Inhibition of cellular respiration by endogenously produced carbon monoxide

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Summary

Endogenously produced nitric oxide (NO) interacts with mitochondrial cytochrome *c* oxidase, leading to inhibition of cellular respiration. This interaction has been shown to have important physiological and pathophysiological consequences. Exogenous carbon monoxide (CO) is also known to inhibit cytochrome *c* oxidase in vitro; however, it is not clear whether endogenously produced CO can inhibit cellular respiration and, if so, what the significance of this might be. In this study, we show that exogenous CO inhibits respiration in a moderate but persistent manner in HEK293 cells under ambient (21%) oxygen concentrations (K_i=1.44 μM). This effect of CO was increased (K_i=0.35 μM) by incubation in hypoxic conditions (1% oxygen). Endogenous CO, generated by HEK293 cells transfected with the inducible isoform of haem oxygenase (haem oxygenase-1; HO-1), also inhibited cellular respiration moderately (by 12%) and this was accompanied by inhibition (23%) of cytochrome *c* oxidase activity. When the cells were incubated in hypoxic conditions during HO-1 induction, the inhibitory effect of CO on cell respiration was markedly increased to 70%. Furthermore, endogenously produced CO was found to be responsible for the respiratory inhibition that occurs in RAW264.7 cells activated in hypoxic conditions with lipopolysaccharide and interferon-γ, in the presence of *N*-(iminoethyl)-L-ornithine to prevent the synthesis of NO. Our results indicate that CO contributes significantly to the respiratory inhibition in activated cells, particularly under hypoxic conditions. Inhibition of cell respiration by endogenous CO through its interaction with cytochrome *c* oxidase might have an important role in inflammatory and hypoxic conditions.

Key words: Hypoxia, Carbon monoxide, Nitric oxide, Respiration, Mitochondria

Introduction

Haem oxygenases (HO) mediate the first step in haem catabolism. These enzymes catalyse the oxidative degradation of haem to biliverdin and carbon monoxide (CO), accompanied by the release of ferrous iron. Three mammalian isoforms of HO have been identified (HO-1, HO-2 and HO-3). HO-2 and HO-3 are expressed constitutively; HO-1 is induced by a variety of stimuli, including haem, heat shock, pro-inflammatory cytokines, hypoxia and bacterial endotoxin, and has a much higher specific activity (Maines et al., 1986; Ryter et al., 2002).

Traditionally, CO has been viewed as a toxic metabolite of haem catabolism. However, it has been shown to be capable of binding to, and subsequently activating, soluble guanylate cyclase (sGC) (Brune and Ullrich, 1987; Ramos et al., 1989; Verma et al., 1993). Under in vitro conditions, the potency of CO as an activator of sGC has, however, been reported to be much lower than that of NO (Stone and Marletta, 1994). In spite of this, several studies have suggested that endogenously produced CO can significantly increase cellular cGMP concentrations (Ingi and Ronnett, 1995; Zakhary et al., 1997; Zufall and Leinders-Zufall, 1997).

In addition to its effects upon sGC, CO has also been shown to increase the activity of calcium-activated potassium channels (K_{Ca}) (Wu et al., 2002). This phenomenon is involved in the relaxation of rat-tail artery strips (Wang and Lu, 1997) and in reversing the hypoxia-induced inhibition of the K_{Ca} channels (Riesco-Fagundo et al., 2001). More recently, it has been demonstrated that HO-2 actually forms part of the channel complex and controls channel activity in hypoxia (Williams et al., 2004).

Exogenously added CO has also been shown to possess anti-inflammatory and anti-apoptotic effects (Otterbein et al., 2000; Brouard et al., 2000). These actions of CO are reported to be due, at least in part, to the activation of the p38 mitogen-activated protein kinase (MAPK) pathway, although the precise mechanism by which CO activates p38 MAPK is still unclear.

Cytochrome *c* oxidase (CCO), the terminal enzyme of the electron transport chain, is known to interact with both CO and NO (Petersen, 1977; Brown and Cooper, 1994). Binding of NO to CCO leads to an oxygen (O_2)-dependent inhibition of mitochondrial respiration, and endogenous NO has been shown to be a physiological regulator of cellular O_2 consumption (Clementi et al., 1999). The interaction of NO with CCO has recently been shown to have various other physiological and pathophysiological consequences (Trimmer et al., 2001; Hagen et al., 2003; Almeida et al., 2004; Xu et al., 2004). Although CO can also bind to CCO and inhibit its catalytic activity

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(Hansen and Nicholls, 1978), it is not clear at present whether endogenously produced CO can modulate cell respiration and what might be the consequences of such an action for cellular function.

We have therefore investigated the effects of exogenous and endogenously produced CO on cell respiration in both ambient (21% O_2) and hypoxic (1% O_2) conditions. In addition, we have evaluated the effects of hypoxia on the activities of the two enzymes HO-1 and inducible nitric oxide synthase (iNOS), both of which are induced in inflammation and generate gases that inhibit CCO.

Results

Inhibition of cellular respiration by exogenous CO

To characterise the inhibition of cellular respiration by CO, O_2 consumption of HEK293 cells was measured with different concentrations of CO (5-20 μM). CO inhibited respiration in a concentration-dependent manner (Fig. 1A, insert). At concentrations of O_2 between 100 μM and 10 μM, control cells respired at a rate of 18.62±0.74 μM/minute (Fig. 1A). However, in cells treated with 20 μM CO, cellular respiration was inhibited by 40% when measured at concentrations of O_2 between 30 μM and 10 μM (from 18.62±0.74 μM/minute in controls to 11.63±0.75 μM/minute, *n*=3; Fig. 1A). By comparison, the release of NO following the addition of 5 μM 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEANO) almost completely inhibited cellular respiration. In addition, the inhibition by CO lasted for at least 30 minutes (Fig. 1B). Following hypoxic (1% O_2) incubation for 10 minutes, the same concentration of CO inhibited respiration by 75% (from 19.14±0.82 μM/minute in controls to 4.92±0.52 μM/minute, *n*=3; Fig. 2). The K_i of CO for CCO in HEK293 cells was calculated to be 1.44 μM and 0.35 μM under ambient and hypoxic conditions, respectively.

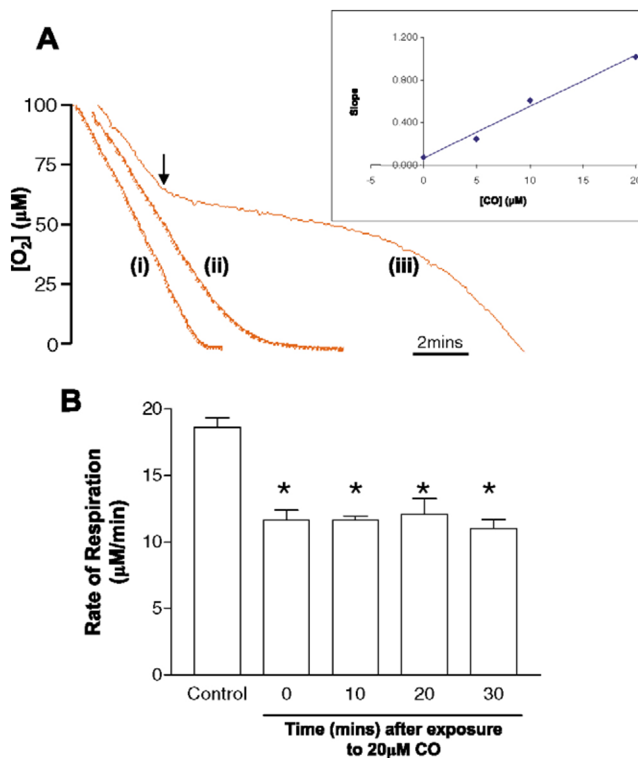

Fig. 1. Inhibition of cellular respiration by exogenous CO. (A) (i) Tracing of O_2 consumption of control HEK293 cells. (ii) Tracing of O_2 consumption of cells treated with 20 μM CO. (iii) Tracing of O_2 consumption showing the effect of 5 μM DEANO (addition indicated by arrow). The rate of respiration recovers after the NO is degraded. Insert: the slopes obtained from the Lineweaver-Burk plot were plotted against [CO] (secondary plot). The points plotted were obtained from the means of at least three independent experiments. The K_i of CO on respiration calculated by interpolation to the *x*-axis was 1.44 μM. (B) The rates of respiration of HEK293 cells were measured 0, 10, 20 and 30 minutes after the addition of 20 μM CO, *n*=3. Tracings are representative of at least three independent experiments. **P*<0.01 compared with control.

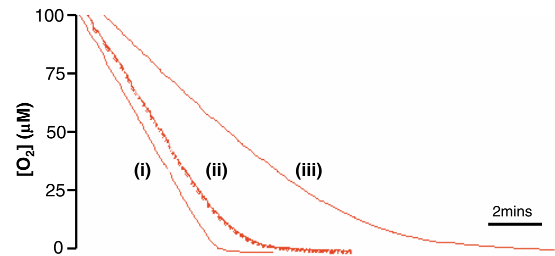

Fig. 2. Effect of hypoxia (1% O_2 for 10 minutes) on the inhibition of cellular respiration by exogenous CO in HEK293 cells. (i) Tracing of O_2 consumption of control cells incubated in hypoxia. (ii) Tracing of O_2 consumption of cells treated with 20 μM CO under ambient O_2 concentrations. (iii) Tracing of O_2 consumption of cells treated with 20 μM CO in hypoxia. No difference was observed between untreated control cells kept under ambient O_2 concentrations and those incubated in hypoxia. The K_i of CO for cells in hypoxia was calculated to be 0.35 μM. Tracings are representative of at least three independent experiments.

Inhibition of cellular respiration by endogenous CO

To study the effects of endogenously produced CO, a HEK293 cell line with stable expression of HO-1 under the control of a tetracycline-inducible promoter was generated (HEK293-HO-1 cells). Tetracycline-induced expression of HO-1 protein was confirmed by western blot analysis (Fig. 3A). In preliminary experiments, maximal expression was observed at 24 hours post-induction using 1 μg/ml of tetracycline. These conditions were used for investigating the effects of endogenously produced CO on respiration.

To characterise our system, we compared the quantity of HO-1 protein expressed by our induced HEK293-HO-1 cells with the amount induced by other stimuli in Hep3B cells. We observed that HO-1 expression in HEK293-HO-1 cells was significant and exceeded that observed in Hep3B cells treated with 50 μg/ml lipopolysaccharide (LPS), 200 μM $CoCl_2$ or 10 μM haemin (Fig. 3A).

When comparing O_2 consumption of induced with non-induced HEK293-HO-1 cells, we observed a 12% decrease (from 18.57±1.06 μM/minute in controls to 16.33±0.87 μM/minute, *n*=3) in the rate of respiration, measured in the O_2 range of 30-10 μM. As with exogenously added CO, hypoxic incubation markedly increased this inhibitory effect to 70% (from 18.35±0.74 μM/minute in controls to 5.24±0.67 μM/minute, *n*=3; Fig. 3B). These results suggest that

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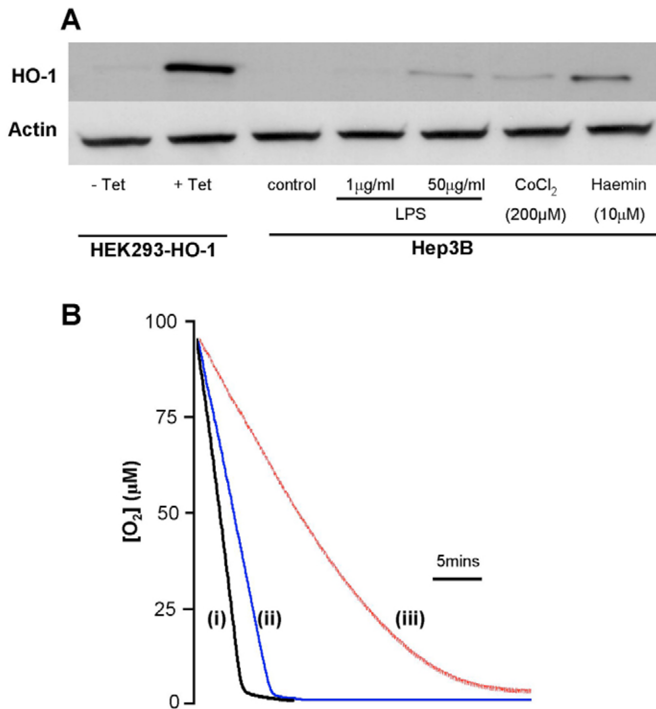

Fig. 3. Induced HEK293-HO-1 cells expressed HO-1 protein and exhibited a decreased rate of respiration. (A) HEK293-HO-1 cells were induced to express HO-1 protein by treatment with 1 μg/ml tetracycline (Tet) for 24 hours. The expression was compared with that of Hep3B cells treated with known inducers of HO-1; LPS (1 μg/ml and 50 μg/ml), $CoCl_2$ (200 μM) and haemin (10 μM). (B) The respiration of induced HEK293-HO-1 cells incubated under ambient O_2 concentrations (ii) and hypoxic conditions (iii) was decreased compared with that of non-induced cells (i). Tracings and western blots are representative of at least three independent experiments.

endogenous CO generated by HO-1 inhibited mitochondrial respiration, most probably as a result of its interaction with CCO. To confirm this, CCO activity was measured and compared in induced and non-induced cells. CCO activity was inhibited by 23% (from 221.7±9.6 nmol/minute/mg in controls to 170.8±9.1 nmol/minute/mg, n=3) in the induced cells incubated under ambient O_2 concentrations. In addition, we determined the redox state of cytochrome a_3 by visual light spectroscopy (Hollis et al., 2003) and observed that it was reduced by 25%, consistent with an inhibition of electron flux by CO.

In agreement with the data obtained with exogenous CO, inhibition of respiration by endogenous CO is enhanced after cell incubation in hypoxia. It is possible that hypoxia alters HO-1 expression or activity directly. To determine whether there is any effect of hypoxia on enzyme expression, we analysed HO-1 protein by western blotting at ambient and 1% O_2. As shown in Fig. 4A, no difference in HO-1 expression was observed in these cells. By measuring the rate of bilirubin synthesis (activity of HO-1) and total extracellular bilirubin, we confirmed that hypoxia did not affect the activity of the enzyme, since no difference was observed in the activity of the enzyme, nor in the bilirubin concentration, when incubated under ambient or 1% O_2 (Fig. 4B,C). Thus, the greater inhibition of respiration after induction of HO-1 under hypoxic conditions is likely to be a direct consequence of the increased binding of CO to CCO at this low O_2 concentration.

Endogenously produced CO does not activate the p38 MAPK pathway

To determine whether endogenously produced CO is able to activate the p38 MAPK pathway, we measured p38 phosphorylation in our HEK293-HO-1 cells by western blotting. The known p38 activators anisomycin (25 μg/ml for 20 minutes) and nitrosoglutathione (GSNO; 1 mM for 8 hours)

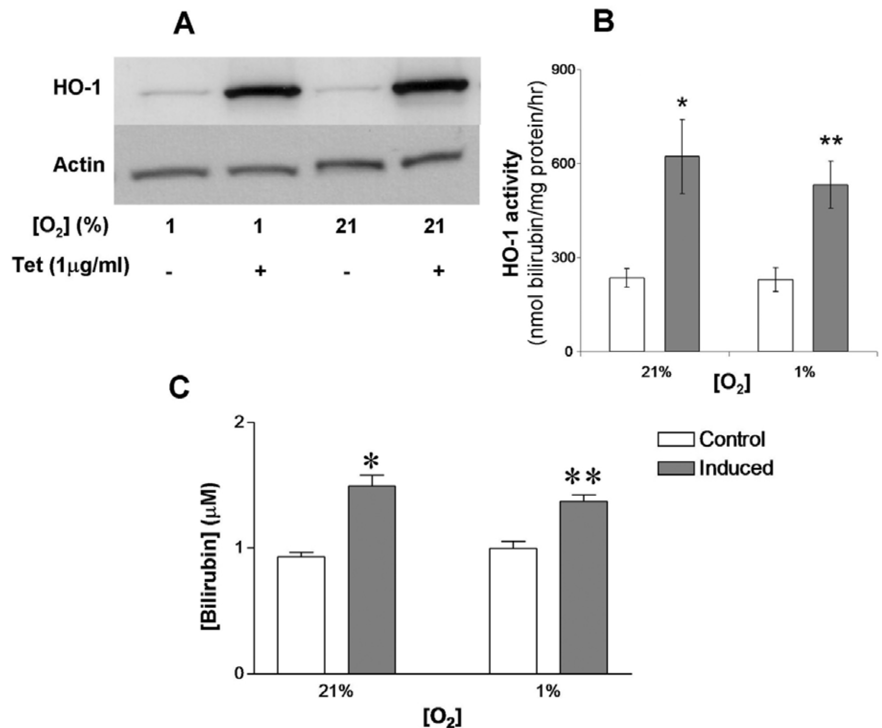

Fig. 4. The expression and activity of HO-1 protein in HEK293-HO-1 cells is not altered by incubation under hypoxic conditions. (A) Western blot of cell extracts from induced (24 hours) and non-induced cells incubated under hypoxic (1% O_2) and ambient (21% O_2) conditions; Tet, tetracycline. (B) HO-1 activity is not affected by incubation under hypoxic or ambient conditions. (C) The concentrations of bilirubin measured in the culture medium. n=3. *P<0.05 compared with control at 21% O_2; **P<0.01 compared with control at 1% O_2.

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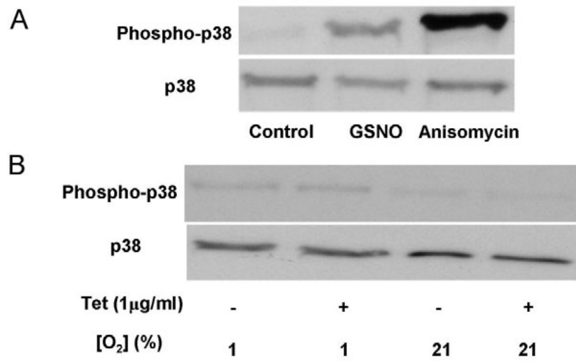

Fig. 5. Endogenously produced CO does not activate the p38 MAPK pathway in HEK293-HO-1 cells. (A) Phosphorylation of p38 (phospho-p38) was observed following treatment with GSNO (1 mM for 8 hours) and anisomycin (25 μg/ml for 20 minutes). (B) p38 phosphorylation was not observed in our induced HEK293-HO-1 cells after 24 hours in either ambient or hypoxic conditions; Tet, tetracycline. Blots are representative of at least three independent experiments.

both induced p38 phosphorylation (Fig. 5A). By contrast, induction of HO-1 under both ambient O_2 and hypoxic conditions was without effect on p38 phosphorylation (Fig. 5B).

The NO-independent inhibition of respiration in activated RAW264.7 cells correlates with the appearance of HO-1 protein

Activation of macrophages is known to lead to induction of iNOS and the generation of NO, which results in inhibition of cellular respiration (Brown et al., 1998). In addition, HO-1 is induced by activation of cells, and our results raise the possibility that CO contributes to the respiratory inhibition under these conditions. To test this, we treated RAW264.7 cells with LPS and interferon-γ (IFNγ) for 12 hours in the absence or presence of 500 μM of the NOS inhibitor *N*-(iminoethyl)-L-ornithine (L-NIO) to distinguish between the effects of CO and NO. Activation of RAW264.7 cells induced the expression of HO-1 and iNOS protein, both in the absence and presence of L-NIO (Fig. 6A). In activated cells, cellular O_2 consumption was almost totally inhibited, but in the presence of L-NIO, which completely blocked NO synthesis, the inhibition of O_2 consumption was reduced to 20% (from 14.27±0.57 μM/minute in controls to 11.57±0.34 μM/minute, *n*=3-4). When the cells were activated at 1% O_2, this NO-independent inhibitory effect was increased to 60% (from 11.33±0.42 μM/minute in non-activated controls to 4.92±0.40 μM/minute in activated cells, *n*=3-4; Fig. 6B). In addition, CCO activity was decreased from 166.60±12.56 to 53.09±13.62 nmol/minute/mg protein (*n*=4) following cell activation for 12 hours in the presence of L-NIO under ambient O_2 concentrations, and from 75.08±19.21 to 25.22±7.74 nmol/minute/mg protein (*n*=4) under hypoxic conditions. Our results suggest that the observed NO-independent inhibition of respiration upon activation of RAW264.7 cells is a result of binding of CO to CCO.

NO synthesis is greatly reduced in hypoxia

To evaluate further the potential contribution of NO and CO to the inhibition of mitochondrial respiration under inflammatory conditions, we investigated the effect of exposure to 1% O_2 on the generation of NO in activated macrophages. As determined by the Griess assay and an NO electrode, we found that NO production was significantly elevated following activation of RAW264.7 cells with LPS and IFNγ under ambient O_2 concentrations. Under hypoxic conditions, the production of nitrite, a metabolite of NO, was reduced by 85% (178.02±8.90 μM to 27.15±2.60 μM; *n*=3-5) in cells activated for 24 hours (Fig. 7A). Hypoxia did not alter the expression of iNOS protein (Fig. 6A), so the decrease in nitrite was likely to be a result of a decrease in enzyme activity. In addition, the concentration of NO in the incubation medium, measured using an NO electrode, was 0.3 μM at 10 μM O_2, compared with 2.3 μM at 100 μM O_2 (Fig. 7B). Furthermore, the K_m of iNOS for O_2 was found to be between 20 and 30 μM in our cells.

Thus, the production of NO is markedly reduced in hypoxia; this contrasts with that of CO, whose synthesis we have shown to be unaffected by hypoxia

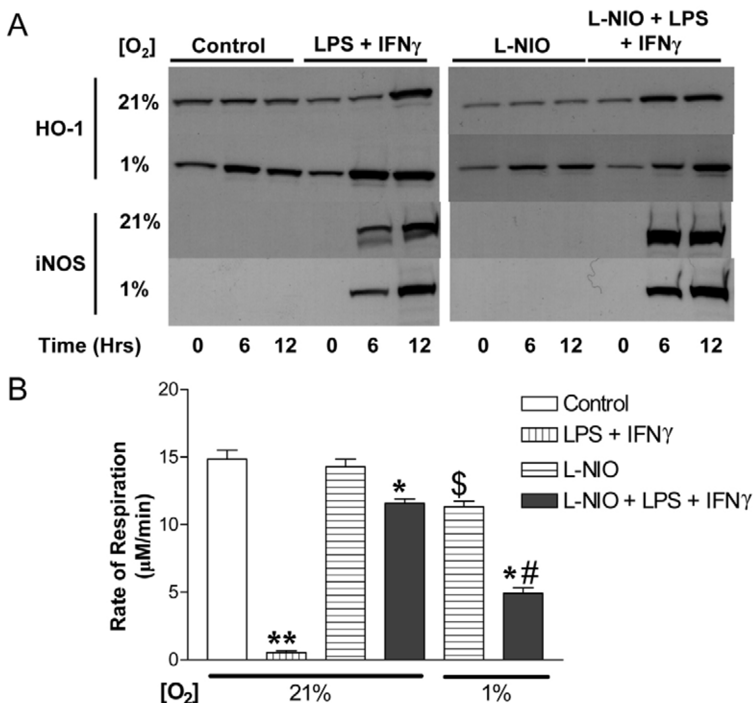

Fig. 6. Effect of activation of RAW264.7 cells on the expression of HO-1, iNOS and the rate of respiration. (A) Cells that were activated for 12 hours in the presence or absence of L-NIO (500 μM) under ambient O_2 or hypoxic conditions expressed HO-1 and iNOS protein. Hypoxia itself induced HO-1 but not iNOS. (B) The respiration of activated cells was almost completely inhibited. Cells activated in the presence of L-NIO did not synthesise NO but still exhibited an inhibition of respiration that was increased by hypoxic incubation. *n*=3-4. $^{**}P<0.01$ compared with control; $^{*}P<0.01$ compared with respective L-NIO-treated controls; $^{\$}P<0.01$ compared with L-NIO-treated cells maintained at 21% O_2; $^{\#}P<0.01$ compared with cells activated in the presence of L-NIO at 21% O_2.

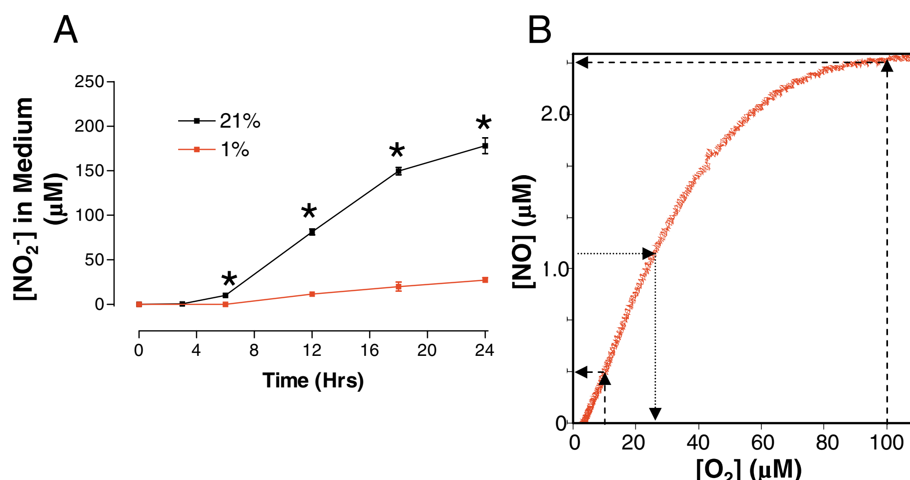

Fig. 7. The synthesis of NO is inhibited by hypoxia. (A) The production of NO by RAW264.7 cells activated for 24 hours in hypoxia was inhibited by 85%, as determined by measurement of its metabolite nitrite (NO_2^-). (B) The synthesis of NO decreased from a concentration of 2.3 μM at 100 μM O_2 to 0.3 μM at 10 μM O_2 (indicated by dashed arrows). The K_m of iNOS for O_2 in RAW264.7 cells was between 20 and 30 μM (indicated by the dotted arrows). *n*=3. **P*<0.01 compared with cells activated at 1% O_2.

in HO-1-expressing HEK293 cells. Our results suggest that the CO-mediated inhibition of respiration becomes particularly significant at low O_2 concentrations.

Hypoxic incubation of RAW264.7 cells induces the expression of HO-1 and is accompanied by a decrease in cellular respiration

Hypoxia has been reported to be a stimulus for the induction of HO-1 (Morita et al., 1995). To investigate this further, RAW264.7 cells were incubated in hypoxic conditions (1% O_2) and HO-1 protein and cellular respiration was measured. The expression of HO-1, but not iNOS, protein was upregulated following hypoxic incubation (Fig. 6A) and this was accompanied by a decrease in cellular respiration in the cells. The rate of respiration, in the range 30-10 μM on the O_2 consumption traces, was initially 11.26±0.91 μM/minute (*n*=3), decreased to 6.68±1.35 μM/minute (41% inhibition; *n*=3) after 12 hours at 1% O_2 and was reduced further to 4.41±1.85 μM/minute (61% inhibition; *n*=3) after 24 hours. CCO activity was also decreased from 157.15±6.03 to 88.25±4.31 nmol/minute/mg protein (44% inhibition, *n*=3-4) by hypoxic incubation for 12 hours. These results are consistent with CO binding to, and inhibiting, CCO under hypoxic conditions.

Discussion

Endogenous NO is capable of inhibiting mitochondrial O_2 consumption by CCO, the terminal enzyme of the electron transport chain, in a manner that is reversible and competitive with O_2 (Clementi et al., 1999). This interaction has several physiological and pathophysiological consequences (Clementi et al., 1999; Trimmer et al., 2001; Hagen et al., 2003; Almeida et al., 2004; Xu et al., 2004). Like NO, CO is produced endogenously and can bind to CCO (Petersen, 1977). However, the effects of exogenous CO on cellular O_2 consumption have not been investigated in detail and the possibility that endogenous CO might also affect respiration has not been reported. Here we show that, although less effective than NO, endogenous CO is able to inhibit cellular respiration under ambient O_2 concentrations. However, this effect is markedly enhanced under hypoxic (1% O_2) conditions.

When HEK293 cells were treated with solutions of CO gas, the rate of O_2 consumption was found, as expected, to decrease in a dose-dependent manner. This is in agreement with the observations of others (Alonso et al., 2003). Furthermore, this inhibition was greater when cells were incubated under hypoxic conditions. From our data we have calculated that the K_i of CO for CCO was 1.44 μM following incubation under ambient O_2 concentrations and this was decreased to 0.35 μM at 1% O_2, indicating that CCO has a greater affinity for CO in hypoxia. These values are similar to those previously reported in purified CCO and for CCO contained in proteoliposomes (Petersen, 1977; Hansen and Nicholls, 1978). They indicate that CO is a much less potent inhibitor of CCO than is NO, which has an IC_{50} of 60 nM at 30 μM O_2 (Brown and Cooper, 1994) and a K_i of 27 nM (Koivisto et al., 1997). This difference in potency between NO and CO could be a result of the redox state of cytochrome a_3. CO is only able to bind to CCO when cytochrome a_3 is reduced (in the ferrous form), whereas NO is capable of binding to CCO when cytochrome a_3 is in the reduced or the oxidized form. Under low O_2 conditions, the electron transport chain is in a more reduced state (Palacios-Callender et al., 2004), which might favour the inhibition of CCO by CO. This, together with a lower O_2 concentration, could explain the increased potency of CO as an inhibitor of CCO in hypoxia.

CO can be synthesised enzymatically by haem oxygenases as a product of haem catabolism. In previous experiments, exogenous CO was used. The concentrations of CO produced endogenously are unknown and it remains unclear whether CO can inhibit respiration in physiological quantities. We have now shown that HEK293 cells expressing the HO-1 protein are capable of producing CO, as indicated by increased bilirubin production (Tenhunen et al., 1969), in sufficient amounts to inhibit cellular respiration. This was accompanied by an inhibition of CCO activity that is consistent with CO binding to, and inhibiting, the enzyme. As with exogenous CO, the inhibition of respiration was also increased by incubation in 1% O_2. Since HO-1 is an O_2-dependent enzyme, we investigated the effect of lowering the O_2 tension to 1% on HO-1 activity. We found that there was no change in HO-1 activity or in the total bilirubin produced. The K_m of HO-1 for O_2 has been reported to be 12 μM (Piantadosi, 2002). However, in our system, we observed no significant change in activity of the enzyme by lowering the O_2 concentration to 1% (10 μM).

CO has been reported to exert anti-apoptotic and anti-

inflammatory effects by activation of the p38 MAPK pathway, which involves the phosphorylation of p38 (Otterbein et al., 2000; Brouard et al., 2000). The concentrations of exogenous CO used in studies in vitro and in vivo to stimulate the p38 MAPK pathway are typically several hundred parts per million (Otterbein et al., 2000; Song et al., 2002; Otterbein et al., 2003). In our transfected cells producing CO, we did not observe p38 phosphorylation at CO concentrations that inhibited cellular respiration. However, it has been suggested that the availability of the substrate haem is the limiting factor for HO-1 activity, and it has been proposed that, under stress, more intracellular haem becomes available, resulting in increased HO-1 activity and the synthesis of more CO (Maines, 1997). It thus remains possible that, under such conditions, endogenously produced CO can lead to the activation of the p38 MAPK pathway.

The expression of both HO-1 and iNOS is induced following cell activation (Wiesel et al., 2000; Connelly et al., 2003; Srisook and Cha, 2004). In our RAW264.7 cells, we observed an inhibition of respiration even when iNOS activity was completely blocked by L-NIO, suggesting an NO-independent mechanism. Several findings support a role of CO in this NO-independent respiratory inhibition: (1) HO-1 protein was induced by cell activation; (2) CCO activity was found to be inhibited in cells activated in the presence of L-NIO; (3) the inhibition of respiration was increased when activated cells were incubated under hypoxic conditions; and (4) unlike NO production, the synthesis of CO was not reduced under conditions of hypoxia. Taken together, these observations suggest that the NO-independent inhibition of respiration in the activated RAW264.7 cells was a result of CO produced by HO-1.

When RAW264.7 cells were activated under ambient conditions in the absence of L-NIO, nitrite, a metabolite of NO, accumulated in the culture medium. The concentration of NO, measured by an NO electrode, produced by the cells was approximately 2 μM at 12 hours post-activation. Under hypoxic conditions, the nitrite produced was 6.5-fold less, indicating that O_2 was limiting for NO synthesis. These results are consistent with previous reports that show that iNOS activity is affected by the availability of O_2. The K_m of iNOS in cells for O_2 has been shown to be 137 μM (McCormick et al., 2000). By contrast, it has been reported that the K_m of the purified iNOS protein for O_2 is much lower, at 6 μM (Rengasamy and Johns, 1996). In our cells, however, we obtain a value of between 20 and 30 μM for the K_m of iNOS for O_2. This difference compared with the value reported by McCormick et al. might be due to the maintenance of our cells in biological stirrer bottles, which could provide a more homogeneous O_2 concentration throughout the medium than in culture plates. The fact that we have shown that HO-1 activity was unaffected by hypoxic incubation would suggest that, at low O_2 tensions, CO produced by HO-1 might be capable of inhibiting mitochondrial respiration, whereas the effects of NO might be less significant as a result of the inactivation of iNOS.

We observed that hypoxic incubation alone induced the expression of HO-1 in our RAW264.7 cells; this observation is in agreement with other reports using a variety of cells (Morita et al., 1995; Panchenko et al., 2000; Garnier et al., 2001). The induction of the protein was accompanied by a decrease in the rate of respiration. This phenomenon was not observed in non-induced HEK293-HO-1 cells, which did not upregulate HO-1 in response to hypoxia, and has not been reported to do so in similar cells. In addition, CCO activity was measured in RAW264.7 cells incubated in hypoxic conditions for 12 hours and was decreased by 44% compared with non-hypoxic controls. This is consistent with our hypothesis that CO inhibits respiration, particularly at low O_2 concentrations, and suggests a possible function under hypoxic conditions. Previous reports have similarly described a decrease in CCO activity in hypoxia (Chandel et al., 1996). This has been interpreted as a direct reaction between O_2 and CCO, whereby the enzyme is allosterically modified. If this were the case, respiration in our non-induced HEK293-HO-1 cells would be reduced following hypoxic incubation. However, this was not observed.

Although others have shown that CO can dose-dependently inhibit CCO activity (Alonso et al., 2003), we have now demonstrated that both exogenously added and endogenously produced CO can inhibit cellular O_2 consumption in a manner that is dependent on the O_2 concentration in the environment. Furthermore, we have investigated the role that NO and CO might have in the inhibition of respiration in activated RAW264.7 cells, a situation that mimics what could occur in inflammation. We found that both these gases have a role in the inhibition of cellular respiration under these conditions and that their contribution differs depending on the O_2 tension, as a result of the differential inactivation of their synthesizing enzymes by low O_2. As yet, the consequences of these effects are unclear. However, our findings raise the possibility that, by inhibiting CCO, CO could elicit similar physiological and pathophysiological effects as have been reported for NO (Clementi et al., 1999; Trimmer et al., 2001; Hagen et al., 2003; Almeida et al., 2004; Xu et al., 2004). For instance, by inhibiting mitochondrial respiration, CO might regulate the endoplasmic reticulum stress response in a similar manner to NO (Xu et al., 2004). It has also been shown that the inhibition of respiration by NO under hypoxic conditions results in the redistribution of intracellular O_2 (Hagen et al., 2003). It is possible that hypoxia-induced upregulation of HO-1, and the subsequent inhibition of respiration by CO, might also be involved in regulating intracellular O_2 availability for other cellular processes. Inhibition of CCO also compromises mitochondrial ATP production and leads to increased production of reactive O_2 species by the mitochondrial electron transport chain. Thus, our results also raise the possibility that CO, like NO, might contribute to mitochondrial dysfunction in inflammatory conditions such as sepsis and to hypoxia-reperfusion injury in ischaemic diseases.

Materials and Methods

Maintenance of cells

HEK293 (human embryonic kidney) and Hep3B (human hepatoma) cells were cultured in DMEM supplemented with 10% heat-inactivated New Zealand foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Cells were grown in 175 cm^2 tissue culture flasks and kept below 90% confluency. The cells were maintained in a humidified incubator set at 37°C with 95% air and 5% CO_2.

RAW264.7 (murine monocyte) cells were obtained from American Type Culture Collection. They were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated New Zealand foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). Cells were maintained in suspension in biological stirrer bottles (Techne) with a continuous stirrer speed of 25 rpm and kept in a humidified incubation chamber set at 37°C with 95% air and 5% CO_2.

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Preparation of CO solutions

Fresh stock solutions of CO gas were prepared each day by the method described by Naseem and Bruckdorfer (Naseem and Bruckdorfer, 1995). Briefly, double-distilled water was boiled for 15 minutes, and allowed to cool to 60°C while being pulled under vacuum into specially adapted gas sampling tubes. 10 ml of the water was then flushed with nitrogen gas (BOC Gases) for 45 minutes and the tubes were then sealed with gas-impermeable rubber septa. CO gas (BOC Gases) was then bubbled into the flushed solutions for 1 hour. The concentration of CO in solution was determined spectrophotometrically by measuring the conversion of deoxymyoglobin to carbon monoxymyoglobin, as previously described (Motterlini et al., 2002).

Determination of K_i values

To calculate the K_i values of CO for CCO, Lineweaver-Burk and secondary plots were constructed. The reaction velocity (rate of O_2 consumption) was measured in the presence of a fixed concentration of inhibitor (CO) and varying substrate (O_2) concentrations. The velocity was then plotted against the substrate concentration in a double reciprocal (Lineweaver-Burk) plot. The slopes obtained were then plotted against the inhibitor concentrations (secondary plot). The K_i was calculated by interpolation to the *x*-axis.

Visual light spectroscopy

Visual light spectroscopy of cytochrome a_3 was performed on cells in suspension as previously described (Hollis et al., 2003). Briefly, cells were suspended at a concentration of 3×10^7 cells/ml in Krebs solution. The redox state of the cytochrome was then detected simultaneously with cellular O_2 consumption. The composition of the Krebs solution was NaCl (118 mM), KCl (4.8 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (1 mM), Hepes (25 mM) and glucose (20 mM). The solution was then adjusted to pH 7.2.

Incubation under hypoxia

Experiments that were performed under hypoxic (1% O_2) conditions were carried out within a humidified anaerobic chamber (Coy Laboratory Products). The chamber was digitally set at the required O_2 concentration and was maintained at 37°C by a thermostatic controller. For experiments involving O_2 consumption, cells were incubated in hypoxia for designated lengths of time and were then reoxygenated immediately prior to measurement of respiratory rates.

Generation of HEK293-HO-1 cells

Full-length human HO-1 was amplified by PCR from human foetal brain cDNA. An *Xho*I restriction site and a Kozak consensus sequence (GACGAG) were included in the PCR primers at the 5′ end, and an *Xba*I site at the 3′ end. The following PCR primers were used: 5′-CACTCGAGGACGAGATGGAGCGTCCGCAACCCG-3′ (forward) and 5′-CATCTAGATTACATGGCATAAAGCCCTAC-3′ (reverse). The PCR product was then gel purified and, after addition of an ‘A overhang’, subcloned into pGEM-T Easy. The sequence was confirmed by DNA sequencing, and the HO-1 gene was transferred to the pcDNA4/TO plasmid, using the *Xho*I and *Xba*I restriction sites. TREX293 cells (Tet-on HEK293 cells) were then transfected with the HO-1-pcDNA4/TO expression plasmid, and stable clones were isolated using zeocin selection.

Measurement of HO-1 activity

The activity of HO-1 in cells was determined as previously described (Motterlini et al., 1996). Briefly, cell extracts were added to a reaction mixture and incubated in the dark at 37°C for 1 hour. The reaction was then halted by the addition of 1 ml chloroform and the concentration of bilirubin produced was calculated by the difference in absorption between 464 and 530 nm, with a molar extinction coefficient of 40 $mM^{-1}cm^{-1}$. The reaction mixture consisted of potassium phosphate buffer (100 mM, pH 7.4) containing NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 units), 3 mg of rat liver cytosol as a source of biliverdin reductase, $MgCl_2$ (0.2 mM) and haemin (20 μM).

Measurement of extracellular bilirubin concentration

In experiments where bilirubin was measured, the culture vessel was wrapped in foil to protect it from light. Treatments were performed and 1 ml aliquots of culture medium were collected at the allotted time points. The aliquots were then centrifuged for 5 minutes at 13,000 rpm to remove any cells or debris. A 0.5 ml sample was collected from the resultant supernatant and added to 250 mg of $BaCl_2$. The mixture was then vortexed vigorously prior to the addition of 0.75 ml benzene. The mixture was vortexed again and then centrifuged for 30 minutes at 13,000 rpm. The upper benzene layer, containing the bilirubin, was then extracted and its absorbance was measured at 450 nm with a reference at 600 nm. The concentration of bilirubin in the sample was then calculated using the molar extinction coefficient of bilirubin dissolved in benzene, ϵ_{450}=27.3 $mM^{-1}cm^{-1}$. A separate sample of fresh culture medium was processed in the same way and used as a blank.

Measurement of cellular respiration

O_2 consumption was measured polarographically using a Clark-type O_2 electrode (Rank Brothers) that was equipped with a water jacket thermostatically set at 37°C. Prior to use, the electrode was calibrated with air-saturated water, assuming an O_2 concentration of 200 μM. Samples (1 ml of cells suspended in incubation medium at a concentration of 10^7 cells/ml) were placed in a gas-tight chamber that was sealed using a plastic plunger. A homogeneous sample was maintained using a magnetic stirrer (Rank Brothers). The decrease of O_2 (O_2 consumption) was recorded using the Duo18 (World Precision Instruments) recording system.

Measurement of cytochrome *c* oxidase activity

CCO activity was determined by the rate of oxidation of reduced cytochrome *c*. Briefly, reduced bovine cytochrome *c* (Sigma) was prepared by dissolving it in phosphate-buffered solution (100 mM, pH 7.0) in the presence of sodium dithionite. The solution was then passed through a sephadex G25 (Sigma) column and the eluent was collected. The concentration of reduced cytochrome *c* was determined spectrophotometrically (ϵ=19.6 $mM^{-1}cm^{-1}$). Cytochrome *c* was added to fresh cellular extracts (prepared by freeze-thaw lysis of cells) and the rate of azide-sensitive oxidation was measured spectrophotometrically.

Activation of RAW264.7 cells

RAW264.7 cells were activated using LPS (100 ng/ml; Sigma) and IFNγ (50 U/ml). Both LPS and IFNγ were prepared in phosphate-buffered saline solution and were added to the cells, which were maintained in biological stirrer bottles.

Western blotting

Following treatment, cells were lysed using ice-cold lysis buffer containing 25 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, 2.5 mM EGTA, 20 mM NaF, 1 mM sodium orthovanadate, 100 mM NaCl, 20 mM sodium β-glycerophosphate, 10 mM sodium pyrophosphate, 0.5% triton, 0.1% β-mercaptoethanol and complete protease inhibitor cocktail. The lysates were centrifuged and the supernatant was analysed for protein concentration. Equal amounts of protein were separated by SDS-PAGE, transferred onto nitrocellulose membranes and immunoblotted. Rabbit polyclonal antibodies were used to detect HO-1 (Stressgen Biotechnologies) and iNOS (BD Transduction Laboratories). An anti-rabbit horseradish peroxidase conjugate (Vector Laboratories) was used as the secondary antibody and the protein band was detected by enhanced chemiluminescence (Amersham Biosciences). Measurement of p38 MAPK activity was determined by detection of phosphorylated p38 by western blotting using a commercially available kit (PhosphoPlus p38 MAP Kinase Antibody Kit; Cell Signalling Technology).

Measurement of NO synthesis

Nitrite, a breakdown product of NO, was measured in the medium of cells to indicate indirectly the production of NO by the Griess assay. Equivolume quantities of 1% sulphanilamide (acidified in 5% phosphoric acid) and 0.1% *N*-(1-napthyl) ethylenediamine dihydrochloride (dissolved in water) were mixed together to form the Griess reagent. Aliquots (100 μl) of this reagent were mixed with 100 μl of sample and the absorbance was measured at 540 nm. Standard solutions containing known nitrite concentrations were also measured to construct a standard curve.

In addition, NO was measured amperometrically using an ISO-NOP NO electrode (World Precision Instruments). The NO electrode was calibrated prior to each experiment by detection of known amounts of NO produced from the reaction between sodium nitrite and potassium iodide under acidified conditions.

Statistical analysis

Data are expressed as the mean ± s.e.m. Differences in measured variables were assessed with Student’s *t* tests. $P<0.05$ was considered to be statistically significant.

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