In vitro studies on the cytoprotective properties of Carbon monoxide releasing molecules and N-acyl dopamine derivatives

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CHAPTER 2

Carbon monoxide: Friend and Foe

Carbon monoxide (CO) toxicity

Carbon monoxise (CO) is a colorless, odorless and tasteless gas that occurs as a byproduct of organic combustion. CO's toxicity is mainly due to its avid binding to hemoglobin (Hb) and the formation of COHb with an affinity approximately 220 higher than that of oxygen. This reduces the oxygen-carrying capacity of the blood thereby leading to tissue hypoxia. In concordance with this, the serum COHb levels correlate with the severity of symptoms. Thus, COHb levels ≤ 10% are asymptomatic, whereas levels between 10% to 30% can produce headache, shortness of breath and dizziness and finally levels above 30% to 50 % can cause confusion, seizures till coma.

As already mentioned 10% COHb constitutes the safest upper limit as up to this level there are no symptoms. However an interesting experimental setting in dogs performed many years ago suggested that CO intoxication may not be a simple function of serum COHb [53]. When dogs were administrated 13% CO gas by inhalation, they died within an hour with an average HbCO levels of 65%. In contrast severe anemic dogs that were in addition transfused with red blood cells containing Hb 80% saturated with CO survived indefinitely. Thus it appears that CO strictly bound to hemoglobin does not compromise its oxygen carrying capacity. On the contrary, administration of CO gas found to be detrimental. These results indicate that it is not the CO bound to hemoglobin that is toxic rather the fraction that escapes it [53, 54]. This dissolved CO gas can subsequently penetrate the tissue and the cells and bind to intracellular targets, i.e. numerous heme-containing proteins in the body, thus interfering with their biological activity.

Against to the dogma that CO is a poisonous gas, Sjöstrand et al found in 1949 that CO is endogenously produced in humans [55]. In 1968 Tenhunen et al identified heme oxygenase, the enzyme responsible for intracellular CO generation [56]. Heme oxygenase catalyzes the first and rate-limiting step in heme degradation yielding equimolar amounts of CO, iron and biliverdin, which is further converted to bilirubin by biliverdin reductase. This process can be easily observed as a bruise develops [53]. The release of oxygenated heme give rise to the red color seen at the beginning. The oxidation of heme by heme oxygenase results in the generation of biliverdin and thus in turn to the blue color seen afterwards. Finally, the bruise turns yellow by the conversion of biliverdin to bilirubin through the action of biliverdin reductase.

Three isoforms of heme oxygenase have been characterized, i.e. HO-1, HO-2 and HO-3. Heme oxygenase 3 has been recently cloned and is not catalytically active [57]. Although HO-1 and HO-2 catalyze the same reaction they have different molecular weights and have different regulation and expression patterns in various tissues. Heme oxygenase 2 is consecutively expressed in many tissues with high activity in testes, liver, kidney and central nervous system [58, 59]. HO-1 is the inducible isoform which is up-regulated upon stress, i.e. oxidative stress, hypoxia, UV-radiation and a variety of physical and chemical stimuli [60-63]. The induction of HO-1 is mediated primarily by transcriptional regulation [64, 65]. The mouse HO-1 (hmox-1) gene 5' regulatory region contains two upstream enhancers that contain sequences homologous to the antioxidant responsive element (ARE) [66-68]. The NF-E2-related factor-2 (Nrf2) represents the major transcriptional regulator of hmox-1 in response to many inducing stimuli [69]. Following cellular stimulation, Nrf2 migrates to the nucleus where it recognizes ARE binding sites in the hmox-1 promoter. Additionally, a number of diverse transcriptional regulators can regulate hmox-1 in a cell-type and inducer-specific fashion.

Over the past decade the function of HO-1 has been expanded from a heme degrading enzyme to a key mediator of tissue protection and host defense and its cytoprotective effects have been described *in vitro* and *in vivo*. Induction of HO-1 and its metabolites has been demonstrated to be beneficial in a variety of different pathologies i.e. ischemia/reperfusion injury, myocardial infraction, type 2 diabetes, induction of tolerance, endotoxic shock and sepsis [70, 71]. Specifically in respect to kidney pathophysiology, pharmacological and genetic approaches aimed at up-regulating HO-1 in renal tissue, have unambiguously proven to be protective in settings of I/R injury [72]. Polymorphism in the (GT)n microsatellite of HMXO1 promoter is considered to be a major factor for the variations seen in the human response to different stimuli and to susceptibility to certain pathologies [73, 74]. Apart from being a fundamental sensor of cell stress that directly drives towards preventing cell damage, the byproducts of HO-1 activity actively participate in cell defense mechanisms.

CO biological activity and molecular targets

Given the remarkable affinity of CO for transition metals, metal-containing proteins compromise the major direct and indirect targets of CO. To this end, typical CO effectors are heme-containing proteins, i.e. soluble guanylyl cyclase (sGC), NO synthase, mitochondria-proteins, heme-containing transcription factors (such as NPAS2 [75]), and heme-containining

potassium channels [76]. As an example, binding of CO to sGC alters its conformation thus positively regulating its activity. Increased sGC activity via CO has been associated with inhibition of platelet activation and aggregation [77], induction of smooth muscle cell proliferation [78], smooth muscle cell relaxation [79], effects on neurotransmission [80]. To this trend, it has been shown that CO is able to inhibit NADPH oxidase thus modulating superoxide production or cytochrome c oxidase hence interfering with electron transport and oxidative phosphorylation.

CO has many biological properties that make it an attractive molecule to use in a variety of pathological conditions. Amongst these, its anti-inflammatory, anti-apoptotic and anti-proliferative effects have been demonstrated in both *in vitro* and *in vivo* models [58, 81, 82]. In this context, it has been demonstrated that CO modulates inflammation by reducing the activation of polymorphonuclear neutrophils and by inhibiting the expression of inflammatory mediators [83-86], through preservation of mitochondrial respiration and biogenesis [87] and attenuation of oxidative stress [88-90]. CO imparts both anti-apoptotic and pro-apoptotic effects. Thus, CO prevents apoptosis of endothelial cells [91-93], hepatocytes [94] and β cells of pancreas [95]. On the contrary, CO exerts pro-apoptotic effects towards T cells [96], cancer cells [97] and hypoproliferative smooth muscle cells [98]. CO also has been shown to exert antiproliferative effects *in vitro*, with respect to the proliferation of vascular smooth muscle cells [78].

CO delivery

Throughout current studies, CO has been delivered to cells or tissues either in a gaseous form or by making use of so-called CO releasing molecules (CORMs). Even though CO inhalation seems to be an easy, straightforward and cost effective application, safety and practical issues constitute serious limitations. First of all administration of CO in its gaseous form is problematic in the clinical setting and in particular for outpatients. In addition no reliable methods have been established yet to achieve safe and effective concentrations of CO in tissues or to monitor CO toxicity [53, 99]. Despite the medical practice of monitoring CO intoxication by serum COHb, it is still not known as mentioned above if COHb constitutes the optimal measure for CO exposure and if COHb simply reflects CO intoxication. Moreover, conflicting data in rodents [100, 101] and the lack of a beneficial effect of CO inhalation in human volunteers on systemic inflammation [102], further discourage the use of CO inhalation and

suggest large inter species differences, making extrapolations of safe and effective CO concentrations to humans difficult. Finally, CO inhalation lacks any tissue specificity and distributes CO throughout the entire body. To this end, the use of CORMs seems to be a more promising approach, as these molecules do not significantly affect COHb levels in vivo [103].

Carbon monoxide-releasing molecules (CORMs)

Based on the avid affinity of CO to transition metals [104] in 2000 Motterlini and his colleagues envisioned the development of transition metal carbonyls as prototypic carbon monoxide-releasing molecules (CORMs) [105]. CORMs are bioactive molecules able to carry and deliver CO to biological systems. These compounds contain a transition metal, such as manganese, iron, cobalt or ruthenium surrounded by a certain number of carbonyl groups as coordinating ligands.

The first CORM developed in 2001 under the name of CORM-1. CORM-1 is a lipid-soluble manganese decarbonyl complex $Mn_2(CO)_{10}$, that requires light to release CO. Isolated rat hearts perfused in the presence of $Mn_2(CO)_{10}$ displayed a marked attenuation in coronary vasoconstriction only after stimulation with light [105]. In the same time the second CORM was developed, namely CORM-2. CORM-2 released CO spontaneously to heme-containing proteins and it was shown to elicit profound vasodilatation in isolated rat aortae [106] and prevented the increase in mean arterial pressure in a rat model of acute hypertension [105]. As organic solvents are not compatible with biological systems, then effort was given for the development of water-soluble compounds such as CORM-3 [107] and CORMA1 [108].

In the following years several groups studied the anti-inflammatory [109-113], the ant-proliferative [114] and cytoprotective [115] properties of CORMs in a variety of disease models i.e. ischemia/reperfusion model [116-121], osteoarthritis [122], sepsis [111, 112, 123], acute cardiac [124, 125], liver [126] and kidney [127-129] injury.

Despite the large collection of CORMs and their beneficial therapeutic effects in a series of animal models, these complexes are lacking an assignable pharmacokinetic profile [130, 131], which hinders their development in the clinic. To mention, most CORMs release CO immediately after dissolution in aqueous buffer with a half-life time of some minutes. In addition they present a poorly understood toxicological profile and despite the promising

preclinical data, current toxicity reports demonstrated contradictory data thus indicating that more studies are needed to evaluate the safety profile of these compounds and their metabolites [132, 133]. Binding and interaction of CORMs with proteins and especially plasma proteins needs also to be further explored [130]. As a tool to help the design of CORMs with the appropriate pharmaceutical properties, Romao and his group proposed a conceptual model [131], made up of three components: a) the metal center responsible for the main properties and the cytotoxic profile of the molecule, b) the coordination sphere (CO and ancillary ligands), which determines the CO rate and the triggers mechanism required to initiate the liberation of CO, c) the drug sphere obtained by modifying the ancillary ligands at their distal sites, thus modulating the desired pharmacological parameters. ALF492, ALF795 and B_{12}-ReCORM-2 are three examples of CORMs with favorable characteristics for in vivo application as described above [130]. Indeed, ALF492 conferred enhanced water solubility and biocompatibility and was preferentially distributed in the liver by targeting asialoglycoprotein receptors [134, 135]. ALF795 also displayed a low toxicity with favorable drug property. This compound delivered also CO in a specific manner of the liver in a model of acetaminophen-induced acute liver failure [135]. Finally, B_{12}-ReCORM-2 has produced based on cyanocobolamin (B_{12}) and even showing cytoprotective properties in an ischemia/reperfusion model this molecule had a poor cellular uptake [136].

In an attempt to achieve tissue targeting, Schmalz and his colleagues introduced acyloxy-butadiene-$Fe(CO)_3$ complexes as potential enzyme-triggered CORMs (ET-CORMs). ET-CORMs are lipid soluble compounds stable in buffer solution under physiological conditions and their tissue selectivity is based on potential differences in cellular enzyme expression rates. Upon exposure of these CO prodrugs to esterases their ester functionality is cleaved, resulting in the formation of labile dienol-iron carbonyl complexes that subsequently disintegrate under oxidative conditions to release CO, iron and the corresponding enone (Figure 1).

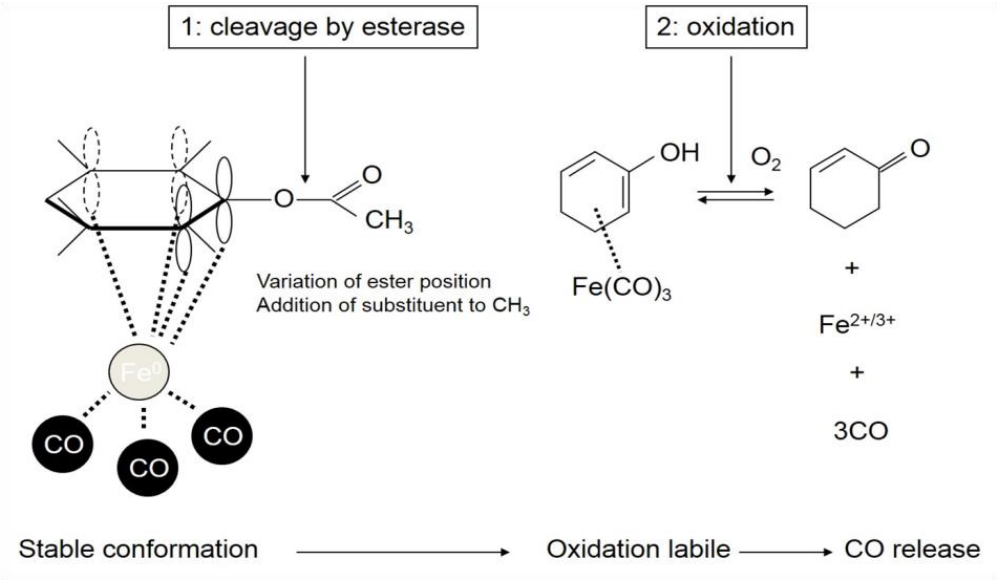

***Figure 1**: Enzymatic cleavage of Enzyme triggred CO-releasing molecules by esterase*

In their initial study Schmalz and his group already shown that ET-CORMs are able to release CO in an esterase dependent manner and that they exert biological effects. Particularly, these complexes inhibited iNOS in a cell-based system using RAW267.4 cells [137]. In the studies presented in this thesis we further investigated the biological properties in relation to their chemical structure, thus offering a better understanding of these molecules.