Reprinted from Journal of Medicinal Chemistry, 1993, 36.

Irreversible Inactivation of Macrophage and Brain Nitric Oxide Synthase by L-N^G-Methylarginine Requires NADPH-Dependent Hydroxylation

5078

Paul L. Feldman*

Glaxo Inc. Research Institute, 5 Moore Drive, Research Triangle Park, North Carolina 27709

Owen W. Griffith

Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Hui Hong and Dennis J. Stuehr†

Department of Immunology, Cleveland Clinic, Cleveland, Ohio 44195, and Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Received September 21, 1992

L-N^G-Methylarginine (NMA) is an established mechanism-based inactivator of murine macrophage nitric oxide synthase (mNOS). In this report, NMA is shown to irreversibly inhibit both mNOS (k_{inact} = 0.08 min^{-1}) and the recombinant constitutive brain NOS (bNOS). For both NOS isoforms, metabolism of NMA parallels that of the natural substrate L-arginine (ARG), in that it undergoes a regiospecific, NADPH-dependent hydroxylation to form L-N^G-hydroxy-N^G-methylarginine (NOHNMA). This intermediate then undergoes further NADPH-dependent oxidation to form L-citrulline (CIT). Authentic NOHNMA, synthesized from L-ornithine, irreversibly inhibited both mNOS (k_{inact} = 0.10 min^{-1}) and bNOS in an NADPH-dependent reaction. The conversion of either NMA or NOHNMA to CIT correlated with irreversible enzyme inactivation. Thus, the data suggest that enzyme inhibition occurs as a consequence of oxidative metabolism of the intermediate, NOHNMA. A unified mechanism is proposed that accounts for NO biosynthesis from ARG, for the inactivation of NOS by NMA and for the intermediacy of hydroxylated ARG or NMA derivatives in these processes.

Introduction

In recent years it has been demonstrated that nitric oxide (NO) is an important and ubiquitous effector molecule that plays a significant role in the regulation of a diverse set of mammalian physiological processes.[1] For example, NO generated from endothelial cells is known to play a critical role in regulating vascular resistance and platelet aggregation via activation of soluble guanylyl cyclase. In activated macrophages, NO acts as a cytostatic and cytotoxic agent by binding to catalytically essential non-heme iron present in enzymes such as ribonucleotide reductase[2] and the iron–sulfur enzymes of the mitochondrial electron transport pathway.[3] Also, NO is now known to be an important messenger in the brain and peripheral nervous system.[1a]

The biological action demonstrated by NO in a particular tissue is critically dependent upon the flux of NO reaching the targeted cells. Therefore, selective control of NO flux via regulation of its synthesis may have therapeutic implications.[4] In mammals, NO is generated by at least two distinct classes of nitric oxide synthase (NOS) enzymes.[1c,5] The constitutive NOS isoforms found in vascular endothelium, brain, and platelets, are Ca^{2+}/calmodulin-dependent enzymes that rapidly generate NO in response to intracellular calcium influx. Inducible NOS, found in cytokine or endotoxin stimulated macrophages, neutrophils, and hepatocytes, is a Ca^{2+}/calmodulin-independent enzyme that generates NO over an extended period of time (approx. 72 h) after its expression.

Several arginine-based irreversible inactivators of the NOS isoforms have been recently described.[1c,6] The inhibitor that has been studied most frequently is L-N^G-methylarginine (NMA)[3], a compound originally identified as a reversible inhibitor that binds competitively with L-arginine (ARG).[3,7] Further studies demonstrated that in the absence of ARG, NMA causes a relatively slow but irreversible inactivation of murine macrophage NOS (mNOS); irreversible inactivation is slowed further in the presence of ARG.[6a] The observation that inactivation by NMA is NADPH-dependent[6a] suggested that NMA might require metabolism to yield a reactive, inactivating species.

We have previously reported that the enzymatic conversion of ARG to NO and L-citrulline (CIT) by mNOS requires an initial NADPH-dependent N-hydroxylation to form L-N^G-hydroxyarginine (NOHARG).[8] NOHARG then undergoes further NADPH-dependent oxidation to generate CIT and the free radical NO (Scheme I). Reasoning that metabolism of NMA might occur through a similar pathway and lead to irreversible inhibition, we synthesized the putative intermediate, L-N^G-hydroxy-N^G-methylarginine (NOHNMA) and characterized its inhibition of both mNOS and constitutive brain (bNOS)[5] isoforms. Additionally, we show that irreversible inhibition of either NOS isoform by NMA depends on its regiospecific N^G-hydroxylation to form NOHNMA as an intermediate. Further NADPH-dependent metablism of NOHNMA to CIT leads to irreversible inactivation of either NOS isoform.

Chemistry

The synthesis of NOHNMA (1) follows closely to the route we described to synthesize L-N^G-hydroxyarginine (NOHARG).[9] Reaction of the known L-ornithine derivative 2 with *N*-methyl-*O*-benzylhydroxylamine[10] at 0 °C

† Case Western Reserve University.

0022-2623/93/1836-0491$04.00/0