

Nitric oxide and biopterin: a study in Chiaroscuro.

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Research Article

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In a painting, chiaroscuro—marked contrasts in light and shade—can heighten the drama and deepen the mystery. Chiaroscuro seems to characterize biopterin's role in the biosynthesis of nitric oxide (NO). A paper in this issue of *The Journal* (1) adds new splashes of light and shade.

Until 1989, biopterin, illumined by neurochemists, nonetheless remained enshrouded by a paradox. Its main known role (in its tetrahydro form) was as a cofactor in the oxidation of aromatic amino acids for biosynthesis of neurotransmitters. Yet the enzymes that conspire to make biopterin, including GTP cyclohydrolase I, the first committed step in the pteridine path, were known to be more widely distributed than biopterin-assisted hydroxylases for phenylalanine, tyrosine, and tryptophan. It seemed there must be more widespread enzymes to whose action biopterin contributes.

Light appeared in a surprising quarter and unfamiliar color. Nitric oxide synthases (NOS's) proved to be biopterin dependent (2, 3). However, NOS's carry out a five-electron oxidation of a guanidino nitrogen of L-arginine, a reaction that differs drastically from the other known functions of biopterin. To this day, we remain in the dark with respect to biopterin's mechanistic role in this reaction (4). Nonetheless, the distribution of NOS's (see below) surely helps explain why so many cells invest GTP in the production of pteridines.

Three NOS's are now distinguished by cloning of their complementary and genomic DNAs in several species, including man (5). Two isoforms are dependent on elevated Ca^{2+} and exogenous calmodulin and are constitutively expressed. One seems confined to endothelium ("endothelial cNOS") while another is largely but not exclusively expressed in peripheral and central neurons ("neural cNOS"). The third isoform, "iNOS," is independent of Ca^{2+} above the level found in resting cells and bears its own calmodulin as a tightly bound subunit (6). iNOS is absent from most cells under normal conditions but can be induced in many by alarm signals from the immune and inflammatory systems. Where investigated, induction of iNOS has largely been transcriptional (5).

Given the participation of NO in the physiology or pathophysiology of nearly every organ system (7, 8), there is intense interest in how NO biosynthesis is or can be regulated physiologically and pharmacologically. Not the least motivation for this interest is evidence that the profound hypotension resulting from the administration of bacterial endotoxin (LPS) or tumor necrosis factor- α (TNF- α) may depend on the induction of iNOS (7, 8). Since endothelial cNOS is critical for suppressing platelet aggregation and adhesion, and neural cNOS may also play important physiologic roles, many investigators have taken it as self-evident that the major challenge is to find a way to selectively inhibit iNOS.

Resting mouse peritoneal macrophages contain enough biopterin to sustain the activity of iNOS in activated cells (3). Hence, at the outset, there was little reason for biopterin to

share the limelight in studies on the regulation of NO biosynthesis. However, four recent papers have revealed a different picture in mouse fibroblasts (9), rat vascular smooth muscle (10), and human endothelial cells (1, 11).

It has been known for some time that interferon- γ (IFN- γ) and TNF- α can induce GTP cyclohydrolase I and augment intracellular levels of tetrahydrobiopterin (for review see reference 11). In 1990, Werner-Felmayer et al. (9) showed that this effect helped explain how the same cytokines could induce NO production in mouse dermal fibroblasts (9). Modulation of tetrahydrobiopterin levels had corresponding effects on NO release. Compounds used for this purpose were 2,4-diamino-6-hydroxypyrimidine, a GTP cyclohydrolase inhibitor; sepiapterin, a precursor of tetrahydrobiopterin via a salvage pathway not dependent on GTP cyclohydrolase; and methotrexate, an inhibitor of the salvage path (9). Even after cytokine-induced elevation of tetrahydrobiopterin, this cofactor was still limiting for the release of NO (9).

In late 1992, Gross and Levi (10) extended this analysis to rat aortic smooth muscle cells at culture passage 10–15. Tetrahydrobiopterin could not be detected in these cells unless they were first treated with LPS and IFN- γ . NO release in response to these stimuli was dependent on their induction of tetrahydrobiopterin synthesis. The authors postulated that iNOS in vascular smooth muscle was likely to be a major source of vasoactive NO in septic shock. If iNOS were more dependent on de novo biosynthesis of tetrahydrobiopterin than cNOS—another postulate—then inhibitors of tetrahydrobiopterin synthesis might offer an avenue to the inhibition of iNOS with relative selectivity over the short term (10).

In early 1993, Werner-Felmayer et al. (11) carried the argument to man and to another isoform, endothelial cNOS. LPS + IFN- γ + TNF- α had no effect on the activity of cNOS in cofactor-repleted cell lysates from umbilical vein endothelial cells in passage 3–8. However, the ability of intact, agonist-triggered cells to increase their cGMP—a reflection of NO synthesis—increased up to 80% after pretreatment with the inflammatory stimuli. This effect was mediated by enhancement of GTP cyclohydrolase I activity (up to 40-fold) and tetrahydrobiopterin levels (up to 14-fold). The authors suggested that cytokine-stimulated tetrahydrobiopterin production might contribute to increased NO production in inflammatory states (11).

Rosenkranz-Weiss et al. (1), using human umbilical vein endothelial cells in passage 3–6, have confirmed the central findings of Werner-Felmayer et al. (11) and added two new observations. First, cNOS activity in cofactor-repleted lysates fell in response to treatment with IFN- γ + TNF- α + interleukin-1 β , in concert with a 94% decrease in endothelial cNOS mRNA. Yet, in the absence of exogenous tetrahydrobiopterin, the apparent activity of endothelial cNOS rose threefold in intact cells and sixfold in their lysates. The latter effect was attributed to a marked induction of GTP cyclohydrolase I and a corresponding increase in the levels of endogenous tetrahydrobiopterin (1). No iNOS could be detected in these cells, though appropriate combinations of cytokines can induce iNOS in endothelial cells, at least in rodents (12). The second new point—which seems to rest precariously on a single experiment—came from comparing biopterin levels in freshly isolated endothelial

cells with those in cultured cells. In the fresh cells, biopterin levels were as high as those achieved by treating the cultured cells with cytokines (1).

Collectively, these studies cast a new light on old presumptions. Endothelial cNOS is constitutive. Paradoxically, however, its activity can be both increased and decreased by cytokines, and both effects can be manifest in the same cell population. Increased activity results from an enhanced supply of endogenous tetrahydrobiopterin, while decreased levels reflect a fall in cNOS mRNA (1), probably due to its destabilization (13). In intact cells, the net effect is biphasic: an early increase in NO production, followed by a later decline. From this viewpoint, perhaps endothelial cNOS deserves to share the spotlight with iNOS for the development of isoform-specific NOS inhibitors with which to attempt to treat septic shock.

Once again, however, part of the picture is in shadow. The precipitous decline in tetrahydrobiopterin with endothelial cell culture (1) raises the possibility that limitation of NOS activity by insufficiency of endogenous tetrahydrobiopterin may be an artifact of cell culture. When cytokines enhance NOS activity by inducing GTP cyclohydrolase I, they may compensate for an unphysiological situation. Perhaps mouse dermal fibroblasts and rat aortic smooth muscle cells also lose tetrahydrobiopterin in prolonged culture, while primary mouse peritoneal macrophages in short-term culture do not.

More puzzles lurk in obscurity. Why do many cells export or import tetrahydrobiopterin? If endothelial cells are normally importers, perhaps they have little need for GTP cyclohydrolase I in vivo. In culture, cut off from their exogenous supply, they may respond to cytokines by commencing endogenous production. Similar questions pertain to the other cell types discussed above. It will be challenging to investigate intracellular and transcellular biopterin metabolism in situ. For each cell type of interest, we need to assess the relative importance of de novo synthesis, the salvage path, and external sources. Is it in fact tenable to block any isoform of NOS selectively in vivo through interference with biopterin metabolism? If so, what aspect of biopterin metabolism should be targeted, and in what cells of origin?

Finally, why do biopterin-producing cells secrete copious neopterin, which differs from biopterin by one hydroxyl on the propyl chain? Neopterin neither sustains nor inhibits the activity of iNOS (3). In fact, no use has ever been found for mammalian neopterin, beyond serving scientists as a fount of publica-

tions, and clinicians as a sign of immune activation. It is hard to accept the current view that neopterin owes its human existence to sloppy enzymology, and has no more significance than to herald the generation of its incontestably useful cousin. Perhaps what has been considered "out of the picture" in the shadow at the edge of the canvas is in fact the beginning of a picture on the other side.

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