Leading article

Inducible nitric oxide synthase: a little bit of good in all of us

Summary

The established dogma regarding the different isoforms of nitric oxide has been that constitutively expressed nitric oxide synthase is an extremely important homeostatic regulator of numerous important physiological processes whereas the inducible form of nitric oxide synthase underlies injury associated with intestinal inflammation. In this brief overview, I review some of the literature that clearly supports this contention, particularly the dramatically beneficial effects of oral L-NAME administration to animals with colitis induced by trinitrobenzene sulphonic acid (TNBS). However, I also highlight some of the gastrointestinal data that does not fit this simple tidy paradigm, particularly with respect to the inducible form of nitric oxide synthase (iNOS). For example, iNOS induced healing of skin and the intestinal mucosa, killing of certain bacteria, regulation of T cell proliferation and differentiation (Th1 *v* Th2), and control of leucocyte recruitment may mask or counter the toxic metabolites that are produced by iNOS. Perhaps it is not surprising that one does not always obtain benefit from inhibiting all iNOS either by gene deletion or by systemic NOS inhibition. I raise some potential flaws in our approaches to studying iNOS. For example, to date no attempts have been made to selectively inhibit iNOS in single cell types. Global inhibition of all iNOS assumes that the large variety of cell types that can produce iNOS have identical functions. Finally, I attempt to highlight areas that require additional investigation and issues that have not been explored.

Introduction

Over the past 10 years more than 30 000 papers have been published on nitric oxide. A major facilitator for this level of activity was the availability of a number of nitric oxide synthesis inhibitors which for the first time provided investigators with the means to inhibit systemic nitric oxide production and examine associated biology. It became apparent that in addition to an important role in homeostatic regulation of the cardiovascular system (blood pressure and blood flow control), nitric oxide played a role in haemostasis and coagulation, innate immunity, and neurotransmission. As early as 1992 it was apparent that in the area of inflammation, nitric oxide might have a dichotomous function as both a beneficial and detrimental molecule. In numerous models of inflammation, inhibition of nitric oxide increased tissue dysfunction or injury whereas in other models of inflammation, inhibition of nitric oxide provided benefit. These paradoxical results led to nitric oxide being labelled with epithets such as "the good, the bad and the ugly" and "Jekyll and Hyde".^{1 2} Initially, different species and models were purported to underlie the discrepant results, but as the body of literature grew these explanations became less satisfying. A new working hypothesis was put forth that the constitutive forms of nitric oxide synthase (cNOS) including neuronal NOS (NOS1 or nNOS) and endothelial NOS (NOS3 or eNOS) were critical to normal physiology and inhibition of these enzymes caused damage, whereas induction of the inducible nitric oxide synthase (NOS2 or

iNOS) was harmful and specific inhibition of this enzyme was beneficial.

Nitric oxide is synthesised from a guanidino group of L-arginine and can be produced by almost all mammalian cells, including endothelium lining the vasculature, neurones of the central and enteric nervous system, and cells of the immune system.^{3 4} As already stated, nitric oxide is produced by three distinct enzyme systems: (1) a constitutive enzyme normally present primarily in endothelium lining the vasculature (eNOS), (2) a neurally associated constitutive nitric oxide synthase found in neurones of the brain and in the enteric nervous system (nNOS), and (3) an inducible enzyme (iNOS) whose expression in endothelium, epithelium, and inflammatory cells requires protein synthesis, is induced by cytokines and lipopolysaccharide (LPS), and produces large amounts of nitric oxide for extended periods of time.⁴ It is the large quantities of nitric oxide produced by iNOS that have associated it with injury whereas the small amounts of NO produced by the constitutive NOSs have been deemed beneficial. Expression patterns have also contributed to this view inasmuch as eNOS and nNOS are present constitutively whereas iNOS is not normally present in tissues but expression is induced in inflammatory conditions and hence often implicated as guilty by association.

Background: the good versus the bad

eNOS appears to be a homeostatic regulator of numerous essential cardiovascular functions. Nitric oxide produced from eNOS (1) maintains adequate perfusion,³ (2) regulates microvascular permeability,⁵ (3) modulates platelet homotypic aggregation and platelet adhesion to vessel walls,⁶ and (4) regulates leucocyte-endothelial cell interactions.⁷ In the gastrointestinal tract, constitutive nitric oxide synthase also appears to regulate epithelial permeability although the isoform responsible remains unidentified.⁸ It is tempting to predict that either eNOS or nNOS regulates this important homeostatic function as they are found in significant quantities in the digestive tract. However, numerous studies have reported small amounts of iNOS message and protein under normal conditions in this organ system^{9 10} and so a role for this isoform cannot be excluded. The presence of iNOS in normal intestine should not be surprising in light of the fact that the epithelium at this mucosal surface is always exposed to foreign antigens and the normal milieu in the colon (bacteria and bacterial byproducts) is a potent stimulus for iNOS induction in tissues.⁴

Inhibition of nitric oxide therefore causes many of the hallmark features of intestinal inflammation including increased neutrophil recruitment, increased oxidative stress, mast cell degranulation, and increased microvascular and epithelial permeability.^{5 7 8 11 12} Moreover, chronic administration of NOS inhibitors also causes intestinal inflammation.^{2 13} Therefore, it is not surprising that an

Abbreviations used in this paper: NOS, nitric oxide synthase; IBD, inflammatory bowel disease; LPS, lipopolysaccharide; TNBS, trinitrobenzene sulphonic acid.

NOS inhibitor exacerbates essentially all acute inflammatory insults either by decreased blood flow, increased leucocyte or platelet adhesion, increased oxidative stress, or by increasing mucosal permeability. Moreover, delivery of exogenous nitric oxide in the form of nitric oxide donors, with few exceptions, reduces the sequelae of very acute inflammation. These studies are summarised in detail in extensive reviews and will not be dwelled upon in this article.

Although it would seem logical that production of large quantities of NO, even if it is from iNOS, would also improve blood flow, reduce leucocyte and platelet recruitment, and hence reduce inflammation, most data do not support this view. Boughton-Smith and colleagues,¹⁴ in a series of studies using endotoxin, clearly demonstrated that although inhibition of nitric oxide within the first few hours of endotoxin administration was detrimental to the intestine, inhibition of NOS activity at four hours resulted in protection against endotoxin damage. These authors proposed that administration of an NOS inhibitor in the early phase of endotoxin injury inhibited constitutive nitric oxide and hence exacerbated injury. In contrast, administration of the same NOS inhibitor in the second phase of endotoxin injury inhibited inducible nitric oxide synthase and functioned in a protective manner.

These same non-specific inhibitors given in drinking water to animals completely prevented mucosal inflammation associated with acetic acid,¹⁵ TNBS,^{2 16} and numerous other models¹⁷ of inflammatory bowel disease (IBD). The results were striking both in terms of magnitude of protection and also the duration (up to seven days). A number of investigators then demonstrated that "more specific" inhibitors of iNOS given orally also provided protection in models of IBD suggesting that indeed iNOS may be the detrimental enzyme in these studies.^{16 18 19} However, not all of the models are iNOS dependent and in most cases the results for a single model are ambiguous. I would like to use the TNBS model of colitis to discuss a number of issues that have not been considered in the literature to any significant degree and that require further investigation. I will use the TNBS model as it has been investigated most thoroughly with respect to nitric oxide and is beginning to shed some light on the mechanisms of action of this ubiquitous molecule.

Oral L-NAME, why is it beneficial?

It is clear that inhibition of NOS by oral administration of non-specific inhibitors in the intestine exposed to TNBS provides dramatic protection and restitution within seven days.² These observations lie in the face of data showing that chronic NOS inhibition in the absence of TNBS causes significant intestinal inflammation.^{2 13} These data suggest that either cNOS loses its protective function and also contributes to the injurious process or that the protective function of cNOS (including antiadhesion, mast cell stabilising effects, and antipermeability effects) is no longer required as other protective molecules are induced that replace the function of cNOS. Prostanoids and sensory neuropeptides may be candidates in this regard.²⁰ However, a more comprehensive review of the literature reveals that the results of NOS inhibition in the TNBS model are not all 100% protective. Studies range from almost complete protection (100%)^{16 21} to partial protection,^{15 19} selective regional protection,²² no protection⁹, and exacerbation of injury.^{23 24} For example, unlike the generalised inhibition of inflammation reported by some investigators, Hogaboam and colleagues²² reported that inhibition of NOS in TNBS induced colitis dramatically reduced neutrophil and macrophage infiltration into the intestine and intestinal hyperplasia (by >90%) but failed to reduce

anorexia, smooth muscle hypertrophy, or myenteric nerve dysfunction. The latter data suggest site specific rather than generalised iNOS induced injury and raise the possibility that iNOS is produced only in certain areas or its toxicity is restricted to certain areas. Consistent with this view is immunohistochemistry of a toxic metabolite of nitric oxide (peroxynitrite) demonstrating cell specific injury.²⁵

A striking similarity among all the studies that reveal protection against TNBS induced injury is the oral route of administration of the NOS inhibitor. To my knowledge no one has reproduced the beneficial effect of non-specific NOS inhibitors in TNBS induced colitis by administering these compounds into the systemic circulation. In fact the opposite result is achieved when NOS inhibitors are given systemically in the TNBS model of colitis. Pfeiffer and Qiu²⁴ reported that subcutaneous osmotic mini pumps which continuously released L-NAME at 0.042, 0.208, 0.417, or 1.667 mg/kg/h were not beneficial in a model of TNBS induced colitis. In fact, the two higher concentrations of continuously infused L-NAME or a single bolus dose of L-NAME (100 mg/kg subcutaneously daily) significantly increased colonic damage. The lowest dose of L-NAME had a trend (insignificant) towards protection and possibly if the authors had tried even lower doses a significant protective effect may have been achieved.

Perhaps these data suggest that too much NOS inhibition achieved by systemic L-NAME administration results in exacerbation of intestinal inflammation and oral L-NAME serendipitously only partially inhibits NOS. This is difficult to assess from the available literature as most investigators have reported luminal rather than plasma nitrite levels as an indicator of degree of NOS inhibition. However, Hogaboam and colleagues²² reported 70% reduction (slightly above baseline) in plasma nitrite levels with L-NAME in drinking water and much of this nitrite could be accounted for by the inflamed intestine. From these data it is tempting to conclude that L-NAME in drinking water has limited access to the systemic circulation and primarily inhibits nitric oxide production in the intestine. However, there is a wealth of evidence that L-NAME in drinking water causes profound chronic hypertension ultimately causing kidney disease,^{26 27} consistent with the view that it does gain access to the general circulation. Moreover, Rachmilewitz and colleagues¹⁵ reported that in the TNBS model, where oral L-NAME was protective, there was rapid and persistent hypertension. It is my opinion that oral administration of L-NAME may be critical to the protective results but the reason(s) underlying this effect is not well established.

One possibility is that these non-specific inhibitors have an effect other than that on NOS inhibition when given in drinking water. In one study in the TNBS colitis model, at one, three, and seven days there were 4.5-, 22-, and 18-fold increases in colonic nitric oxide synthase activity which were reduced by 0%, 15%, and 50% at these time points. Yet neutrophilic infiltrate and intestinal injury was reduced by more than 60% at three days despite only 15% reduction in iNOS activity at this time. Either even subtle (15%) inhibition of NOS was sufficient to significantly impact upon intestinal inflammation or the full spectrum of anti-inflammatory effects of oral L-NAME and other NOS inhibitors remains unappreciated. In fact, Miller and colleagues²⁸ reported that oral L-NAME does not result in sustained suppression of NOS because of compensatory expression of inducible nitric oxide synthase. At oral concentrations of L-NAME previously given to animals with TNBS, the investigators demonstrated that in healthy animals this inhibitor induced the iNOS gene. Although plasma nitrite levels were not increased above control lev-

els in that study, a marked increase in NO production from the vasculature was noted. These data raise important questions about the function of L-NAME, particularly if iNOS is paradoxically increased in normal intestine as well as perhaps in the TNBS model of colitis.

iNOS deficient mice: what insights have they provided?

I was sure that iNOS deficient (iNOS^{-/-}) mice would bypass many of the aforementioned problems of iNOS inhibitors and provide insight into the role of iNOS in the intestinal inflammatory process. Perhaps my enthusiasm should have been tempered by the first description of mice lacking iNOS. Indeed, MacMicking and colleagues²⁹ reported that the lethal dose of *Listeria* was 10-fold lower in iNOS^{-/-} mice than wild-type mice, an observation reproduced for other infectious agents (reviewed by Nathan³⁰). However, the results for responses to LPS (*Escherichia coli*) were equivocal. The iNOS^{-/-} mice were more resistant to 1 mg/kg of LPS than their wild-type littermates, but at 10 mg/kg of LPS cytokines (TNF, IL-1, IL-6) and liver enzymes (index of liver injury) increased in the serum equivalently in both iNOS^{-/-} and iNOS^{+/+} mice. Similarly, iNOS^{-/-} mice pretreated with heat killed *Propionibacterium acnes* and challenged with LPS died at the same rate as wild-type mice. A second study by this group revealed that in response to *Salmonella typhimurium* LPS, genetic deficiency of iNOS was associated with no protection or a reduction in survival³¹ suggesting different susceptibilities to LPS from different strains. Two other groups of investigators created iNOS^{-/-} mice. Whereas one group³² observed resistance in response to LPS (12.5 mg/kg) in iNOS^{-/-} mice, the other group³³ saw no resistance to LPS (*E. coli*) at 25 or 12 mg/kg. Clearly, the dichotomous results with iNOS^{-/-} mice has provided more confusion than enlightenment.

Not surprisingly, the LPS studies have foretold the results of iNOS^{-/-} mice in models of IBD. Zingarelli and colleagues²¹ reported that TNBS induced 90% mortality in wild-type mice and 38% mortality in iNOS^{-/-} counterparts. Associated with these results was the observation that at four days after colitis mice had a remarkably reduced level of colitis and by day 7, 100% resolution of colitis, whereas the wild-type mice that survived continued to have severe mucosal damage at seven days of TNBS. Neutrophil infiltrate was not different between the mutant and wild-type mice. The authors also detected intense immunostaining of nitrotyrosine and concluded that iNOS derived nitric oxide contributes to the nitrosative and oxidative changes associated with TNBS induced colitis. McCafferty and colleagues⁹ reported that within the first 72 hours of TNBS induced colitis the iNOS^{-/-} mice had approximately 50% greater damage scores and increased neutrophilic infiltrate. As the model entered the chronic phase of inflammation by seven days (mast cell hyperplasia, macrophage and lymphocyte infiltration) there was no difference in the various parameters of injury measured between iNOS^{-/-} and iNOS^{+/+} mice. Zingarelli and colleagues²¹ observed complete prevention of the chronic phase of TNBS induced colitis in iNOS^{-/-} mice whereas McCafferty and colleagues⁹ reported severe chronic inflammation in iNOS^{-/-} mice identical to iNOS^{+/+} mice in response to TNBS.

At first glance these two models may appear identical. However, differences exist which may provide an opportunity to identify potentially important regulators of iNOS benefit versus iNOS toxicity. Firstly, an obvious but often ignored parameter is the housing environment. Aside from two different institutions, mice from the McCafferty study⁹ were derived in a specific pathogen free facility and moved

to a conventional housing facility two weeks before induction of TNBS induced colitis. Mice from Zingarelli and colleagues²¹ study were taken directly from specific pathogen free conditions and given TNBS (Zingarelli, personal communication). Based on the far greater sensitivity of the iNOS^{-/-} mice to a number of pathogens, iNOS^{-/-} mice may be more susceptible to injurious agents in conventional housing facilities. Therefore, iNOS, much like antibiotics,³⁴ could be beneficial in TNBS induced colitis in an unrestricted housing environment.

Noteworthy is the 90% mortality in response to TNBS in one study²¹ and no mortality in the other study⁹ in wild-type mice. In fact, Zingarelli and colleagues²¹ used 50% ethanol to break the mucosal barrier and allow TNBS to penetrate the submucosa whereas McCafferty and colleagues⁹ used 30% ethanol to avoid lethality in their model. Although 50% ethanol is the concentration generally used in rats, this concentration is lethal in mice, as demonstrated by Zingarelli and colleagues,²¹ and explains the striking difference in mortality between the two studies. Clearly, iNOS^{-/-} but not iNOS^{+/+} mice are resistant to high levels of ethanol in combination with TNBS but at lower concentrations the inflammatory response follows a similar path. A final difference is that the targeting vectors for the making of the iNOS^{-/-} mice were not the same. However, as both forms of mutant mice had no detectable iNOS by a myriad of assays, the approaches used to delete the iNOS gene are unlikely to account for the differences in the varying phenotypes in response to TNBS. My personal opinion is that environmental differences is the dominant determinant in the gastrointestinal tract.

Future directions

I feel that a number of issues need further exploration. First and foremost, iNOS is treated as a single entity in terms of collective action (either good or bad) in spite of the fact that there may be as many as 15 different types of cells that can express iNOS.⁴ For example, extravascular resident leucocytes (macrophages), intravascular and/or infiltrating leucocytes (neutrophils and monocytes), endothelium, and parenchymal cells, including intestinal epithelium, are all capable of expressing iNOS. Is it really appropriate to assume that iNOS functions in the same way in all of these cells? For example, if nitric oxide from iNOS combines with superoxide to form toxic molecules such as peroxynitrite, then the iNOS produced from an oxidant producing cell such as the neutrophil has the potential to generate significantly more peroxynitrite than a cardiac or epithelial cell that produces far less oxidants. Indeed, Dijkstra and colleagues²⁵ reported peroxynitrite staining on the surface of leucocytes but not epithelium suggesting site specific production of peroxynitrite from iNOS. An initial approach to begin to tease apart the importance of various iNOS producing cells may be to make chimeric mice (iNOS^{+/+} bone marrow transplanted into iNOS^{-/-} mice and vice versa) so that only the leucocytes or only non-leucocytic cells have the capacity to produce iNOS. This approach may reveal cell specific roles for iNOS in TNBS induced colitis. Ideally, cell or tissue restricted iNOS deficient mice will be the optimal experimental tool.

In summary, iNOS has been shown to have multiple biological effects. It is essential for normal healing in the skin³⁵ and intestinal mucosa,³⁶ for killing of certain bacteria,³⁰ is potentially important in regulating T cell proliferation and differentiation (Th1 v Th2),³² and for regulation of leucocyte recruitment, perhaps by affecting adhesion molecule expression or leucocyte activation.³⁷ Countering these effects is the fact that iNOS can produce toxic metabolites, albeit perhaps from only a subset of cells. Therefore, it is not surprising that one does not always get

benefit from inhibiting all iNOS either by gene deletion or by systemic NOS inhibition. In fact, delivery of iNOS to skin using adenoviral vectors improved healing of this tissue in iNOS^{-/-} mice, suggesting that iNOS may be therapeutically useful if given in appropriately regulated amounts at specific sites.³⁵ I think that the hypothesis that cNOS is good and iNOS is bad is far too simplistic and no longer explains the majority of data generated by the scientific community. Afterall, if there wasn't a little bit of good in iNOS would the principles of evolution not have applied and selected against this molecule many millions of years ago?

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