EMBO reports

EMBO open

scientific report

CO_2 exacerbates oxygen toxicity

Benjamin Ezraty, Maïalène Chabalier, Adrien Ducret, Etienne Maisonneuve & Sam Dukan[+]

Aix Marseille Université, Laboratoire de Chimie Bactérienne (UPR 9043), Institut de Microbiologie de la Méditerranée (IFR88), CNRS, Marseille, France

Reactive oxygen species (ROS) are harmful because they can oxidize biological macromolecules. We show here that atmospheric CO_2 (concentration range studied: 40–1,000 p.p.m.) increases death rates due to H_2O_2 stress in *Escherichia coli* in a dose-specific manner. This effect is correlated with an increase in H_2O_2-induced mutagenesis and, as shown by 8-oxo-guanine determinations in cells, DNA base oxidation rates. Moreover, the survival of mutants that are sensitive to aerobic conditions (Hpx*$^-$ *dps* and *recA fur*), presumably because of their inability to tolerate ROS, seems to depend on CO_2 concentration. Thus, CO_2 exacerbates ROS toxicity by increasing oxidative cellular lesions.

Keywords: carbon dioxide; DNA; Fenton reaction; oxidative stress; mutagenesis

EMBO *reports* (2011) **12**, 321–326. doi:10.1038/embor.2011.7

INTRODUCTION

CO_2 levels have become a major point of focus of the global community, because of their contribution to the greenhouse effect (Cox *et al*, 2000). Levels are currently 389 p.p.m. (0.039%), and worst-case climate projections predict an increase in CO_2 concentration to 1,000 p.p.m. (0.1%) by 2100 (Nakicenovic *et al*, 2000). The best known effect of increasing CO_2 concentration is global warming, but large increases in CO_2 concentration (to 1% or 10%) are also known to affect cellular biochemical reactions, leading to an increase in intracellular oxidative stress in human neutrophils (Coakley *et al*, 2002), pulmonary inflammation in mouse (Abolhassani *et al*, 2009; Schwartz *et al*, 2010) and increased virulence or bactericidal activities of various pathogenic bacteria (Visca *et al*, 2002; Karsten *et al*, 2009). However, current and predicted concentrations are not of this order of magnitude; hence, the probable, direct effects of CO_2 on living organisms at the predicted concentrations remain unclear.

Aix Marseille Université, Laboratoire de Chimie Bactérienne (UPR 9043), Institut de Microbiologie de la Méditerranée (IFR88), CNRS, 31, Chemin Joseph Aiguier, 13402 Marseille, France

[+]Corresponding author. Tel: +33 4 91 16 46 01; Fax: + 33 4 91 71 89 14; E-mail: sdukan@ifr88.cnrs-mrs.fr

Received 4 June 2010; revised 13 December 2010; accepted 14 January 2011; published online 25 February 2011

In cells CO_2 is a main by-product of metabolism. It also constitutes the main physiological pH-buffering system in higher eukaryotic organisms and is required for the growth of many microorganisms (Walker, 1932). Atmospheric CO_2 is in equilibrium in liquid with dissolved CO_2, bicarbonate ion (HCO_3^-) and carbonate ion (CO_3^{2-}; equation (1)).

$$(1)\quad CO_{2(g)} \leftrightarrow CO_{2(d)} + H_2O \leftrightarrow H_2CO_3 \overset{pK_{a1}}{\longleftrightarrow} HCO_3^- + H^+ \overset{pK_{a2}}{\longleftrightarrow} CO_3^{2-} + H^+$$

$$pK_{a1} = 6.4, pK_{a2} = 10.3\ (25^\circ C)$$

Reactive oxygen species (ROS) are produced by aerobic metabolism. The most common ROS are the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$; Imlay, 2008). ROS can oxidize all biological macromolecules including DNA, thereby generating highly mutagenic lesions.

Interestingly, it has been shown that the oxidation of amino acids and arsenic(III) by the Fenton reaction (equation (2)) is dependent on the presence of the bicarbonate ion (Berlett *et al*, 1990; Stadtman & Berlett, 1991; Hug & Leupin, 2003).

$$(2)\quad H_2O_2 + Fe^{2+} \rightarrow HO^\bullet + HO^- + Fe^{3+}\ k = 4\times10^2 M^{-1}s^{-1}$$

It has been suggested that this dependence is due to the generation of the carbonate radical ($CO_3^{\bullet -}$), a new potentially toxic radical generated by the reaction between HCO_3^- or CO_3^{2-} and $HO^\bullet$ (equations (3) and (4); Augusto *et al*, 2002; Medinas *et al*, 2007).

$$(3)\quad HCO_3^- + HO^\bullet \rightarrow CO_3^{\bullet -} + H_2O\ k = 8.5\times10^6 M^{-1}s^{-1}$$

$$(4)\quad CO_3^- + HO^\bullet \rightarrow CO_3^{\bullet -} + HO^-\ k = 3\times10^8 M^{-1}s^{-1}$$

Indeed, although it has a lower oxidizing potential than $HO^\bullet$ ($E_{HO^\bullet/H_2O} = 2.3\,V$), $CO_3^{\bullet -}$ ($E_{CO_3^{\bullet -}/HCO_3^-} = 1.7\,V$) is a strong oxidant. *In vitro* studies have shown that $CO_3^{\bullet -}$ rapidly and more specifically oxidizes guanine residues in DNA, as well as amino-acid residues including tryptophan, cysteine, tyrosine, methionine and histidine (Stadtman & Berlett, 1991; Shafirovich *et al*, 2001). Finally, Liochev and Fridovich (2004) showed *in vitro* that CO_2 is converted to $CO_3^{\bullet -}$ by the peroxidase activity of Cu,ZnSOD. The second-order rate constants of $CO_3^{\bullet -}$ reactions

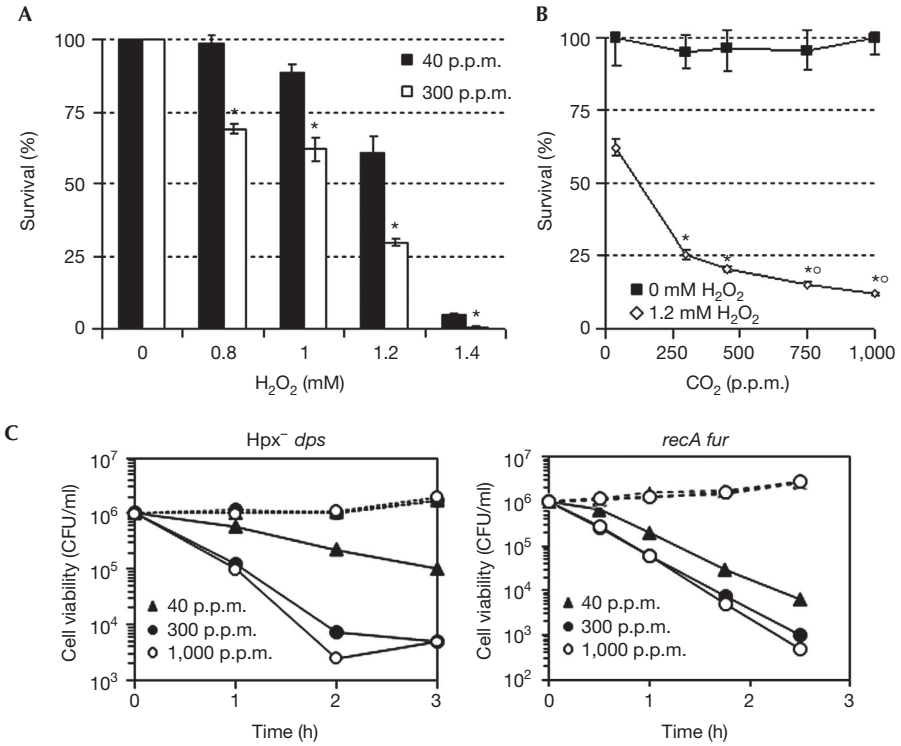

Fig 1 | Synergistic effects of atmospheric CO_2 concentration and H_2O_2 induce bacterial cell death. (**A**) *Escherichia coli* was collected at an $OD_{600} = 0.5$ and plated in the presence of various concentrations of H_2O_2 (0–1.4 mM). LB agar plates were incubated in atmospheres containing two concentrations of CO_2: 40 p.p.m. (black bars) and 300 p.p.m. (white bars; see Methods section). Means ± s.d. for three experiments are shown. Mann–Whitney *U*-tests were used for statistical analysis. Significantly higher survival rates were recorded for cells in atmospheres containing 40 p.p.m. than for cells in atmospheres containing 300 p.p.m. CO_2, in the presence of H_2O_2 (asterisk). (**B**) *E. coli* was harvested at an $OD_{600} = 0.5$ and plated in the presence of various concentrations of CO_2 (40, 300, 450, 750 and 1,000 p.p.m.), with (empty diamond) and without (filled square) H_2O_2 (1.2 mM). Means ± s.d. for three experiments are shown. Significantly higher survival rates were observed at 40 p.p.m. CO_2 than at 300, 450, 750 and 1,000 p.p.m. CO_2 in the presence of H_2O_2 (asterisk) and at 300 p.p.m. than at 750 and 1,000 p.p.m. CO_2 in the presence of H_2O_2 (circle). After 48 h, LB agar plates initially containing H_2O_2 were no longer toxic for cell growth. (**C**) Low concentrations of CO_2 (40 p.p.m.) rescued growth after the shift from anaerobic to aerobic conditions for strains susceptible to aerobic conditions. Hpx⁻ *dps* and *recA fur* strains were cultured in anaerobiosis and shifted to aerobiosis with various atmospheric CO_2 concentrations (40, 300 and 1,000 p.p.m.), as described in the Methods section, in the absence (solid line) or presence (dotted line) of 2,2′-dipyridyl (250 μM). Atmospheric CO_2 levels had no effect on the growth of the MG1655 parental strain after the shift from anaerobic to aerobic conditions (supplementary information online). Representative results are presented in the figure and each analysis was repeated three times. CFU, colony-forming units; LB, Luria–Bertani.

with biological molecules are well-known and are of biological relevance (10^6–$10^9\ M^{-1}s^{-1}$; Medinas *et al*, 2007).

Moreover, H_2O_2 might directly react with dissolved CO_2 to generate peroxymonocarbonate (HCO_4^-; equation (5)), another strong oxidant ($E_{HCO_4^-/HCO_3^-} = 1.8$ V); Richardson *et al*, 2003). *In vitro*, HCO_4^- has been shown to oxidize methionine and sulphides or tertiary amine more rapidly (100–400 times faster) than H_2O_2 alone (Richardson *et al*, 2003; Balagam & Richardson, 2008).

$$(5)\quad CO_{2(d)} + H_2O_2 \rightarrow HCO_4^- + H^+ \quad K = 0.33$$

All of these *in vitro* observations led us to speculate that CO_2 might be an unexpected factor in oxidative stress *in vivo*. Oxidative stress is ubiquitous and has important consequences in almost all biological systems (Roberts *et al*, 2010). We therefore hypothesized that the atmospheric CO_2 concentration might modulate oxidative stress *in vivo*. We used *Escherichia coli* as a unicellular model organism in this study.

RESULTS

CO_2 exacerbates H_2O_2 toxicity in *E. coli*

We measured the effect of CO_2 concentration (range: 40–1,000 p.p.m.; current atmospheric concentration: 389 p.p.m.) on the tolerance of *E. coli* to H_2O_2. *E. coli* cells were spread on Luria–Bertani (LB) agar plates containing various concentrations of H_2O_2 and incubated in the presence of either 40 p.p.m. (sufficient for optimal *E. coli* growth, with no difference in intracellular pH and metabolism observed between 40 and 1,000 p.p.m. of CO_2; see supplementary information online) or 300 p.p.m. CO_2. Regardless of the H_2O_2 concentration tested, cell viability was significantly more affected at 300 p.p.m. CO_2 than at 40 p.p.m. CO_2 (Fig 1A; $P < 0.05$). Moreover, whereas no effect was observed at 40 p.p.m.

in the presence of the lower concentration of H_2O_2 (0.8 mM), cell viability was already affected in the presence of 300 p.p.m. CO_2, suggesting that CO_2 exacerbated the toxicity of H_2O_2 in *E. coli*. To confirm this synergistic effect of H_2O_2 and CO_2, we also measured cell viability in the presence of H_2O_2, at increasing levels of CO_2. No effect was observed in the absence of H_2O_2 (Fig 1B), but cell viability was affected in a dose-dependent manner by increases in CO_2 concentration (Fig 1B). Thus, CO_2 exacerbates the toxicity of H_2O_2 in *E. coli* in a dose-dependent manner.

CO_2 increases HO• toxicity

Next, we evaluated the effect of CO_2 on *E. coli* mutants sensitive to aerobic growth conditions. The Hpx⁻ *dps* mutant lacks the three enzymes responsible for all *E. coli* peroxide-scavenging activity (catalases KatE and KatG and the peroxidase AhpC) and Dps, a ferretin-like protein that sequesters iron and protects the chromosome in stress conditions (Park *et al*, 2005). An anaerobic culture of Hpx⁻ *dps* mutant cells was used to inoculate fresh LB broth, which was then incubated under aerobiosis for 3 h in the presence of three CO_2 concentrations (40, 300 and 1,000 p.p.m.). Aerobiosis decreased cell viability in the presence of CO_2 concentrations of 300 and 1,000 p.p.m. (from approximately 10^6 to approximately 10^3 colony-forming units (CFU)/ml after 3 h of aerobiosis) (Fig 1C). However, cell viability was less affected at 40 p.p.m. CO_2 (from approximately 10^6 to approximately 10^5 CFU/ml). As the sensitivity to oxygen of the Hpx⁻ *dps* mutant has been attributed to the DNA damage caused by Fenton reaction-based HO• production (Park *et al*, 2005), these finding suggest that CO_2 exacerbates HO•-induced DNA damage.

We tested this hypothesis by examining the effect of CO_2 concentration on the *recA fur* mutant, which cannot grow in aerobic conditions because it lacks RecA—a regulator of the SOS response involved in DNA strand-break repair—and Fur, the main iron homeostasis regulator in *E. coli* (Touati *et al*, 1995). The effects were less marked than those for the Hpx⁻ *dps* mutant, but we observed that the cell viability of the *recA fur* mutant was also less affected at a concentration of 40 p.p.m. CO_2. As the oxygen sensitivity of the *recA fur* mutant is also due to HO•-mediated DNA damage (Touati *et al*, 1995), this result supports the hypothesis that CO_2 exacerbates HO• toxicity.

We sought further support for the conclusion that CO_2 directly increases oxygen toxicity, by modulating the steady-state concentrations of H_2O_2 and/or the HO• radical by using exogenous catalase, iron chelator (2,2′-dipyridyl), anaerobiosis or a radical-trapping reagent (5,5-dimethyl-1-pyrroline N-oxide—DMPO). The synergistic effect of CO_2 on oxygen toxicity disappeared in these conditions, providing further evidence for the hypothesis that CO_2 directly exacerbates HO• toxicity (Fig 1C; supplementary information online).

CO_2 increases H_2O_2-dependent mutation frequency

We investigated whether CO_2 exacerbated HO• toxicity by examining the effect of CO_2 concentration on mutation frequency. The pPY98 plasmid carries the P22 *mnt* repressor gene, which confers sensitivity to tetracycline, the reversal of which is a direct function of the cell mutational rate (Lucchesi *et al*, 1986).

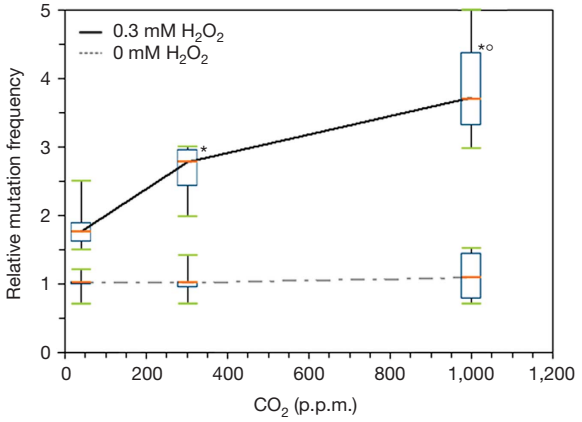

Fig 2 | Atmospheric CO_2 levels affect the frequency of H_2O_2-induced mutation in *Escherichia coli*. *E. coli* cells harboring pPY98 were prepared as described in the Methods section. Box plot of the relative mutation frequency observed with increasing concentrations of CO_2, in the absence (dashed line) or presence (solid line) of H_2O_2. The orange bar indicates the median for 10 experiments. Mann–Whitney *U*-tests were carried out for statistical analysis. Significantly higher relative mutation frequencies were observed for 300 and 1,000 p.p.m. CO_2 than for 40 p.p.m. CO_2 in the presence of H_2O_2 (asterisk), and for 1,000 p.p.m. CO_2 than for 300 p.p.m. CO_2 in the presence of H_2O_2 (circle).

We grew *E. coli* cells harboring pPY98 in the presence of various concentrations of CO_2 (40, 300 and 1,000 p.p.m.) and determined mutation frequencies. Mutation frequencies in the absence of H_2O_2 were similar at all atmospheric CO_2 concentrations used (Fig 2). The basal frequency of mutation (as indicated by the several tetracycline-resistant clones) was approximately 10^{-7}, consistent with the findings of Lucchesi *et al* (1986). Interestingly, mutation frequencies increased significantly ($P<0.05$) on exposure to H_2O_2 in the presence of higher CO_2 levels (Fig 2).

CO_2 increases H_2O_2-dependent 8-oxo-guanine DNA damage

We directly quantified DNA lesions by immunofluorescence-based detection of 8-oxo-guanine *in situ*. In the absence of H_2O_2, no difference in fluorescence intensity was found between samples grown in atmospheres containing the three CO_2 concentrations tested (40, 300 and 1,000 p.p.m.; Fig 3). However, in cells exposed to H_2O_2, fluorescence intensity and several DNA lesions were positively correlated with CO_2 concentration (Fig 3). These experiments demonstrate that increases in CO_2 concentration aggravate oxidative DNA damage.

CO_2 decreases H_2O_2-dependent carbonyl content

We then quantified the carbonyl protein content, which is a marker for irreversible oxidative damage to proteins. In the absence of H_2O_2, no difference was found between samples grown in the two atmospheres tested (40 and 1,000 p.p.m. CO_2; Fig 4). However, in cells exposed to H_2O_2, the carbonyl protein content increased, as expected (Dukan *et al*, 2000), but was negatively correlated with CO_2 concentration (Fig 4).

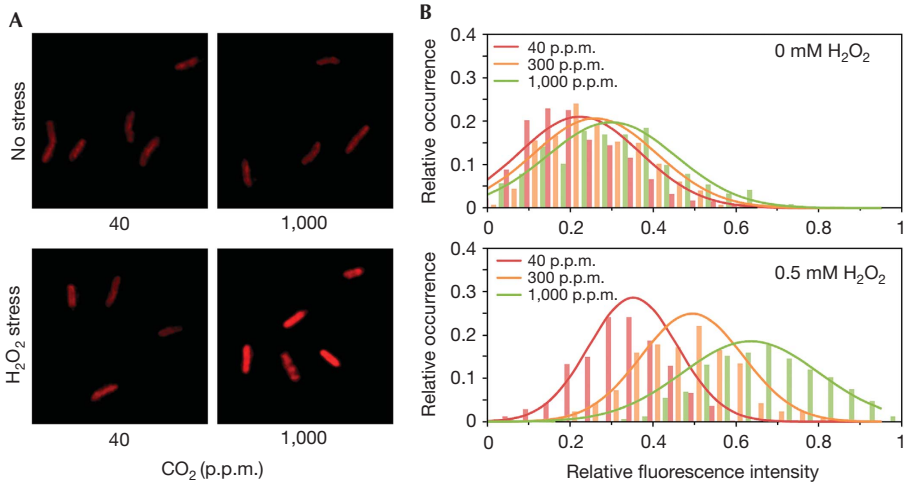

Fig 3 | Increases in atmospheric CO_2 concentrations are associated with an increase in DNA damage, as estimated by 8-oxo-guanine levels. *Escherichia coli* cells were prepared as indicated in the Methods section. Cells were fixed and *in situ* immunofluorescence studies were carried out with a goat 8-OHdG (8-oxo-guanine) polyclonal antibody (Millipore). (**A**) Representative fluorescence images obtained at 40 and 1,000 p.p.m. CO_2 with or without stress (0.5 mM H_2O_2). (**B**) Distribution of fluorescence intensity retrieved by quantitative analysis of immunofluorescence staining of cells ($N = 1{,}500$).

DISCUSSION

It is widely accepted that oxidative stress is caused by exposure to ROS, which can damage proteins, nucleic acids and cell membranes. By ruling out other possibilities, we can infer from our data that CO_2 probably reacts with ROS *in vivo*, such as $HO^{\bullet}$ or H_2O_2, to exacerbate oxidative stress.

Several lines of evidence suggest that, as has been shown *in vitro* (Augusto *et al*, 2002; Richardson *et al*, 2003; Medinas *et al*, 2007; Balagam & Richardson, 2008), $HO^{\bullet}$ and/or H_2O_2 reacts *in vivo* with CO_2, mostly generating $CO_3^{\bullet-}$. We show here that (i) CO_2 exacerbates the toxicity of H_2O_2 in a dose-dependent manner; (ii) the aerobic lethality of *recA fur* and Hpx$^-$ *dps* mutants, thought to be mediated by $HO^{\bullet}$, is CO_2 dependent; (iii) H_2O_2-induced mutagenesis and 8-oxo-guanine levels are CO_2 dependent; and (iv) carbonyl content on H_2O_2 exposure is CO_2 dependent. We also show that anaerobiosis or a decrease in ROS concentrations abolishes CO_2-dependent toxicity. Furthermore, the range of CO_2 concentrations used in this study had no effect on intracellular pH, general metabolic pathways or protein turnover, suggesting that indirect effects of CO_2 on the cells are probably not involved in this phenomenon. These findings are thus consistent with the occurrence of direct reactions between CO_2 and ROS *in vivo*.

Finally, taken together, our results are consistent with a direct reaction between CO_2 and ROS. $HO^{\bullet}$ reacts in the environment in which it is generated, whereas Shafirovich *et al* (2001) have shown that $CO_3^{\bullet-}$ is more selective, oxidizing guanine residues in DNA more specifically than $HO^{\bullet}$, for example. The selective reactivity of $CO_3^{\bullet-}$ with guanine rather than the other three DNA bases is a consequence of the thermodynamic and kinetic characteristics of this radical (Shafirovich *et al*, 2001). The strong correlation between the increase in 8-oxo-guanine levels within the cell and CO_2 levels during oxidative stress is consistent with this idea. Moreover, the amounts of carbonyl derivatives formed by the oxidation of proline, arginine, lysine and threonine are

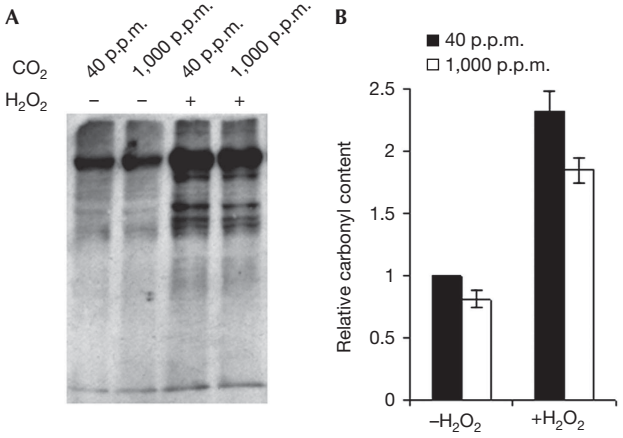

Fig 4 | Increasing atmospheric CO_2 levels are associated with a decrease in carbonylated protein levels on exposure to H_2O_2 in *Escherichia coli*. *E. coli* cells were prepared as described in the Methods section. (**A**) Protein carbonylation pattern after one-dimensional protein electrophoresis. (**B**) Relative carbonylated protein levels (black bars, 40 p.p.m.; white bars, 1,000 p.p.m.) were quantified with Quantity One software. The data shown are the means ± s.d. of four independent experiments.

negatively correlated with CO_2 concentration. Interestingly, the products of $HO^{\bullet}/CO_2$ reactions have a lower reactivity than $HO^{\bullet}$ alone with these amino-acid side chains (Stadtman & Berlett, 1991). Consequently, the titration of $HO^{\bullet}$ with CO_2 might decrease protein carbonylation.

The $CO_3^{\bullet-}$ radical seems to have a central role in the chemistry of CO_2 and ROS. However, in the light of a recent study demonstrating extremely rapid recombination between $HO^{\bullet}$ and $CO_3^{\bullet-}$ leading to the formation of HCO_4^- (equation (6); Haygarth *et al*, 2010), another strong oxidant ($E_{HCO_4^-/HCO_3^-} = 1.8\,V$), we

cannot exclude the possibility that HCO_4^-, rather than $CO_3^{\bullet-}$ reacts with biological molecules *in vivo*.

(6) $CO_3^{\bullet-} + HO^{\bullet} \rightarrow HCO_4^- \; k = 6\times10^9 M^{-1}s^{-1}$

It will be a challenge to detect such molecules (HCO_4^- and $CO_3^{\bullet-}$) *in vivo*. The most commonly used method for studying short-lived species involves the spin trapping of radicals. For instance, $CO_3^{\bullet-}$ can react with DMPO, leading to the formation of DMPO-OH (Villamena *et al*, 2007). However, since 1980, DMPO has been used to trap $HO^{\bullet}$ *in vivo*, also resulting in the formation of DMPO-OH (Buettner, 1987). Thus, the DMPO-OH detected *in vivo* might originate from either or both $HO^{\bullet}$ and $CO_3^{\bullet-}$. The design of specific spin traps for $CO_3^{\bullet-}$ and $HO^{\bullet}$ is a key challenge limiting further investigation.

In 2000, the Intergovernmental Panel on Climate Change published its *Special Report on Emissions Scenario*, predicting that the atmosphere in 2100 will contain 1,000 p.p.m. CO_2 (Nakicenovic *et al*, 2000). More recently, in his essay, Schneider (2009) described an increase of this magnitude as the 'worst-case scenario' and explored what a world with 1,000 p.p.m. CO_2 in its atmosphere might look like, in terms of society, economics and environment. This study provides the first evidence that oxidative stress is exacerbated by increasing atmospheric CO_2 concentrations. This exacerbation might be of great ecological concern, with important implications for life on Earth.

METHODS

Cell growth experiments at various atmospheric CO_2 concentrations. Cell growth experiments were carried out in sealed-flow chambers with Crystal Mix (Air Liquide) containing N_2/O_2 (80/20%) and CO_2 at concentrations of 0–1,000 p.p.m.

Lethality studies on LB agar plates. *E. coli* (MG1655) was grown aerobically in liquid LB broth, at 37 °C, with shaking at 160 r.p.m. When the OD_{600} reached 0.5, cells were exposed to various concentrations of CO_2 (40, 300, 450, 750 or 1,000 p.p.m.). LB agar plates with and without H_2O_2 in the medium were allowed to equilibrate for 20 h at the CO_2 level to be tested (40, 300, 450, 750 or 1,000 p.p.m.). Serial dilutions of cell suspensions in phosphate buffer (0.05 M, pH 7.4) were prepared and aliquots (150–200 cells) were spread onto the LB agar plates. Colonies were counted after incubation at the CO_2 concentration tested for 16 h at 37 °C. After this period of incubation, no extra colonies were observed.

Aerobic cell growth and viability. MG1655, Hpx$^-$ *dps* and *recA fur* strains were cultured twice (anaerobic chamber containing 40 p.p.m. CO_2), in anaerobic LB broth supplemented with 0.2% glucose, to an OD_{600} of 0.3. They were then mixed with fresh aerobic medium to yield an OD_{600} of 0.003 and incubated in atmospheres containing various concentrations of CO_2 (40, 300 and 1,000 p.p.m.). The aerobic medium was filter-sterilized and allowed to equilibrate in an atmosphere containing the concentration of CO_2 tested for 3 h before use. This equilibration process had no detectable effect on the pH of the LB broth. Viability was assessed by mixing cells at various time points with anaerobic phosphate buffer (0.05 M, pH 7.4) and spreading them on anaerobic LB agar plates. Colonies were counted the next day.

Mutation frequency. *E. coli* harboring pPY98 was cultured to an OD_{600} of 0.2 in LB broth supplemented with ampicillin (20 μg/ml), in an atmosphere containing 40 p.p.m. CO_2, at 37 °C. The culture was then split into six subcultures. Two subcultures each were equilibrated in the presence of 40, 300 and 1,000 p.p.m. CO_2. We then subjected one subculture for each set of CO_2 conditions to oxidative stress (0.3 mM H_2O_2) for 5 min. These challenge conditions induced no detectable bacterial cell death. The cells were then centrifuged and washed twice in phosphate buffer (0.05 M, pH 7.4). We then plated 100 μl (approximately 10^8 cells) of the cell suspension on an LB agar plate containing ampicillin (20 μg/ml) and tetracycline (3.5 μg/ml) and determined the number of CFU after incubation for 16 h at 37 °C in an atmosphere containing 40, 300 or 1,000 p.p.m. CO_2.

8-oxo-guanine detection by immunofluorescence. Overnight aerobic cultures of *E. coli* grown in an atmosphere containing 40 p.p.m. CO_2 were mixed (1/100) with LB broth, equilibrated with an atmosphere containing 40 p.p.m. CO_2 for 1 h, and then subcultured twice to yield an OD_{600} of 0.3 in an atmosphere containing 40 p.p.m. CO_2. The cells were then transferred to LB broth that had been previously equilibrated at the atmospheric CO_2 level tested (40, 300 and 1,000 p.p.m.) for 3 h. This equilibration process had no detectable impact on the pH of the LB broth. H_2O_2 was added to a concentration of 0.5 mM when the OD_{600} was 0.2 and the suspension was incubated for 10 min. These challenge conditions induced no detectable bacterial cell death. Cells were then centrifuged, washed twice in phosphate buffer (0.05 M, pH 7.4) and diluted 1:10 in the same buffer. 8-oxo-guanine detection by immunofluroescence was performed as indicated in the supplementary information online.

Carbonylation assays. Cells were prepared as described by using the 8-oxo-guanine immunofluorescence procedure. H_2O_2 was added to a concentration of 0.5 mM when the OD_{600} was 0.2, and the culture was incubated for 10 min. These challenge conditions induced no detectable bacterial cell death. The cells were then centrifuged, washed twice in phosphate buffer (0.05 M, pH 7.4) and lysed by passage through a French press. Carbonylated proteins were detected with an OxyBlot™ protein oxidation detection kit (Chemicon International), as described previously (Dukan *et al*, 2000).

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank H. Aguilaniu, F. Barras, E. Bouveret, J.M. Claverie, F. Denizot, M. Deutscher, A. Galinier, V. Geli, I. Matic, T. Mignot, F. Taddei, A. Vergnes and M. Zundel for helpful comments on the paper. We especially thank I. Fridovich, J. Imlay, P. Moreau, M. Toledano and D. Touati for scientific discussions and comments on the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Abolhassani M, Guais A, Chaumet-Riffaud P, Sasco AJ, Schwartz L (2009) Carbon dioxide inhalation causes pulmonary inflammation. *Am J Physiol Lung Cell Mol Physiol* **296:** 657–665

Augusto O, Bonini MG, Amanso AM, Linares E, Santos CC, De Menezes SL (2002) Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. *Free Radic Biol Med* **32:** 841–859

Balagam B, Richardson DE (2008) The mechanism of carbon dioxide catalysis in the hydrogen peroxide N-oxidation of amines. *Inorg Chem* **47:** 1173–1178

Berlett BS, Chock PB, Yim MB, Stadtman ER (1990) Manganese(II) catalyzes the bicarbonate dependent oxidation of amino acids by hydrogen

peroxide and the aminoacid facilitated dismutation of hydrogen peroxide. *Proc Natl Acad Sci USA* **87:** 389–393

Buettner GR (1987) Spin trapping: ESR parameters of spin adducts. *Free Radic Biol Med* **3:** 259–303

Coakley RJ, Taggart C, Greene C, McElvaney NG, O'Neill SJ (2002) Ambient pCO_2 modulates intracellular pH, intracellular oxidant generation, and interleukin-8 secretion in human neutrophils. *J Leuk Biol* **71:** 603–610

Cox PM, Betts RA, Jones CD, Spall SA, Totterdell IJ (2000) Acceleration of global warming due to carbon-cycle feedbacks in a coupled climate model. *Nature* **408:** 184–187

Dukan S, Farewell A, Ballesteros M, Taddei F, Radman M, Nystrom T (2000) Protein oxidation in response to increased transcriptional or translational errors. *Proc Natl Acad Sci USA* **97:** 5746–5749

Haygarth KS, Marin TW, Janik I, Kanjana K, Stanisky CM, Bartels DM (2010) Carbonate radical formation in radiolysis of sodium carbonate and bicarbonate solutions up to 250 °C and the mechanism of its second order decay. *J Phys Chem* **114:** 2142–2150

Hug SJ, Leupin O (2003) Iron-catalyzed oxidation of arsenic(III) by oxygen and by hydrogen peroxide: pH-dependent formation of oxidants in the Fenton reaction. *Environ Sci Technol* **37:** 2734–2742

Imlay JA (2008) Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* **77:** 755–776

Karsten V, Murray SR, Pike J, Troy K, Ittensohn M, Kondradzhyan M, Low KB, Bermudes D (2009) msbB deletion confers acute sensitivity to CO_2 in *Salmonella enterica serovar Typhimurium* that can be suppressed by a loss-of-function mutation in zwf. *BMC Microbiol* **9:** 170

Liochev SI, Fridovich I (2004) CO_2, not HCO_3^-, facilitates oxidations by Cu,Zn superoxide dismutase plus H_2O_2. *Proc Natl Acad Sci USA* **101:** 743–744

Lucchesi P, Carraway M, Marinus MG (1986) Analysis of forward mutations induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine in the bacteriophage P22 mnt repressor gene. *J Bacteriol* **166:** 34–37

Medinas DB, Cerchiaro G, Trindale DF, Augusto O (2007) The carbonate radical and related oxidants derived from bicarbonate buffer. *IUBMB Life* **59:** 255–262

Nakicenovic N, Alcamo J, Davis G, de Vries B, Fenhann J, Gaffin S, Gregory K, Grübler A, Jung TY, Kram T (2000) *Special Report on Emissions Scenarios*. Cambridge, UK: Cambridge University Press

Park S, You X, Imlay JA (2005) Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx- mutants of *Escherichia coli*. *Proc Natl Acad Sci USA* **102:** 9317–9122

Richardson DE, Regino CA, Yao H, Johnson JV (2003) Methionine oxidation by peroxymonocarbonate, a reactive oxygen species formed from CO_2/bicarbonate and hydrogen peroxide. *Free Radic Biol Med* **35:** 1538–1550

Roberts RA, Smith RA, Safe S, Szabo C, Tjalkens RB, Robertson FM (2010) Toxicological and pathophysiological roles of reactive oxygen and nitrogen species. *Toxicology* **276:** 85–94

Schneider S (2009) The worst-case scenario. *Nature* **458:** 1104–1105

Schwartz L, Guais A, Chaumet-Riffaud P, Grévillot G, Sasco AJ, Molina TJ, Abolhassani M (2010) Carbon dioxide is largely responsible for the acute inflammatory effects of tobacco smoke. *Inhal Toxicol* **22:** 1–9

Shafirovich V, Dourandin A, Huang W, Geacintov NE (2001) The carbonate radical is a site-selective oxidizing agent of guanine in double-stranded oligonucleotides. *J Biol Chem* **276:** 24621–24626

Stadtman ER, Berlett BS (1991) Fenton chemistry. Amino acid oxidation. *J Biol Chem* **266:** 17201–17211

Touati D, Jacques M, Tardat B, Bouchard L, Despied S (1995) Lethal oxidative damage and mutagenesis are generated by iron in delta fur mutants of *Escherichia coli*: protective role of superoxide dismutase. *J Bacteriol* **177:** 2305–2314

Villamena FA, Locigno EJ, Rockenbauer A, Hadad CM, Zweier JL (2007) Theoretical and experimental studies of the spin trapping of inorganic radicals by 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). 2. Carbonate radical anion. *J Phys Chem A* **111:** 384–391

Visca P, Fabozzi G, Milani M, Ascenzi P (2002) Nitric oxide and *Mycobacterium leprae* pathogenicity. *IUBMB Life* **54:** 95–99

Walker HH (1932) Carbon dioxide as a factor affecting lag in bacterial growth. *Science* **76:** 602–604