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Molecular Pharmacology

Perspective

No NO for HO-1 from SNP

By

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Running Title:

SNP, iron and HO-1

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Abbreviations:

NO, nitric oxide;
SNP, sodium nitroprusside;
HO-1, heme oxygenase-1;
CO, carbon monoxide;
SIN-1, linsidomine;
SNAP, S-nitroso-*N*-acetyl-DL-penicillamine;
IRP, iron regulatory protein;
PKA, protein kinase A;
MAPK, mitogen-activated protein kinase;
ERK, extracellular regulated kinase;
JNK, c-Jun *N*-terminal kinase;
ROS, reactive oxygen species

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ABSTRACT

Nitric oxide (NO) and NO donors where among the first reported inducers of the tissue protective protein heme oxygenase-1 (HO-1) with a potential for eventual use in humans. Besides other clinically established NO releasing drugs, sodium nitroprusside (SNP) has frequently been employed as an experimental tool to explore effects of NO on HO-1 and other biological targets. In this issue of *Molecular Pharmacology (Mol Pharmacol…..),* Kim et al. demonstrate that the effects of SNP on expression of HO-1 are mainly due to free iron released from SNP in aqueous solution whereas NO plays a negligible role, if any, as the mediator of response to SNP. Downstream effects of iron, after being dissociated from SNP, include increases in intracellular cAMP that are causally linked to subsequent phosphorylation of specific MAPK targets and enhanced HO-1 protein levels. Based on the data by Kim et al., the use of SNP as an experimental tool to mimic intracellular effects of NO should be avoided in the future. This work not only helps revise concepts in NO and HO-1 research but also may direct future efforts to the role of iron and reactive oxygen species in the regulation of adenylyl cyclase.

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In recent years, the inducible stress protein heme oxygenase-1 (HO-1, HSP 32) has been recognized as a central player in maintaining antioxidant and anti-inflammatory defense mechanisms. Antisense and knock-out studies, as well as clinical investigations, have clearly shown that induction or increased expression of HO-1 is followed by, and causally related to, tissue protective actions that lead to inhibition of atherogenic processes in the cardiovascular system. Cytoprotective actions of HO-1 are not confined to the vasculature and have been reported to occur in various tissues including heart, kidney, and neuronal cells (Ryter et al., 2002; Wagener et al., 2003; Maines, 2004). Thus and in addition to its general anti-inflammatory function, HO-1 prevents chronic rejection or arteriosclerosis of transplants (Soares et al., 1998) and enhances the resistance of pancreatic islet cells to cytokine-mediated injury (Ye and Laychock, 1998). Moreover, the first human case of HO-1 deficiency, which occurs secondary to a genetic disorder, shows severe, persistent endothelial damage and increased tissue vulnerability to oxidant injury besides growth retardation and anemia (Yachie et al., 1999)

The reaction catalyzed by HO-1 results in the degradation of heme and yields biliverdin and carbon monoxide (CO), two metabolites that were initially considered mere waste products of heme catabolism without physiological function. This view has dramatically changed in recent years. Biliverdin is subsequently transformed to bilirubin by biliverdin reductase. Bilirubin, which is formed from biliverdin by biliverdin reductase, exerts strong antioxidant effects at physiological plasma concentrations. High-normal plasma levels of bilirubin were reported to be inversely related to atherogenic risk and to provide protection against endothelial damage (Hopkins et al., 1996; Mayer 2000). Risk reduction by bilirubin was comparable to that of HDL cholesterol (Hopkins et al., 1996). CO is likewise known to produce anti-apoptotic and cytoprotective actions. In addition, CO activates the soluble

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guanylyl cyclase/cGMP system, and is thought to function, through this signaling pathway and in close resemblance to nitric oxide (NO), as a smooth muscle relaxing agent and neurotransmitter (Ryter et al., 2002; Wu and Wang, 2005).

A third component generated during heme catabolism is free iron, which in turn activates translational expression of ferritin. Ferritin has been shown to provide marked antioxidant cellular protection by rapidly sequestering free cytosolic iron, the crucial catalyst of oxygen-centered radical formation via the Fenton reaction in biological systems (Balla et al., 1992). Thus, in addition to HO-1, ferritin plays an important role as a fast acting endogenous cytoprotectant in cellular antioxidant defense mechanisms.

The unique combination of tissue protective and smooth muscle relaxing properties makes HO-1 an interesting target for treatment of cardiovascular diseases, including atherosclerosis, and other inflammatory disorders, among them neurodegenerative processes such as Alzheimer's and Parkinson's (Ryter et al, 2002; Maines, 2004). Support for the importance of HO-1 in human disease comes from clinical investigations demonstrating that HO-1 promoter polymorphisms with longer (GT)n repeats are associated with lower transcriptional activity as well as diminished vascular protection from atherogenic insults (Alam et al, 2003; Exner et al., 2004). In addition, HO-1 seems crucial for keeping the human uterus in a relaxed state during pregnancy and a reduced expression of placental HO-1 has been associated with a higher risk for pre-eclampsia (Bainbridge and Smith, 2005). Thus, therapeutic strategies aimed at moderately increasing tissue expression of HO-1 are potentially beneficial in a number of disease states. However, until recently, known inducers of HO-1, such as cadmium chloride and other heavy metals, did not have great promise for eventual therapeutic use in humans.

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NO and NO donors were the first reported "benign" inducers of HO-1; these agents includedlong-established drugs in cardiovascular therapy such as glyceryl trinitrate or molsidomine and its active metabolite linsidomine (SIN-1). Those agents were shown to enhance, via cGMP-dependent pathways, expression of HO-1 at the mRNA, protein and catalytic level and, in addition, to prolong the half-life of HO-1 mRNA through cGMP-independent interactions of NO with the transcript (Yee et al., 1996; Durante et al., 1997; Polte et al., 2000; Bouton and Demple 2000). Meanwhile, it has become clear that other known HO-1 inducers such as aspirin can act via increasing endogenous NO formation from L-arginine (Grosser and Schröder 2002; Grosser et al. 2003; Bach, 2005).

Sodium nitroprusside is a "spontaneous" NO donor, releasing NO (and NO^+) upon dissolution in aqueous solvents and, in contrast to nitric acid esters, does not require enzymatic reduction or hydrolysis for this process. However, degradation of SNP generates two other biologically active products besides NO, notably cyanide and free iron. Therefore, the use of SNP as experimental tool for investigation of the effects of NO in biological systems is, to say the least, problematic. Despite being such a "messy" NO donor, SNP has been used (and still is) in studies where it is supposed to act *solely* as donor of NO (which it does not) and where the effects observed are interpreted as being the consequence of NO release from SNP,which based on the numbers of active mediators generated from SNP has only ~ 33% chance of being the correct explanation.

Using RAW 264.7 murine macrophages, Kim et al. clearly demonstrate that, in case of HO-1 induction by SNP, NO has, at best a negligible role as a mediator of this action of SNP. In a number of well-designed experiments and by using appropriate controls such as the "true" donor of NO, SNAP, Kim et al. provide compelling evidence that SNP's NO or NO^+ contribute minimally to HO-1 induction. Thus, SNP turns out to be a much stronger HO-1

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inducer than SNAP although it possesses only 13% of SNAP's NO donating capacity under these conditions. Also, scavengers of NO as well as inhibitors of its downstream signaling target, soluble guanylyl cyclase, are without effect on HO-1-induction by SNP in RAW macrophages.

Kim and co-workers conclude that free iron and not NO is the driving force behind the observed increases in HO-1 protein following exposure to SNP. Induction of HO-1 by SNP but not SNAP are blocked upon co-incubation with the iron binding compound deferoxamine and the authors are able to mimic the effect of SNP on HO-1 by use of exogenous iron (ferricyanide or ferric ammonium citrate). Cyanide, the third component released during SNP dissociation, does not function as an HO-1 inducer, since a cyanide inhibitor does not alter the SNP effect on HO-1. This is in agreement with earlier observations by Motterlini and co-workers who found that in contrast to other NO donors, SNP was cytotoxic at high concentrations due to the release of catalytic iron (Motterlini et al., 1996).

How does iron induce HO-1? The answer given by Kim et al. is a very simple and unexpected one, based on the generally accepted concept of iron working through iron regulatory and binding proteins such IRP1 and IRP2. Using an inhibitor approach, Kim et al. demonstrate that iron released from SNP induces HO-1 via adenylyl cyclase activation and enhanced formation of cAMP: Exogenous iron as well as SNP increase both intracellular cAMP and HO-1 levels. HO-1 induction by both SNP and iron is almost completely blocked by inhibitors of protein kinase A (PKA). This series of experiments also reveals that the NO/cGMP system tends to play a minor role since inhibitors of protein kinase G cause a small, non-significant reduction of HO-1 induction by SNP. Ultimately however, this secondary signaling pathway seems to converge with the adenylyl cyclase/cAMP cascade since cGMP can activate expression of cAMP-sensitive cytoprotective pathways by inhibiting

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cAMP breakdown (Polte and Schröder 1998). The role of cAMP in mediating the effect of SNP/iron on HO-1 becomes even more convincing in the light of another series of experiments aimed at assessing the phosphorylation of PKA targets within the group of mitogen-activated protein kinases (MAPK). In RAW macrophages, extracellular regulated kinase (ERK1/2) and c-Jun *N*-terminal kinase (JNK) are reponsive to SNP and iron whereas p38 MAPK seems to be of no relevance for the induction of HO-1. SNP and 8-Br-cAMP produced the same pattern of MAPK phosphorylation, notably of ERK1/2 and JNK, which in case of SNP is antagonized by both H89 (a PKA inhibitor) and deferoxamine (an iron chelator). Of various MAPK inhibitors employed by the authors, only those interfering with ERK and JNK are able to reverse HO-1 induction by SNP. The sheer number of control experiments at different levels within this signaling cascade is impressive and provides a very solid basis for this novel SNP-iron-cAMP-PKA-MAPK-HO-1 pathway proposed by Kim and co-workers.

There is one step in this pathway, however, where the authors remain remarkably vague about potential bridging mechanisms, namely the link between iron release from SNP and increased cAMP levels. Although they acknowledge that iron is known to be involved in the regulation of genes and proteins, particularly those with pro- or anti-inflammatory functions, no attempt is made to explain the stimulatory effect of iron on the adenylyl cyclase/cAMP system.

However, considerable evidence has accumulated over the past decades that free iron can increase adenylyl cyclase activity, either alone or in synergy with other stimuli (Baba et al., 1981; Sponsel et al., 1996; Tan et al., 1995). The underlying signaling mechanisms apparently involve the formation of reactive oxygen species (ROS) via iron-catalyzed free radical formation (Fenton reaction). Oxygen-derived free radicals are thought to enhance cAMP

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formation via tyrosine kinase-mediated effects on the catalytic subunit of adenylyl cyclase (Tan et al., 1995).

Based on such results, one could ask whether the inhibitor approach chosen by the authors to elucidate signaling events downstream of adenylyl cyclase would have been equally successful to explore the missing upstream link connecting iron and adenylyl cyclase. Based on the ROS concept of adenylyl cyclase regulation, antioxidants such as N-acetylcysteine should prevent the iron-dependent increases in cAMP observed by the authors. For now, there is enough evidence from the literature to add a hypothetical iron/ROS link to the upper part of the authors' elaborate signaling scheme (Fig. 1). It may take only one or two experiments to make the question mark disappear!

Based on the data by Kim et al., the use of SNP as an experimental tool to mimic intracellular effects of NO should once and for all be avoided, particularly since numerous "clean" NO donors are available as alternatives. The importance and value of this work lies in the detailed and extensive approach by which the authors establish a clear causal link between iron (released from SNP) and its downstream effects on cAMP, MAPK and HO-1, effects which were previously, and apparently mistakenly, attributed to SNP-derived NO. Perhaps as importantly, this paper may not only lead to revised concepts in NO and HO-1 research but could redirect attention to the not fully explored role of iron and free radicals in the regulation of adenylyl cyclase.

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FIGURE LEGEND

Figure 1:
Reactive oxygen species generated via the Fenton reaction may be the missing link between iron released from SNP and increased adenylyl cyclase activity, i.e. elevated cAMP levels. Original diagram from Kim et al. *(Mol Pharmacol…),* modifications in red color.

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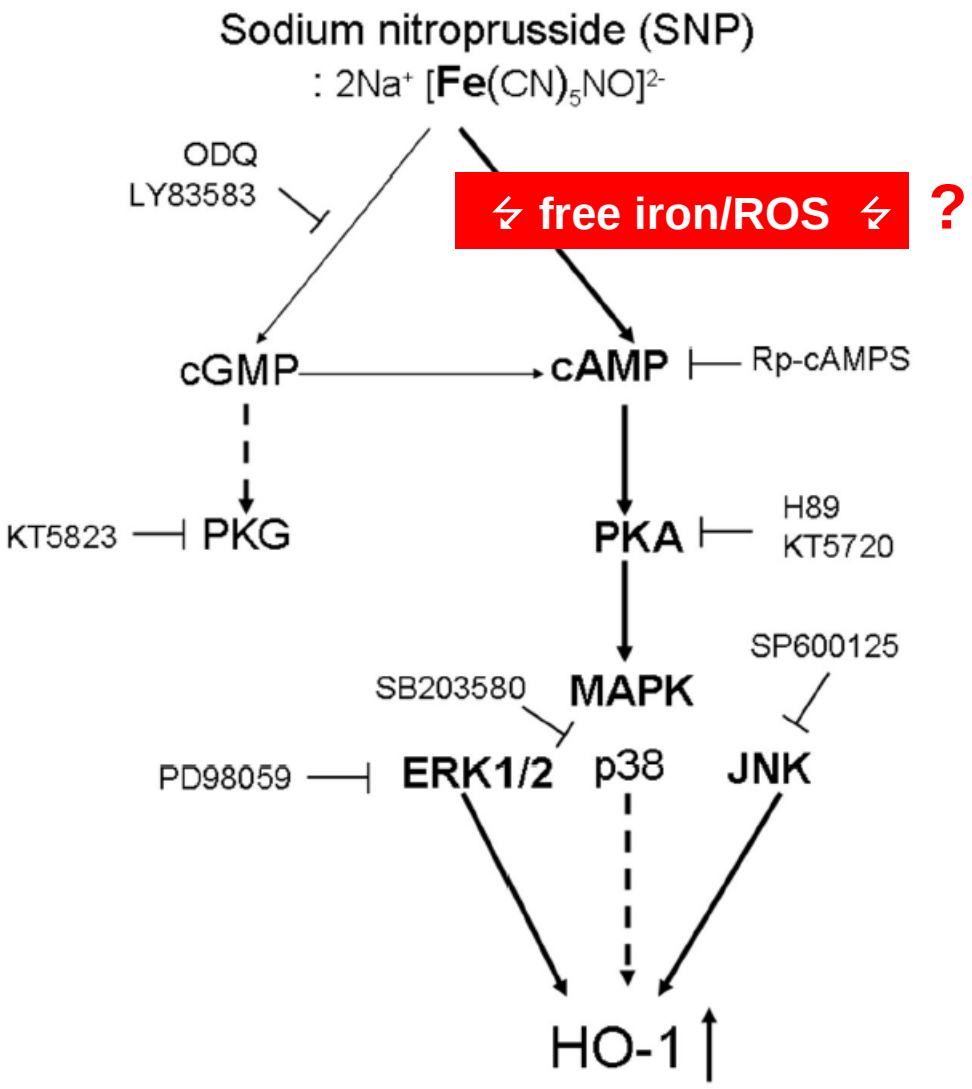

Figure 1

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