Final report on the grant

Unravelling the mechanism of carbon monoxide toxicity

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Summary

According to the research plan we have studied the role of reactive oxygen species (ROS) and oxidative stress in the mechanism of neuronal cell death induced by carbon monoxide.

In agreement with others we have found that:

- CO induces the inhibition of mitochondrial respiration and a decrease in mitochondrial membrane potential in primary neurons and astrocytes;
- CO triggers calcium signalling in these cells.
- CO stimulate production of Nitric oxide (NO)
- Exposure of neurons and astrocytes to CO induce:
    - a fast and significant increase in ROS production which is generated by various intracellular sources.

Uniquely, we have found using a novel system, that:

Although CO inhibit mitochondrial respiration and ATP production, the death of neurons and astrocytes during CO exposure/ reoxygenation is not induced by ATP depleting and energy deprivation.

Importantly, the removal of CO and the reintroduction of oxygen to these cells (so mimicking current treatment options of CO poisoning), induced even higher rates of ROS production.

- We have identified the main producers of ROS during CO exposure and reoxygenation,
- We have identified inhibitors which successfully reduced CO-induced oxidative stress (so decreasing the level of endogenous antioxidant glutathione and an increase in lipid peroxidation).
    - These inhibitors and antioxidants also protected neurons against CO-induced neuronal cell death, specifically the inhibitor of NADPH oxidase AEBSF.

Thus, we have confirmed, CO-induced ROS production and oxidative stress play an important role in the mechanism of carbon monoxide neurotoxicity. We have established that inhibition of this process can be used for development of a neuroprotective therapeutic strategy. We have identified inhibitors: mitochondrially targeted antioxidants, inhibitors of xanthine oxidase and a general antioxidant that have the potential to work with the greatest effect, either singularly or in combination in conjunction with the provision of O_2, that will prevent the production of harmful ROS associated with both CO poisoning, current treatment methods, and neurological injury.

Future work: to establish, as a priority, the combination of inhibitors that work with the greatest effect, (whether singularly or in combination), in conjunction with the provision of O_2, to deliver optimum patient outcomes. This will assist in preventing neurological damage in patients and expedite the development of scientifically evidence-based treatment guidelines for CO-poisoned patients for the NHS, NICE and National Poisons Information Service (NIPS).

Full report

Carbon monoxide (CO) poisoning is one of the most common lethal poisonings, with neurological or psychiatric sequelae occurring in up to 67% of survivors. CO is induced dissociation with hemoglobin (to form carboxyhaemoglobin) that induce severe hypoxia in tissues. Carbon monoxide also inhibits cellular respiration by binding to cytochrome c oxidase, a component of the mitochondrial electron transport chain that also can be accepted as a chemical hypoxia. These conditions are a main reason for toxicity of CO in tissues and reintroduction of the oxygen is vitally essential for recovery. Consider this, treatment with hyperbaric oxygen (HBO) was recommended for long time because it reduces carboxyhemoglobin dissociation half-life from more than four hours at room air or 45 minutes on 100% oxygen to 23 minutes at 2.5 atmospheres absolute (ATA). However, post CO re-oxygenation by HBO or by atmospheric oxygen induce neurotoxicity. This neurotoxicity, in parts, can be explained by the same mechanisms of neuronal cell death which induced by anoxia or hypoxia. Hypoxia induced cells death is multifactorial process which is affected by overproduction of reactive oxygen species leading to oxidative stress which is enhanced in the time of re-oxygenation (Abramov et al., 2007).

With support from Gas Safety Trust we studied the effect of reactive oxygen species and oxidative stress in mechanism of CO induced neurotoxicity.

Firstly, we established if conditions (induction of the toxic CO release from chemical compound CORM-401) have effect on the mitochondrial respiration and could induce chemical hypoxia. Effect of CO on mitochondrial respiration in live primary neurons and astrocytes was assessed using autofluorescence NADH (Bartolome, Abramov, 2015). NADH is produced in Krebs's cycle in the matrix of mitochondria and used as a substrate and donor of electrons in complex I electron transport chain. Inhibition of respiration should reduce consumption of the NADH in mitochondria and increase the level of NADH. In our experiments, application of the 50 μM CORM-401 induced slow and progressive increase in NADH fluorescence (N=8 experiments; Figure 1A). Application of mitochondrial uncoupler FCCP to these cells did not induce activation of respiration and decrease in NADH fluorescence (Figure 1A). However, inhibition of respiration with 1 mM NaCN in the end experiment still induced rise in NADH signal. *These results strongly suggest that CO induce profound but not complete inhibition of mitochondrial respiration in primary co-culture of neurons and astrocytes.*

Mitochondrial membrane potential is the indicator of mitochondrial function and health. We measured the effect of CO on mitochondrial membrane potential using Rhodamine 123 as fluorescent indicator. We have found that application of 50 μM CORM-401 induce depolarization of mitochondria in both neurons and astrocytes (Figure 1 B-C). Importantly, CO induce significant but not complete depolarization after 10 and 20 min of exposure of neuros and astrocytes to CO (it dropped to 21±3% in 20 min of basal potential; N=8 experiments; Figure 1 B). *Thus, toxic concentrations of CO induce inhibition of respiration and dramatically decrease mitochondrial membrane potential. However, this is not a complete inhibition and drop of potential that can be induced by anoxic conditions.*

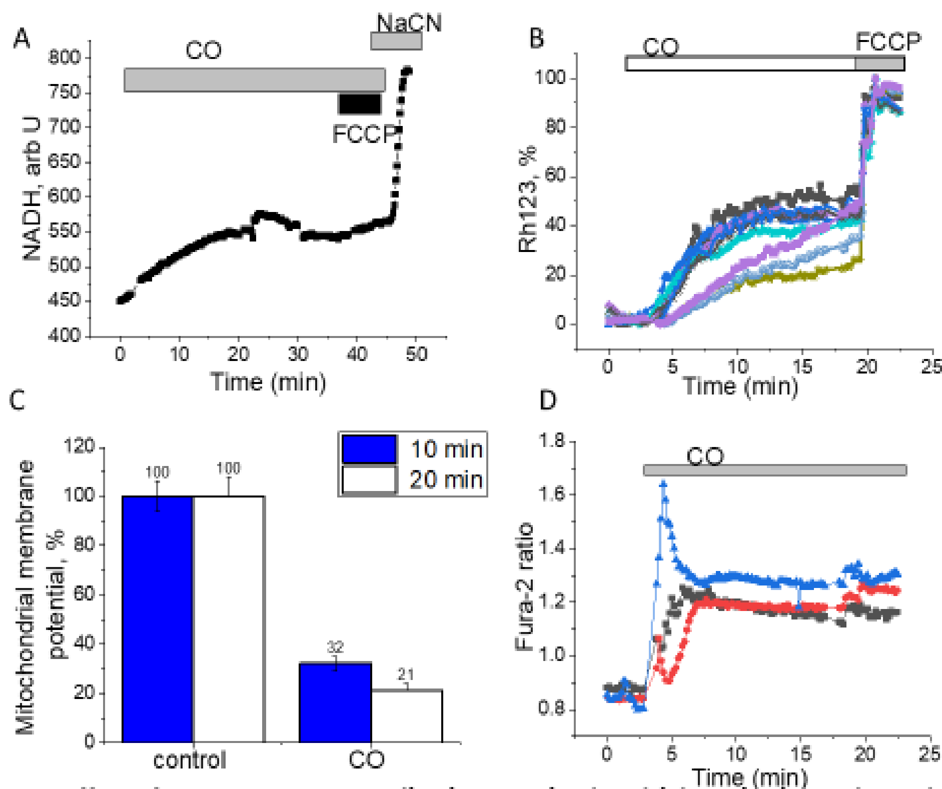


Figure 1. Effect of CO or CORM 401 application on mitochondrial respiration and membrane potential. A- CO inhibit mitochondrial respiration that increase NADH autofluorescence in neurons and astrocytes; B, C – CORM 401 decrease mitochondrial membrane potential (Rh123 fluorescence). FCCP in the end of experiment induce complete depolarisation. D-CO induce calcium signal in primary neurons

Anoxia and hypoxia are known to be inductors for calcium signal in neurons and astrocytes (Angelova et al., 2016). In our experiments CO induced calcium signal in neurons and astrocytes (N=6 experiments; Figure 1D). Mechanisms of this calcium elevation most likely induced by release of glutamate and could be a subject for further investigation. CO-induced calcium signal can be trigger for multiple processes in neurons and astrocytes including activation of enzymes involved in enzymatic production of reactive oxygen species (ROS).

CO-induced reactive oxygen species production is multiphase and generated from multiple sources:

The main aim of this study is unraveling the role of ROS and oxidative stress in CO - induced neurotoxicity. In our experiments we have found that incubation of the primary cortical co-culture of neuron and astrocytes with CO or by adding to solution CORM-401 (which release CO) induce 5-fold increase in the rate of ROS production (Figure 2 A-B).

Importantly, the rate of ROS in neurons and astrocytes was changing during CO exposure and further increase in the time of re-oxygenation (N=14 experiments; Figure 2A). Different phases of this ROS increase can be reduced by mitochondrially targeted antioxidants (MitoTEMPO, SkQ, MitoQ), and inhibitors of xanthine oxidase (allopurinol, oxypurinol) and NADPH oxidase AEBSF (Figure 2, AB), and partially by general antioxidant Trolox. Interestingly, inhibitor of monoamine oxidase (MAO, selegiline) also reduced CO-induced ROS production in neurons and astrocytes.

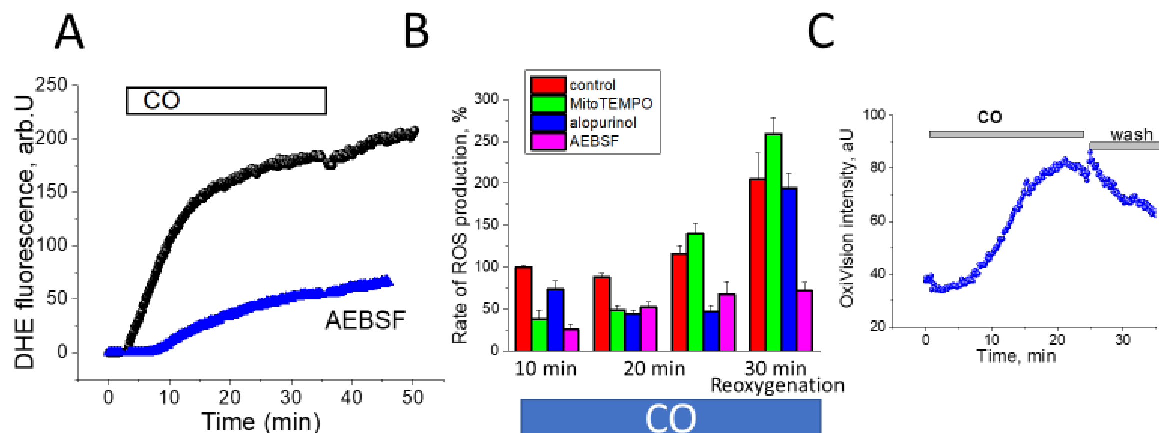


Figure 2. Effect of CO on ROS production in primary cortical neurons and astrocytes. A - CO-incubation (or application of CORM-401) increase the rate of dihydroethidium oxidation and increase of fluorescence, that correspond to ROS production. Preincubation (10 min) cell with inhibitor NOX 20 μM AEBSF inhibit ROS production (blue line). B- effect of 1 μM MitoTEMPO, 20 μM allopurinol and 20 μM AEBSF on CO-induced ROS production in different time points. CO induced ROS production in first 10 min was taken as 100%.

C-CO induced only moderate effect on H2O2 production suggesting that superoxide anion is a main type of ROS produced under CO exposure and reperfusion

Thus, CO induced ROS production in neurons and astrocytes from various sources including the most pronounced NADPH oxidase. However, production of ROS may not induce oxidative stress and to be toxic.

We have studied the effect of CO and inhibitors of various sources of CO-induced ROS production on oxidative stress by measurement of level of major endogenous antioxidant in central nervous system – glutathione and level of lipid peroxidation.

Importantly, carbon monoxide can induce production of nitric oxide (NO, Figure 2i). Interestingly, the activation of NO production is dramatically increase on after 10 min of CO exposure and become stable with no changes in the time of reoxygenation.

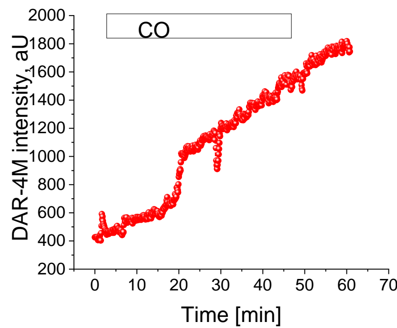
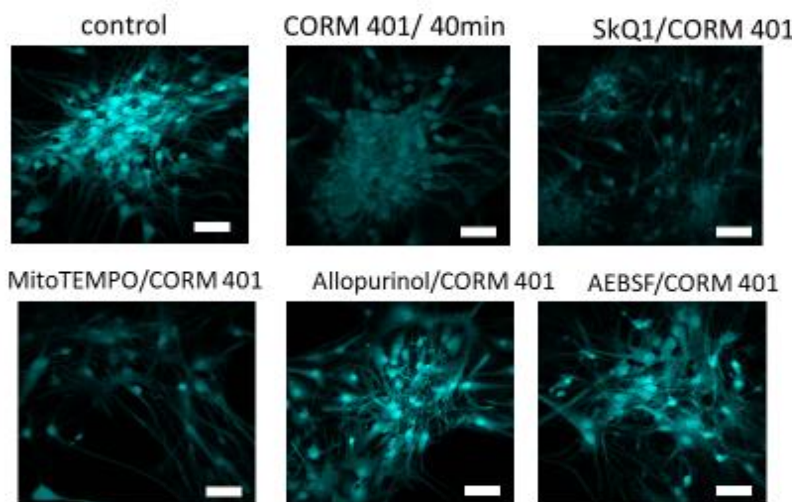


Figure 2i. Carbon monoxide induce production of nitric oxide. NO was measured using DAR-4M as fluorescent indicator.

GSH. 40 min incubation of primary co-culture of neurons and astrocytes with 50 µM CORM-401 induced depletion of endogenous antioxidant glutathione (GSH, Figure 3A). Importantly, inhibitors of CO- induced ROS productions and specific antioxidants effectively protected cells against glutathione decrease and oxidative stress (Figure 3 B).



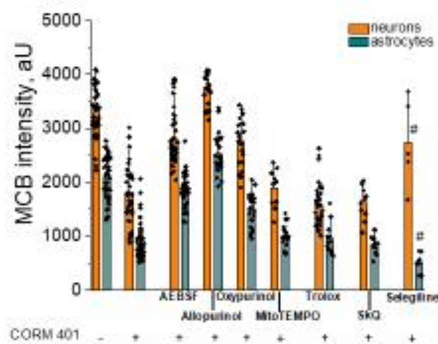


Figure 3. CO induce oxidative stress and glutathione depletion in neuro-glial co-cultures A- Effect of CORM-401 incubation (40 min, 50 uM) on monochorobimine (MCB) fluorescence. B-Incubation of the cortical neurons and astrocytes with CO decrease level of GSH, measured with MCB fluorescence.

CO induced significant increase in the rate of lipid peroxidation in neurons and astrocytes (Figure 4 A-C). Importantly, inhibitors and antioxidants which successfully inhibited CO-induced ROS production but have no direct effect on lipid peroxidation (except water soluble analogue of vitamin E- Trolox) also significantly reduced effect of CO on lipid peroxidation (Figure 4 B-C). Thus, we identify the sources of ROS production and oxidative stress induced by CO.

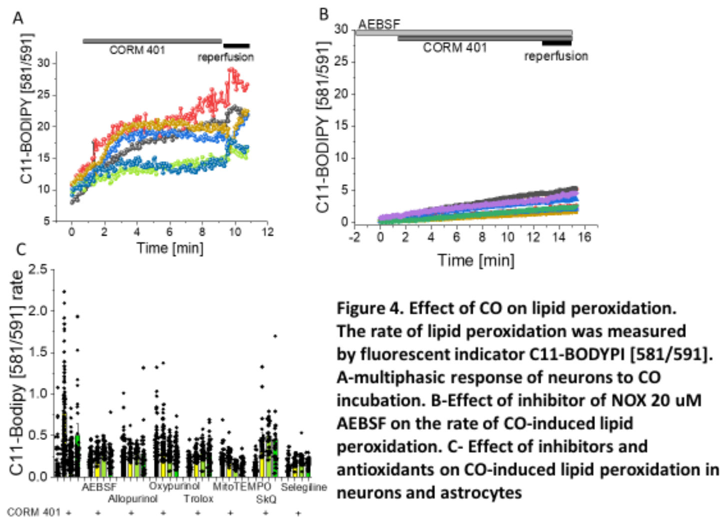


Figure 4. Effect of CO on lipid peroxidation. The rate of lipid peroxidation was measured by fluorescent indicator C11-BODYPI [581/591]. A-multiphasic response of neurons to CO incubation. B-Effect of inhibitor of NOX 20 uM AEBSF on the rate of CO-induced lipid peroxidation. C- Effect of inhibitors and antioxidants on CO-induced lipid peroxidation in neurons and astrocytes

In order to investigate if ROS overproduction and oxidative stress are involved in mechanism of CO-induced neurotoxicity we tested how various antioxidants and inhibitor of ROS production affect cell death.

We found that 30 min incubation of the primary cortical co-cultures with CO induced cell death in more than 90 % of neurons (Figure 5). We have found that inhibitor of NADPH oxidase ABSCF was the most protective and reduced the number of dead neurons from 90% to 24%. Importantly, mitochondrial antioxidants and inhibitors of XO and monoamine oxidase also had a protective effect, although it was much lower (Figure 5).

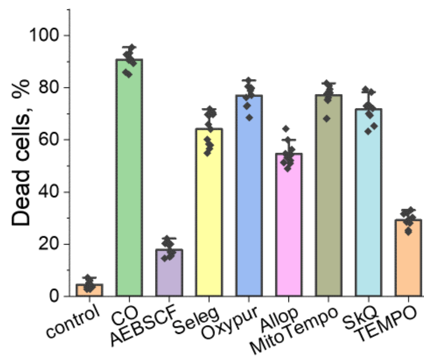


Figure 5. Effect of CO on percentage of dead cells in primary cortical co-cultures on neurons and astrocytes. Mitochondrial antioxidants (SkQ, 1 μM, MitoTEMPO) and inhibitors of enzymatic ROS production allopurinol (20μM), oxypurinol, 20 μM, AEBSF, 20 μM and selegiline, 20 μM) protect cells against cell death and GSH depletion.