus, resulting in the activation of transcription factors. The three systems identified, extracellular regulated kinase

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(ERK), p38, and c-Jun N-terminal kinase (JNK), can serve to transduce the effects of inflammatory cytokines in a variety of cell types, including neutrophils, macrophages, and endothelial cells. We postulated that HO, acting through CO, could exert a modulating effect on the MAP kinase pathway, and thereby influence the inflammatory response. Earlier work in our laboratory has shown that gene transfer of HO-1 to rat lung is highly protective in a model of lung inflammation (7). Therefore, we used primary cultures of rat pulmonary artery endothelial cells (RPAEC) stimulated by tumor necrosis factor-α (TNF-α) to study the effects of CO, and adenovirally overexpressed HO-1, on the three MAP kinase pathways.

MATERIALS AND METHODS

Cell culture

Primary cultures of RPAEC, passages 12–22, were grown to confluence on cell culture dishes in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were placed in DMEM containing 0.5% FBS for at least 18 h prior to initiation of experiments. Immediately after the addition of TNF-α (GibcoBRL, Grand Island, NY, U.S.A.) to the culture medium in a final concentration of 50 ng/ml, the cells were incubated in chambers with gaseous CO at the desired concentration for the required duration, and harvested (see below).

Assay for phosphorylated MAP kinases

ERK1/2, p38, and JNK kinases were assayed with the Western Immunoblot kit (New England Biolabs Inc., Beverly, MA, U.S.A.). Confluent cell cultures were rinsed twice with ice-cold phosphate-buffered saline, then harvested with lysis buffer containing 62.5 m*M* Tris-HCl, 2% sodium dodecyl sulfate, 10% glycerol, 50 m*M* dithiothreitol and 0.1% bromphenol blue. The lysate was transferred to a microfuge tube on ice, sonicated to reduce sample viscosity, heated to 95–100°C for 5 min, and microcentrifuged for 5 min. Fifteen to 20 μl of this lysate was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane. Equal loading was confirmed by protein staining. After transfer, the membrane was washed with Tris-buffered saline containing 0.1% Tween 20, blocked with 5% nonfat dry milk in Tris-buffered saline/0.1% Tween 20, washed again, and incubated overnight at 4°C with primary antibody diluted 1:1,000 in Tris-buffered 5% bovine serum albumin. The primary antibodies used were specific for the phosphorylated forms of the three MAP kinases. After washing again, the membrane was probed with horseradish peroxidase-conjugated secondary antibody, and phosphorylated MAP kinase levels visualized by chemiluminescence technique. Equal loading was confirmed by probing the samples with nonphosphorylated MAP kinase antibody, supplied with the immunoblotting kit.

Assay for HO-1

The same protocol for western blotting as described for the MAP kinase assay was used, except that the primary antibody was a polyclonal anti-rat HO-1 antibody (Amersham, Piscataway, NJ, U.S.A.), used at a 1:4,000 dilution.

Adenoviral transfer of HO to rat pulmonary endothelial cells

Adenovirus vectors encoding HO-1 were added to monolayer cultures of RPAEC in serum-free DMEM at 100 pfu/cell and incubated with continuous shaking for 2–3 h, and then washed with serum-free DMEM and incubated at 37°C with DMEM containing 10% FBS for 18–22 h. These adenovirally infected cells overexpressing HO-1 were then stimulated with TNF-α, and assayed for phosphorylation of the MAP kinases.

CO exposures

Cell cultures were exposed to a gaseous mixture of CO (range of 10,000 ppm to 250 ppm), 5% carbon dioxide, and balance air in a plastic exposure chamber. Gas mixtures were obtained from AirGas, (Hartford, CT, U.S.A.).

Chemical inhibitors

The chemical inhibitors of cyclic guanosine monophosphate (cGMP), LY-83583 and ODQ [1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one] (Calbiochem, San Diego, CA, U.S.A.), were used at final concentrations of 20 μ*M* and 10 μ*M*, respectively. RPAEC cultures were pretreated for 1 h with the inhibitors, followed by stimulation with TNF-α and exposure to CO. The phosphatase inhibitor sodium orthovanadate (Calbiochem) was used at a final concentration of 1 m*M*. *N*-Acetylcysteine (Sigma, St. Louis, MO, U.S.A.) was used at a final concentration of 10 m*M*.

RESULTS

Stimulation of monolayer cultures of RPAEC with TNF-α resulted in time-dependent activation of all three MAP kinases (Fig. 1). Basal levels of ERK1/2 were very high in these cells, as others have noted, but increased by 30 min, peaking between 60 and 120 min. The p38 system was phosphorylated by 7.5 min, peaked by 15 min, and returned to basal levels by 30 min. The JNK MAP kinase was also activated in the same time frame. Time points of 15 and 60 min were used to evaluate the effects of exposure to 1% CO on TNF-α-induced stimulation of these MAP kinase pathways. Exposure to CO resulted in marked attenuation of ERK1/2 phosphorylation at 60 min, without any effect on the TNF-α-induced phosphorylation of the JNK pathway (Fig. 2, upper and lower panels). Conversely, exposure to CO resulted in *increased* phosphorylation of the p38 MAP kinase in response to TNF-α (Fig. 2, middle panel). Importantly, these effects were also noted, though to a significantly lesser extent, upon exposure to 250 ppm of CO (Fig. 3). In addition, exposure to CO was also noted to reduce the TNF-α-induced phosphorylation of the MAP kinase kinase (MEK) 1/2 kinase upstream of ERK (Fig. 4).

Overexpression of HO-1 in RPAEC also modulated TNF-α-induced stimulation of the MAP kinase pathway in a similar manner. Phosphorylation of ERK1/2 was reduced at 60

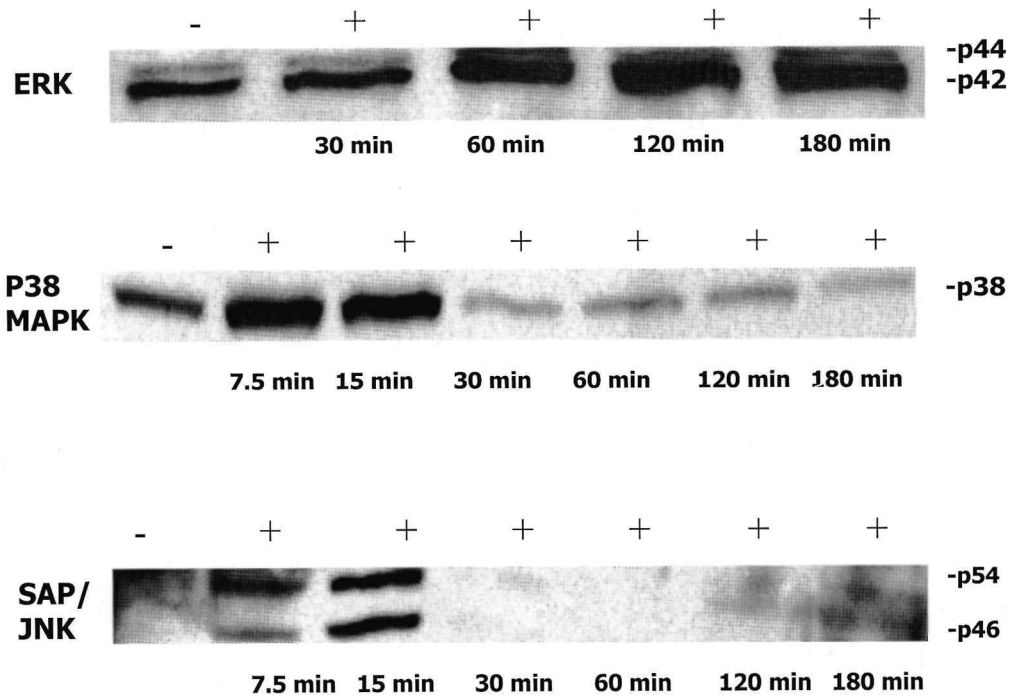

FIG. 1. Effect of TNF-α on phosphorylation of MAP kinases in RPAEC. Confluent monolayers of RPAEC were incubated with 50 ng/ml TNF-α (+) or without (−), for the durations indicated. A representative western blot of the time course of induction of phosphorylated ERK, p38, and stress-activated protein (SAP)/JNK kinases is shown.

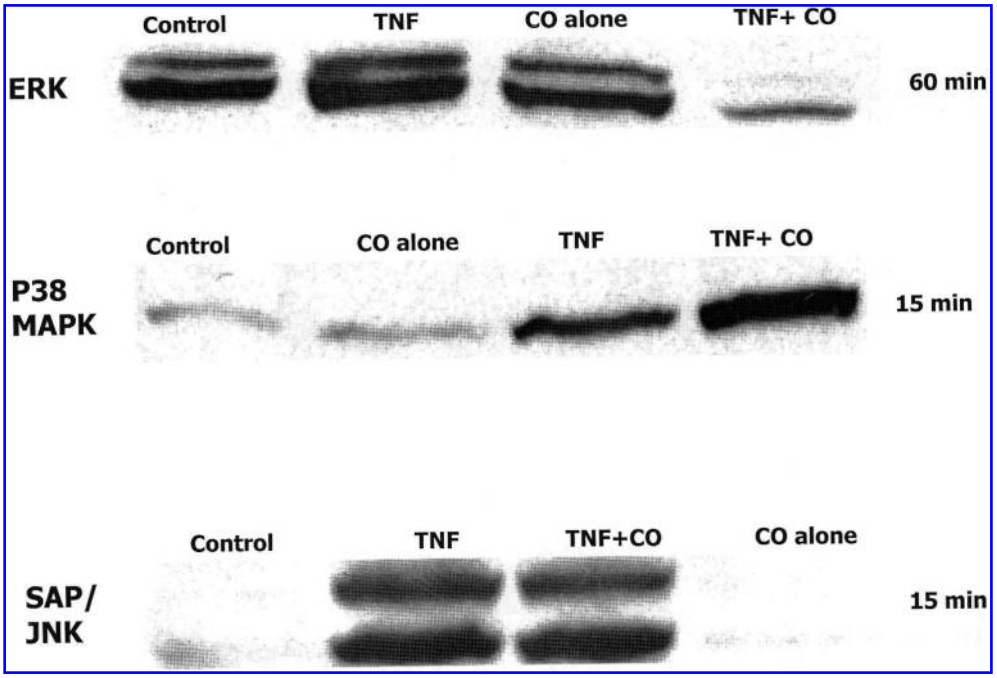

FIG. 2. Effect of 1% exogenous CO on the phosphorylation of MAP kinases after treatment with TNF-α in RPAEC. Confluent monolayers of RPAEC were incubated with TNF-α and exposed to either 1% CO in 5% CO_2 balance air, or 5% CO_2 balance air alone for the durations indicated. Control cells were not manipulated with TNF-α or exposure to CO. Confluent monolayers exposed to CO alone without treatment with TNF-α are indicated. A representative western blot of the phosphorylation of ERK, p38, and stress-activated protein (SAP)/JNK kinases is shown.

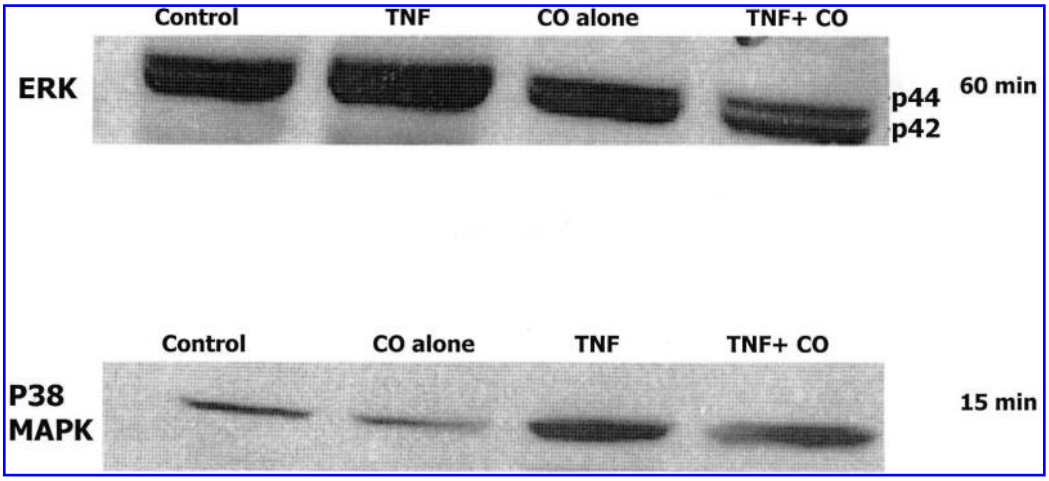

FIG. 3. Effect of 250 ppm exogenous CO on phosphorylation of ERK and p38 MAP kinases after treatment with TNF-α in RPAEC. Confluent monolayers of RPAEC were incubated with TNF-α and exposed to either 250 ppm CO in 5% CO_2 balance air, or 5% CO_2 balance air alone for the durations indicated. Control cells were not manipulated with TNF-α or exposure to CO. Confluent monolayers exposed to CO alone without treatment with TNF-α are indicated. A representative western blot of the phosphorylation of ERK, and p38 kinases, is shown.

min, in the cells overexpressing HO-1 as compared with cells overexpressing β-galatosidase (Fig. 5, upper panels). Phosphorylation of p38 in response to TNF-α was increased at 15 min in cells overexpressing HO-1 as compared with cells overexpressing β-galactosidase, with minimal phosphorylation of p38 in unmanipulated cells (control) (Fig. 5, lower panel).

To investigate the mechanisms by which exposure to CO was able to modulate the MAP kinase systems, we studied the effects of pretreatment with the phosphatase inhibitor orthovanadate. Basal levels of both phosphorylated ERK and p38 were elevated in the presence of orthovanadate, and the effects of CO on reduction of TNF-α-induced ERK phosphorylation were inhibited (Fig. 6). Orthovanadate exhibited an additive effect on the ability of TNF-α and CO to induce p38 phosphorylation. In the presence of two inhibitors of the cGMP pathway, different patterns of activation were noted (Fig. 7). Pretreatment with LY-83583 alone elevated basal concentrations of both p38 and ERK1/2 MAP kinases, implying that these pathways are tonically under the inhibitory control of cGMP. After pretreatment with LY-83583, exposure to CO could not attenuate the elevated phosphorylation of ERK1/2. Pretreatment with LY-83583 augmented the phosphorylation of p38 by TNF-α and CO to the high levels seen with LY-83583 pretreatment alone. In the presence of ODQ, another inhibitor of cGMP, basal levels of phosphorylated ERK were elevated, and could not be significantly attenuated by CO. ODQ pretreatment did not significantly affect the augmented phosphorylation of p38 in response to TNF-α and CO. Finally, pretreatment of rat pulmonary artery cells with *N*-acetylcysteine attenuates TNF-α-induced phosphorylation of ERK1/2 to a similar extent as CO, suggesting that this effect of CO may be mediated by reduction of intracellular oxidative stress (Fig. 8).

DISCUSSION

HO-1 was described in 1968 as the enzyme responsible for the oxidative catabolism of the α-methene bridge of the heme moiety, liberating equimolar amounts of CO, biliverdin, and iron. The free iron is rapidly sequestered in fer-

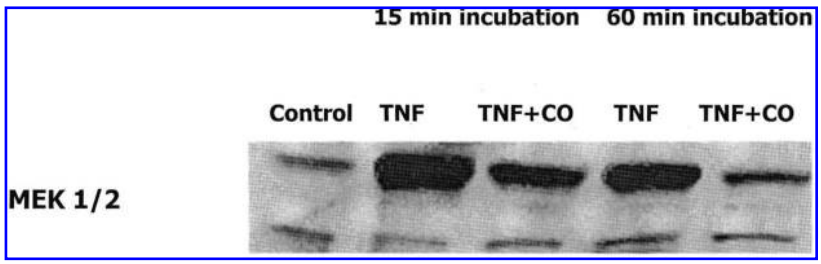

FIG. 4. Effect of 1% exogenous CO on phosphorylation of MEK 1/2 kinase after treatment with TNF-α in RPAEC. Confluent monolayers of RPAEC were incubated with TNF-α and exposed to either 1% CO in 5% of CO_2 balance air or 5% CO_2 balance air alone for the durations indicated. Control cells were not manipulated with TNF-α or exposure to CO. A representative western blot of the phosphorylation of MEK 1/2 kinase is shown.

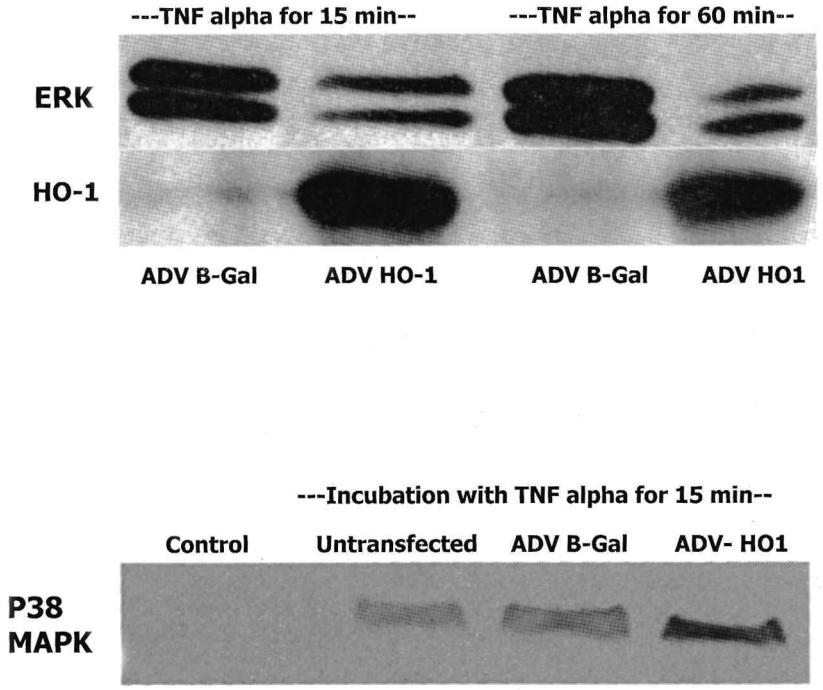

FIG. 5. Effect of adenoviral vector-mediated overexpression of HO-1 in RPAEC on phosphorylation of ERK and p38 kinases. Confluent monolayers of RPAEC were transfected with adenoviral vectors containing HO-1 and β-galactosidase (control) inserts, and then analyzed by western blotting for content of HO-1 and phosphorylated MAP kinases, under conditions of stimulation with TNF-α. Representative western blots are shown.

ritin, whereas biliverdin is subsequently reduced to bilirubin, also a potent antioxidant. The biological importance of this enzyme is demonstrated by its highly conserved structure (rat and human HO-1 are 90% identical) and its presence in all forms of aerobic life, including mammals, plants, algae, and bacteria. The role of inducible HO-1 in protecting against hyperoxia, inflammation, and transplant rejection has become known only recently, but the mechanism by which this protection is exerted remains elusive. CO, however, has long been known for its toxicity, binding with high affinity to hemoglobin and thus impairing oxygen transport in animals with a cardiovascular system, and poisoning cytochrome a_3.

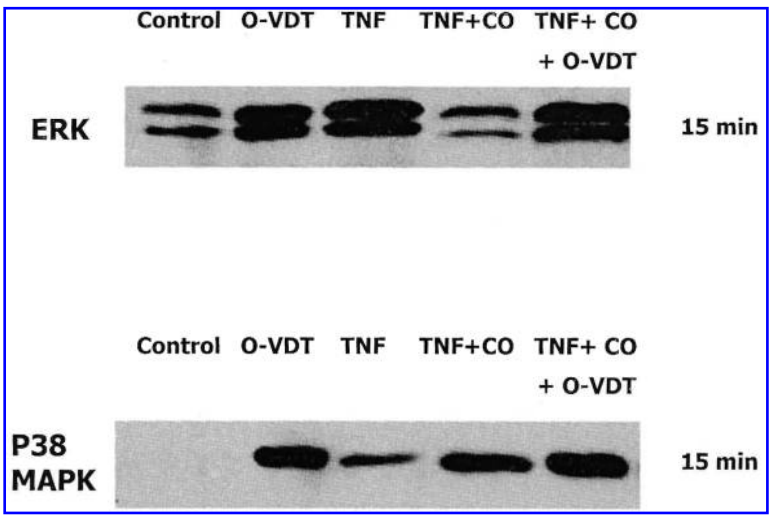

FIG. 6. Effects of pretreatment for 1 h with orthovanadate (O-VDT) on the phosphorylation state of the p38 and ERK kinases after exposure to TNF-α and CO. Confluent cell cultures of RPAEC were treated with orthovanadate to inhibit phosphatase activity, and then treated with TNF-α and exposed to CO. Representative western blots of ERK and p38 kinase phosphorylation state are shown.

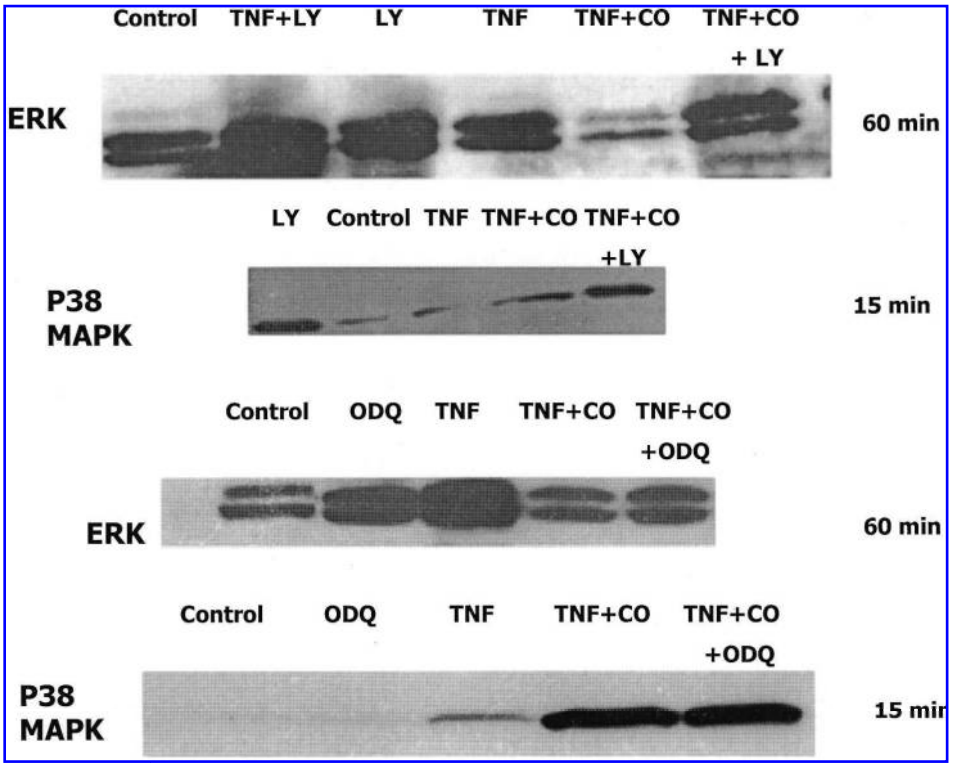

FIG. 7. Effects of pretreatment for 1 h with LY-83583 and ODQ on the phosphorylation of ERK and p38 kinases after exposure to TNF-α and CO. Confluent cell cultures of RPAEC were pretreated with LY-83583 (**upper panel**) and ODQ (**lower panel**) to inhibit cGMP, and then exposed to TNF-α and exogenous CO. Representative western blots of phosphorylation of ERK and p38 kinases are shown.

CO can be detected in elevated concentrations in the exhaled air of animals and humans suffering from a wide variety of inflammatory disorders, raising the possibility that, in lower than toxic concentrations, the gas may mediate some or all of the antiinflammatory effects of HO-1.

The MAP kinases constitute hierarchical phosphorylation cascades responsible for transducing inflammatory signals from the cell surface through to the nucleus, resulting in cellular activation and the production of cytokines. Thus, for example, in human neutrophils the ERK family of MAP kinases, activated by n-form-met-leu-phe, is involved in a broad array of activation responses, including calcium influx, superoxide production, and granule enzyme release (10). Similarly, lipopolysaccharide (LPS) stimulates the p38 MAP kinase, resulting in TNF-α release. *In vivo,* selective chemical inhibition of p38 abrogates TNF-α release and acute lung injury in a rat model of pancreatitis-associated lung injury (15). Therefore, modulation of the MAP kinase system is one potential mechanism for the ability of HO-1 or CO to exert an antiinflammatory effect, a hypothesis borne out by the data presented in this article.

CO has recently been shown to reduce production of TNF-α from macrophages in the presence of LPS, by up-regulating p38 MAP kinase, thus acting in an anti-inflammatory manner (8). The inhibition of the phosphorylation of ERK1/2, shown here, explains how CO can oppose the effects of TNF-α in yet another manner. Phosphorylation of ERK1/2 is known to induce the nuclear activating factors activator protein-1 and nuclear factor-κB, both of which induce the expression of adhesion molecules on endothelium and induce the production of cytokines such as interleukin-8 from endothelial cells. Therefore, by suppressing ERK phosphorylation by TNF-α,

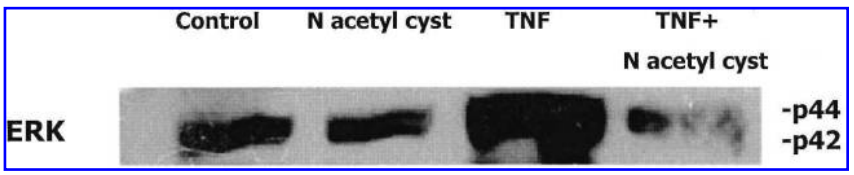

FIG. 8. Effects of pretreatment for 1 h with *N*-acetylcysteine on the phosphorylation of ERK in response to stimulation with TNF-α. Confluent cell cultures of RPAEC were pretreated with *N*-acetylcysteine to scavenge oxidant radicals, and then exposed to TNF-α. Western blots of phosphroylated ERK are shown.

CO has the capacity to greatly down-regulate TNF-α-induced inflammation. The data from our experiments do not necessarily extend to other cell types and stimuli, however. For example, in our experiments with RPAEC, CO was able to attenuate the TNF-α-induced phosphorylation of ERK1/2, in contrast to RAW cells, where LPS-induced phosphorylation of ERK1/2 was unaffected by CO (8). This pattern of stimulus- and cell-specific regulation of the MAP kinases is well described (3). Further, the downstream effects of activation of the MAP kinases also differ in a cell- and stimulus-specific manner.

It is unclear if the differential effects of CO on the TNF-α-stimulated MAP kinase pathways noted in this article constitute toxic, pharmacological, or potentially physiological effects. The concentration of CO used for the earlier experiments, 1%, is unequivocally toxic, but the reproducibility of the same effects with lower doses of CO (250 ppm) in subsequent experiments suggests that these effects are biologically significant. These lower concentrations are well within the nontoxic range for small animals. For example, rats exposed to 500 ppm of CO for prolonged periods demonstrate no adverse physiological effects (12). Moreover, the ability of CO to increase the phosphorylation of TNF-α-stimulated p38 kinases, while suppressing the phosphorylation of ERK1/2 and leaving phosphorylation of JNK unaffected, argues against a generalized CO-poisoning effect on cellular function.

No explanation exists at present for the mechanism by which CO exerts these effects on the MAP kinases. None of these serine-threonine proteases contains a heme group that would bind to CO, but cGMP does contain a heme group, is known to modulate the MAP kinases, and could be a potential target (2). Our experiments with chemical inhibition of cGMP cannot confirm that this is the case, especially in view of the ability of these inhibitors to independently raise basal levels of MAP kinase phosphorylation. Other data from our laboratory show that, in RAW cells, CO-augmented phosphorylation of p38 in response to LPS is cGMP-independent (8). In our experiments, exposure of the cell cultures to CO within a half minute after the addition of TNF-α to the media brought about the effects noted, which represents a surprising and significant departure from the paradigm that pretreatment with an inhibitor is needed to abrogate the effect of a rapidly acting inflammatory stimulus like TNF-α *in vitro*. This finding is likely indicative of the potency of CO, its rapid diffusion into cells, and potentially, that it acts to up-regulate an as yet unknown, counterinflammatory system that opposes the effects of TNF-α. Such a counterinflammatory system could be set in place by TNF-α and augmented by exposure to CO, a paradigm that could help explain the absence of an effect of CO alone, in the absence of TNF-α, on the MAP kinases. Alternatively, CO could inhibit an ongoing injurious effect of TNF-α that stimulates the MAP kinase system, such as free-radical generation, as suggested by our experiments with *N*-acetylcysteine, a free radical scavenger. These pathways remain to be elucidated.

Finally, overexpression of HO-1 in cultured endothelial cells by *in vitro* adenoviral transfer had the same effect on the MAP kinase systems as did CO. This constitutes the strongest evidence yet that CO may, indeed, be the effector molecule for at least some of the antiinflammatory effects of HO-1, specifically those exerted by modulating the MAP kinases.

Conclusion

In summary, our data indicate that CO can differentially modulate signaling through each of the three families of MAP kinases, effects that help explain the ability of this gaseous molecule to suppress cytokine production and the inflammatory effects induced by TNF-α. The ability to produce these effects in concentrations that are well tolerated by animals and possibly nontoxic in humans as well, and the lack of a need for pretreatment to produce these effects, bring the future therapeutic use of the gas to suppress inflammation into the realm of possibility.

ABBREVIATIONS

cGMP, cyclic guanosine monophosphate; CO, carbon monoxide; DMEM, Dulbecco's modified Eagle medium; ERK, extracellular regulated kinase; FBS, fetal bovine serum; HO-1, heme oxygenase-1; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; ODQ, 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one; RPAEC, rat pulmonary artery endothelial cells; TNF-α, tumor necrosis factor-α.

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