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Role of antioxidants in the protection of the nitrergic neurotransmitter

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Abstract

There is now compelling evidence that the L-arginine/nitric oxide (NO) pathway generates the non-adrenergic non-cholinergic (NANC) neurotransmitter which mediates smooth muscle relaxation in a variety of nitrergically-innervated tissues. However, one strange aspect of this nitrergic neurotransmission process is that certain drugs (i.e. superoxide generators and NO-scavengers) powerfully inhibit relaxations to exogenous NO, but have little or no effect on relaxations to electrical field stimulation. This thesis examined the possibility that in the nitrergically-innervated gastric fundus of the pig tissue antioxidants present in the neuroeffector junction might protect the endogenous nitrergic neurotransmitter (free radical NO) from attack by superoxide anions and scavenging activity, while exogenous NO would still be vulnerable before it reaches the nitrergic synapses within the tissue.

We found that several antioxidants (in casu Cu/Zn superoxide dismutase, reduced glutathione, bilirubin) exerted a partial or complete protection of the relaxation induced by exogenous NO against the differentiating drugs under investigation. A close interrelationship between the endogenous nitrergic neurotransmitter and the antioxidants Cu/Zn superoxide dismutase and bilirubin (produced by the heme oxygenase/biliverdin reductase system) was corroborated by immunohistochemical data showing the presence of these latter defense systems in all nitrergic neurons. Pharmacological depletion further established a role for Cu/Zn superoxide dismutase in peripheral nitrergic neurotransmission. For glutathione, only a partial depletion could be obtained and this did not influence nitrergic neurotransmission.

Introduction

The free radical nitric oxide (NO), derived from *L*-arginine by the catalytic activity of the family of NO-synthase (NOS) isozymes, has emerged in the last decade as a key molecule in most fields of life sciences. The endothelial NOS (eNOS) – derived NO has been implicated in a vast array of cardiovascular functions including regulation of blood pressure, endothelial permeability and platelet aggregation ; inducible NOS (iNOS) – derived NO exerts antimicrobial, antitumoral and immunoregulatory effects ; neuronal NOS (nNOS) – derived NO has been established as a messenger molecule in signal transduction in both the central and peripheral nervous systems.

In the central nervous system NO was first characterized as the intercellular messenger mediating the increase in cGMP levels that follows activation of glutamate receptors (Garthwaite *et al*., 1989). Although nNOS positive neurons represent roughly only 1% of cell bodies in the cerebral cortex, virtually every neuron in the cortex is exposed to nNOS nerve terminals. nNOS can mediate anterograde and retrograde transmission and is particularly involved in neuronal signaling, neurotoxicity, modulation of behavioural pathways such as learning or expression of pain, and in synaptic plasticity (long-term potentiation and long-term depression).

There is also general consensus that NO fulfills a principal role in peripheral neurotransmission as inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitter producing relaxation of smooth muscle in the respiratory, urogenital, cardiovascular and gastrointestinal systems (Rand & Li, 1995a). Among the gastrointestinal motility patterns directed by NO-releasing nitrergic motor neurons localized in the myenteric plexus of the enteric nervous system, the relaxation of the proximal stomach during food intake represents one of the most extensively studied and best documented models.

Identity of the peripheral nitrergic neurotransmitter : NO or not ?

The appreciation of NO, a simple free radical gas mediating synaptic transmission as faithfully as acetylcholine and noradrenaline, as neurotransmitter has weakened the importance of the classical criteria for a substance to become accepted as a neurotransmitter. These criteria are the following : (1) the substance should be synthesized by the neuron and moved to the sites of storage in the nerve terminals ; (2) the substance should be released (Ca^{2+}-dependent) from the nerve terminals ; (3) postjunctional (postsynaptic) responses to exogenous

application of the putative neurotransmitter should mimic the responses to nerve stimulation ; (4) there should be an inactivation process for the proposed neurotransmitter (a specific mechanism for uptake or redistribution of the substance or its breakdown products) ; (5) drugs that block or potentiate the responses to the exogenous putative neurotransmitter should have parallel effects on responses to nerve stimulation. Free radical NO does not meet the criteria (1) and (4), and in that sense differs from classical neurotransmitters. Still, one would expect that the fifth criterion is fulfilled. Although in the peripheral nervous system exogenous NO (administered as an aqueous solution of nitric oxide gas) indeed produces relaxations of nitrergically-innervated smooth muscle tissues that are very similar to the effects of nitrergic nerve stimulation, the fifth criterion has been challenged by the following pharmacological observation : certain drugs can potently reduce or even abolish relaxations of smooth muscle to exogenous NO, but at the same time have no effect on relaxations induced by the nitrergic neurotransmitter, released by electrical stimulation of nitrergic nerves, in the same tissues (Barbier & Lefebvre, 1992). These drugs, collectively called NO-inhibitors and discriminating between relaxations to exogenous NO (blockade) and those to nitrergic nerve stimulation (no effect), can be subdivided into two different categories : (1) the superoxide anion generators and (2) the NO-scavengers.

The superoxide anion generators produce the one-electron reduced chemical form of oxygen i.e. the superoxide anion ($O_2^{\bullet -}$) ; when produced in proximity, this superoxide anion radical and free radical NO can chemically interact at an almost diffusion-limited rate resulting in the abolition of the biological activity of NO and the formation of peroxynitrite. The mode of action of the NO-scavengers is through a direct binding (scavenging) of free radical NO. Since the nitrergic neurotransmitter has to diffuse from its neuronal site of synthesis to its target enzyme soluble guanylyl cyclase in the neighbouring effector smooth muscle cells, it is during this journey vulnerable to superoxide attack and NO-scavenging activity ; if the nitrergic neurotransmitter is free radical NO, this is expected to result in a decreased bioavailability of the nitrergic neurotransmitter and hence a reduction (or complete blockade) of smooth muscle relaxation. So, although it is recognized that the peripheral nitrergic neurotransmitter, released from nitrergic nerves, is synthesized by and requires the functional integrity of nNOS in the nerves (Förstermann & Kleinert, 1995), there still remains doubt on the exact biochemical identity of the peripheral nitrergic neurotransmitter (Rand & Li, 1995b). This debate has inspired a number of possible explanations, but the most elegant and experimentally the best supported theory is the ‘antioxidant theory’.

Role of antioxidants in peripheral nitrergic neurotransmission

Several lines of evidence support the hypothesis that natural tissue antioxidants, present in the neuroeffector region and/or released by nitrergic nerve terminals, might function as ‘guardian angel’ for free radical NO : they may provide a protective shield fencing off radical attack and scavenging activity thereby creating a correct redox environment for NO to fulfill its function as endogenous nitrergic neurotransmitter. Reactive oxygen species (ROS) are indeed produced by all living organisms using molecular oxygen as fuel for energy generation (i.e. aerobic metabolism). ROS (such as the superoxide anion, hydrogen peroxide and the hydroxyl radical) are capable of chemically altering virtually all major classes of biomolecules (e.g. lipids, proteins, nucleic acids) with concomitant changes in structure and function. For this reason, all aerobic organisms have evolved a variety of mechanisms to protect themselves from the potentially deleterious effects of ROS. These include antioxidant enzymes such as superoxide dismutase (SOD) and catalase, as well as non-enzymatic water- and lipid-soluble antioxidants such as reduced glutathione, ascorbic acid (vitamin C) and α-tocopherol. Due to the above mentioned antioxidant systems, which are also integrated in nitrergically-innervated tissues, the chemical interaction between free radical NO and ROS will be limited ; this enables free radical NO to perform a role as endogenous nitrergic neurotransmitter. This hypothesis also explains the observed discriminatory action of superoxide generators, without the need to invoke a transmitter other than free radical NO : the antioxidant systems protect the endogenously released nitrergic neurotransmitter against attack by superoxide anions and scavenging activity, while on the other hand exogenous NO, applied in the organ bath, is still vulnerable to inactivation before reaching the protective antioxidant environment of the nitrergic synapses.

The group of W. Martin in Scotland was the first to test this theory to reality. In the bovine retractor penis muscle, Martin *et al.* (1994) confirmed that relaxations in response to nitrergic nerve stimulation were resistant to several superoxide generators (pyrogallol, 6-anilino-5,8-quinolinedione and xanthine/xanthine oxidase), while those to exogenous NO were significantly reduced. However, when they pretreated the tissue with the copper chelator diethyldithiocarbamate (DETCA), which irreversibly inhibits both extra- and intracellular Cu/Zn dependent SOD, the superoxide generators did now inhibit the responses elicited by nitrergic nerve stimulation ; furthermore, this inhibition could be reversed by administration of exogenous Cu/Zn SOD. As it is well-known that the enzyme superoxide dismutase (either copper/zinc SOD or

manganese SOD) dismutates superoxide anions to hydrogen peroxide, which is subsequently converted to molecular oxygen and water by catalase, and thus regulates the tissue level of superoxide anions, the authors proposed that high levels of endogenous Cu/Zn SOD were protecting the nitrergic transmitter at the neuroeffector region from the inactivating action of superoxide anions ; exogenously added NO could then still be inactivated before reaching the Cu/Zn SOD-protected areas in the tissue. Similar results were obtained in other gastrointestinal nitrergically-innervated tissues (Lilley & Gibson, 1995 ; Lefebvre, 1996). However, results that do not fit within the antioxidant hypothesis were also reported in the literature : inhibition of Cu/Zn SOD with DETCA did not alter the differential action of superoxide generated by hypoxanthine/xanthine oxidase and pyrogallol in respectively the rat gastric fundus (Lefebvre, 1996) and the rat anococcygeus muscle (La & Rand, 1999). Furthermore, the NO-scavenging agents carboxy-PTIO in the mouse anococcygeus (Lilley & Gibson, 1996) and hydroxocobalamin in the rat gastric fundus (Lefebvre, 1996) held their discriminating action between exogenous NO and the endogenous nitrergic neurotransmitter even in DETCA-pretreated tissue. This indicates that the presence of Cu/Zn SOD does not provide a complete answer for the discriminatory effect of superoxide generators, and certainly not for the NO-scavengers ; additional factors must thus be involved.

Of course, Cu/Zn SOD represents only one of several crucial antioxidant systems exploited by aerobic tissue to withstand oxidative stress (Halliwell, 1994). Experimental work in mouse and rat anococcygeus muscle by Lilley & Gibson (1996, 1997) has demonstrated that these other natural tissue antioxidants (reduced glutathione, ascorbic acid and α-tocopherol) also might participate in the defense of neuronally released free radical NO against superoxide and scavenging inactivation. Indeed, their findings in mouse anococcygeus that reduced glutathione, α-tocopherol and ascorbic acid protected against one or more of the battery of NO-inhibitors tested (i.e. duroquinone, xanthine/xanthine oxidase, hydroquinone and carboxy-PTIO) (Lilley & Gibson, 1996), leans direct support to the notion that these antioxidants might also secure the endogenous nitrergic neurotransmitter. Moreover, one of these postulated protective antioxidants, ascorbic acid, was released from the rat anococcygeus along with the nitrergic transmitter upon nitrergic nerve depolarization (Lilley & Gibson, 1997). Further support corroborating a role of antioxidants in nitrergic neurotransmission was added by Liu *et al.* (1997) : they examined by immunohistochemistry the tissue distribution of Cu/Zn SOD in rat anococcygeus muscle and concluded that there was a selective colocalization of Cu/Zn SOD and nNOS within the neurons of the myenteric plexus, suggestive of a functional relationship between both enzymes.

Taken all together, the above results raise the possibility that several antioxidant mechanisms may act in concert to protect free radical NO as nitrergic neurotransmitter on its journey from the nerve terminal, across the synaptic cleft, towards the target protein within the postjunctional smooth muscle effector cell. This antioxidant redox environment may prove to be the key factor responsible for the inability of several NO-inhibitors to affect nitrergic neurotransmission.

It was therefore the aim of the present study to further elucidate the role of antioxidants in preserving the bioavailability of free radical NO as the endogenous nitrergic neurotransmitter versus NO-inhibitors using the nitrergically-innervated porcine gastric fundus as experimental model.

Experimental work

In a first series of experiments on isolated circular smooth muscle preparations of porcine gastric fundus, we studied the influence of a number of NO-inhibitors, respectively one superoxide generator (6-anilino-5,8-quinolinedione ; LY83583), one NO-scavenger (hydroxocobalamin ; HC) and hydroquinone (HQ ; superoxide generator or NO-scavenger depending on the experimental tissue), on nitrergic relaxations elicited by exogenously added free radical NO or by nitrergic nerve stimulation (Colpaert & Lefebvre, 2000). The results demonstrate that LY83583, HQ and HC exert their discriminatory action on exogenous free radical NO and the endogenous nitrergic transmitter, as cited in the literature, also in the porcine gastric fundus indicating that our experimental model is adequate for further investigation of a role for antioxidants in nitrergic neurotransmission. Subsequently, we have studied the influence of exogenous administration of antioxidants on short-lasting relaxant stimuli in the presence and absence of LY83583, HQ and HC. Some of these antioxidants (e.g. Cu/Zn superoxide dismutase, reduced glutathione and bilirubin) protect (partially or completely) the relaxation to exogenous free radical NO against inactivation by one or more of the NO-inhibitors ; this suggests that these antioxidants might play a role in the protection of the endogenous nitrergic neurotransmitter against inactivating agents.

In order to further sustain the hypothesis that antioxidants protect the endogenous nitrergic neurotransmitter against NO-inhibitors, we tried to deplete our experimental preparations of endogenous tissue antioxidants and to determine the sensitivity of the endogenous nitrergic transmitter (released by nitrergic nerve stimulation) towards superoxide generators and NO-scavengers in these

conditions of depletion (Colpaert *et al*., 2002a). First of all, we have focused on Cu/Zn superoxide dismutase (Cu/Zn SOD) and on reduced glutathione (GSH), since they exhibit a broad protection pattern of exogenous free radical NO against the NO-inhibitors tested in this study. By use of the copper chelator diethyldithiocarbamate (DETCA) we can almost completely inhibit the Cu/Zn dependent SOD. Indeed, spectrophotometric analysis of the Cu/Zn SOD activity in homogenates of DETCA-pretreated preparations shows a reduction of 95% of the specific activity when compared with untreated controls. Functional in vitro organ bath studies clearly demonstrate that, when pre-incubating the smooth muscle preparations with DETCA (i.e. inhibiting Cu/Zn SOD), the relaxation to the endogenous nitrergic neurotransmitter, which is released from the nitrergic nerve endings upon electrical stimulation, becomes strongly sensitive towards the superoxide generator LY83583 and the NO-scavenger HC ; the amplitude of relaxation is indeed approximately halved. The potential protection of the biological activity of the nitrergic transmitter by Cu/Zn SOD is further corroborated by an immunohistochemical study in porcine gastric fundus. Using indirect immunohistochemical techniques, we could demonstrate that there is 100% colocalization of nNOS and Cu/Zn SOD. To eliminate the antioxidant potential of GSH in tissue, several methods have been proposed in the literature. We have tested the efficiency of three different depletion strategies. These include : 1. inhibition of γ-glutamyl-cysteine synthetase, the rate limiting enzyme in the biosynthesis of GSH, via buthionine sulphoximine (BSO) ; 2. inhibition of GSH reductase, thereby preventing the reduction of oxidized GSH to GSH via carmustine ; 3. complexation of GSH and formation of an adduct with ethacrynic acid. Only for the BSO depletion method, we could find a significant reduction in tissue GSH content : spectrophotometric analysis of the GSH content in homogenates of smooth muscle preparations pretreated with BSO reveals a 40% reduction in GSH content when compared with the value found in untreated controls. Yet, pretreatment with BSO does not render the relaxations to nitrergic nerve stimulation sensitive to superoxide generators and NO-scavengers. Based on the above data, we can conclude that Cu/Zn SOD plays an important role in nitrergic neurotransmission in porcine gastric fundus. Before excluding that this does not hold for GSH, we should be able to investigate the influence of a more pronounced reduction of the GSH content on nitrergic neurotransmission.

Bilirubin is the third antioxidant we further elaborated on. It is derived from heme via two consecutive enzymatic steps : heme oxygenase (HO) converts heme to biliverdin, which is then subsequently reduced to bilirubin by biliverdin reductase (BVR). Since relatively little is known about the localization of heme oxygenase-2 (HO-2, the constitutive isoform of HO) in the gastrointestinal tract and no data can be found on the gastrointestinal distribution of BVR, we decided to first investigate immunohistochemically the localization of HO-2 and BVR in the gastric fundus of both pig and human (Colpaert *et al*., 2002b). Immunoreactivity for HO-2 and BVR can be detected in the epithelium of the gastric fundus, in endothelial cells lining intramural blood vessels, in interstitial cells of Cajal and in intrinsic neurons of both the submucous and myenteric plexus. Moreover, there is a perfect overlap between HO-2 and BVR immunofluorescence, indicating the possibility that heme-derived biliverdin can indeed be reduced to bilirubin. This part of the study was also extended to human gastric tissue ; we can conclude that there is no interspecies difference between pig and human regarding the localization of HO-2 and BVR in the gastric fundus.

Further immunohistochemical analysis on frozen sections of porcine gastric fundus, clearly demonstrates that all intrinsic myenteric neurons containing nNOS also stain for HO-2 and BVR, meaning that all nitrergic neurons possess the enzymes necessary for bilirubin biosynthesis. In order to interfere with the production of bilirubin, we tested tin protoporphyrin (SnPP). SnPP is commonly used to inhibit the production of bilirubin as it is a competitive inhibitor of the HO-enzyme ; furthermore, SnPP has been proposed to be the most selective HO-inhibitor for gastrointestinal research. However, incubation of the smooth muscle preparations with SnPP does not render the relaxation to nitrergic nerve stimulation sensitive to the superoxide generator LY83583 ; spectrophotometric analysis of the HO-activity in homogenates of SnPP-pretreated smooth muscle preparations reveals no difference when compared with untreated controls. This indicates that, at least in porcine gastric fundus, SnPP is unable to inhibit HO-activity. Due to this non-effect of SnPP, the possible role of bilirubin as endogenous protectant of the nitrergic neurotransmitter can not be further substantiated.

Conclusion

In general, our results strongly suggest that endogenous tissue antioxidants play a role in the protection of the endogenous nitrergic neurotransmitter against inactivation by both superoxide generators and NO-scavengers in the porcine gastric fundus. Cu/Zn superoxide dismutase and bilirubin and possibly other antioxidants might form a correct redox environment enabling free radical NO to fulfill its role as nitrergic neurotransmitter. Such an antioxidant redox environment might be of importance in all peripheral organs with a nitrergic component in the NANC neurotransmission system.

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