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Alkaloids, Nitric Oxide, and Nitrite Reductases: Evolutionary Coupling as Key Regulators of Cellular Bioenergetics with Special Relevance to the Human Microbiome

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Typical alkaloids expressed by prokaryotic and eukaryotic cells are small heterocyclic compounds containing weakly basic nitrogen groups that are critically important for mediating essential biological activities. The prototype opiate alkaloid morphine represents a low molecular mass heterocyclic compound that has been evolutionarily fashioned from a relatively restricted role as a secreted antimicrobial phytoalexin into a broad spectrum regulatory molecule. As an essential corollary, positive evolutionary pressure has driven the development of a cognate 6-transmembrane helical (TMH) domain μ3 opiate receptor that is exclusively responsive to morphine and related opiate alkaloids. A key aspect of "morphinergic" signaling mediated by μ3 opiate receptor activation is its functional coupling with regulatory pathways utilizing constitutive nitric oxide (NO) as a signaling molecule. Importantly, tonic and phasic intra-mitochondrial NO production exerts profound inhibitory effects on the rate of electron transport, H+ pumping, and O2 consumption. Given the pluripotent role of NO as a selective, temporally-defined chemical regulator of mitochondrial respiration and cellular bioenergetics, the expansion of prokaryotic denitrification systems into mitochondrial NO/nitrite cycling complexes represents a series of evolutionary modifications of existential proportions. Presently, our short review provides selective discussion of evolutionary development of morphine, opiate alkaloids, μ3 opiate receptors, and NO systems, within the perspectives of enhanced mitochondrial function, cellular bioenergetics, and the human microbiome.

MeSH Keywords: Alkaloids • Evolution, Molecular• Microbiota • Mitochondria • Morphine • Nitric Oxide • Nitrite Reductases

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Background

Pathophysiological expression of aberrant cellular and molecular events in response to prolonged excitatory processes has been functionally linked to the initiation and persistence of human sensory and metabolic disorders[1]. Within this context, the historical primacy of opium and its biologically active compounds, codeine and morphine, as medicinal agents with potent inhibitory/calming actions at the behavioral and cellular level has reinforced the importance of opiate alkaloids as frontline analgesic agents [2]. From a teleological perspective, morphine represents a relatively low molecular mass heterocyclic alkaloid that has been evolutionarily fashioned from a relatively restricted role as a secreted antimicrobial phytoalexin into a broad spectrum regulatory molecule mediating diverse physiological responses in animal cellular systems. As an essential corollary, requisite expansion of stereo-selective signaling complexes responsive to morphine and its chemical congeners has reinforced the biological roles of opiates as potent inhibitory regulators of diverse excitatory processes in peripheral organ systems and in the CNS.

Outside the realm of pain and analgesia, it has also been established that opiate regulatory processes extend to homeostatic maintenance of basal levels of cellular and organ-specific excitation required for optimal realization of essential metabolic processes linked to efficient energy utilization[2–4]. A key aspect of "morphinergic" signaling mediated by mu opiate receptor activation is realized as a functional coupling with regulatory pathways utilizing constitutive nitric oxide (NO) as a signaling molecule. Amplification and biochemical diversification of opiate action via recruitment of NO signaling systems provides fine-tuned, temporally dependent, regulation of cellular micro-domains in differentially defined activation states [3,4]. Relatively recent lines of investigation have introduced an additional level of regulatory complexity into a developing model of opiate action whereby "morphinergic" stimulation of NO expression is mediated by activation of L-arginine-dependent NO synthase (NOS) and/or by enzymatic reduction of nitrite by cellular and mitochondrial nitrite reductases.

Evolution of Nitric Oxide as a Pluripotent Chemical Intermediate and Signaling Molecule

It has been previously hypothesized that the spectrum of nitrogenous chemicals found in the primordial atmosphere that includes elemental N2, ammonia, nitrogen oxides, and the short-lived free radical NO, was dynamically modified by the increasing presence of O2 generated by the evolving biosphere [5]. Consistent with the physiological demands of developing biological processes, limited abiotic generation of NO from N2 and O2 via lightning discharge [6] was superseded by multiple enzymatic pathways of nitrification and denitrification by primordial microbial systems. Interestingly, evolutionary transition of largely anaerobic to microbial-based aerobic nitrogen cycles by the late Archean period approximately 2.5 billion years ago may have preceded significant atmospheric O2 accumulation generated by Cyanobacteria before the great oxygenation event at the end of Proterozoic period [7,8].

Evolutionary development of major enzymatic components of microbial denitrification and aerobic respiration drives NO and N2O formation by nitrite reductase (NIR) and nitric oxide reductase (NOR) activities, respectively [8]. Furthermore, co-evolution of denitrification and aerobic respiratory complexes within hybrid prokaryotic respiratory chains provide strong putative evidence for the operationally-dependent amplification of the chemical diversity of NO as a pluripotent effector molecule within eukaryotic biological systems [9], including its potential role as a chemical mediator of microbial symbioses with diverse eukaryotic hosts [10]. Accordingly, NOR mediating a key step in anaerobic denitrification is suggested to provide an evolutionary prototype for the development of mitochondrial cytochrome C oxidases (COXs) on based shared amino acid sequence homology [11]. The preeminence of nitrate, nitrite, and NO and as oxidizing substrates within the prokaryotic nitrogen cycle most likely facilitated evolutionary development of eukaryotic enzyme-mediated aerobic respiratory chains employing O2 reduction by heme/copper dioxygen reductases as the terminal enzymatic step [12,13].

Mechanistically, functional adaptation of NO as an important signaling molecule/chemical effector capable of reversible post-translational protein modification may be illustrated by biochemical and molecular studies of the Rrf2 family transcription factor NsrR in a wide range of bacteria following exposure to cytotoxic concentrations of NO [14–16]. Interesting, studies of Streptomyces coelicolor NsrR (ScNsrR), a transcriptional regulator of hmpA gene expression encoding NO-detoxifying flavohemoglobins, indicate that ScNsrR evolved as a specialized regulatory protein focused on NO detoxification [14]. Selective high affinity binding of ScNsrR to receptive sequences within promoter regions of these same target genes was abolished following NO exposure via formation of reversible, iron-nitrosyl, chemical species within critically important iron-sulfur [4Fe-4S] clusters [15]. In sum, the reversible loss of high affinity binding of ScNsrR to targeted DNA sequences with subsequent elimination of transcriptional repression and selective activation of NO-responsive genes was empirically determined to be functionally dependent on the chemical integrity of critically important iron-sulfur [4Fe-4S] clusters within the primary protein sequence [15,16]. Finally, relatively recent studies have established NO as a significant epigenetic regulatory molecule via its observed effects on posttranslational modifications of

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histones, DNA methylation, and selective recruitment of regulatory microRNAs [17].

Focal Modulation of Cellular Bioenergetics within the Mitochondrial Inner Membrane by Nitric Oxide: Critical Significance of Nitrite Reductases

Requisite enhancement of eukaryotic cellular energy requirements indicates a convergence of metabolic processes within the mitochondrial matrix for optimal synthesis of ATP from ADP and inorganic phosphate via chemiosmotic H+ gradient formation and utilization. Accordingly, positive evolutionary pressure has apparently provided overwhelming existential advantages to the host eukaryotic cell by segregating this physiochemical process to the organelle's inner membrane via coordinate expression of complexes I, III, and IV of the respiratory chain [18]. Mitochondrial rotor-stator type ATP synthases (F-ATPases) require a defined membrane potential to achieve a transductive proton-motive force across the inner membrane that effectively drives high efficiency ATP production [19] and recent elegant work has demonstrated an enhanced efficiency of 2.7 *vs.* 3.3–5 protons per synthesized ATP molecule by eukaryotic *vs.* prokaryotic F-ATPases, respectively [20]. Complex regulatory mechanisms based on temporally variable physiological demands are required to operationally define states of cellular excitation to titratable rates of mitochondrial ATP production. As discussed above, it appears that the fundamental chemical reactivity of NO within narrow spatial and temporal domains supported its evolutionary retrofit of from a transiently expressed intermediate substrate within prokaryotic nitrogen cycling pathways into a pluripotent chemical effector/regulator of flavonoid-, quinone-, and cytochrome-catalyzed electron transport within eukaryotic mitochondria [19].

Within this functional context, empirical studies have demonstrated that tonic and phasic intra-mitochondrial NO production exerts profound inhibitory effects on the rate of electron transport, H+ pumping, and O2 consumption [18,19,21] by engendering reversible post-translational modification of discrete subunits of complexes I, III, and IV of the respiratory chain [22]. For example, the inhibitory effects of NO on complex I activity are selectively mediated by reversible S-nitrosation of Cys39 exposed on the surface of the ND3 subunit with a relatively slow onset of greater than 60 seconds [23–25]. In contrast, the reaction of NO with Complex IV/COX proceeds with a rapid onset of milliseconds to seconds and results in a reversible blockade of a critical binuclear heme a3/CuB active site within the COX complex via formation of nitrosyl- or nitrite-heme adducts that are preferentially driven by the intra-mitochondrial redox state [23–25]. Mechanistically, reverse oxidation of heme-bound NO to nitrite within the COX binuclear heme a3/CuB active site proceeds via formation of heme-Fe(III)-O-Cu(II) complexes. COX-mediated NO oxidation provides a molecular mechanism for retention of physiologically important NO equivalents within a dynamically changeable intra-mitochondrial free nitrite pool that is critically important for maintaining cellular bioenergetics parameters during periods of severe physiological stress [26,27]. Conversely, enzymatic reduction of nitrite to NO within the reduced COX heme a3/CuB active site COX provides a significant physiological advantage during hypoxic/anoxic environmental conditions via O2 sparing that is sufficient to maintain normalized electron transport within compromised mitochondria [26].

In addition to COX, the critical role of molybdenum-dependent nitrite reductases in maintaining intra-mitochondrial NO/nitrite cycling has been established, as previously reviewed [28]. A key example is xanthine oxidoreductase (XO), a multipurpose enzyme that catalyzes the two main hydroxylation steps involved in the metabolism of purines, *i.e.*, hypoxanthine to xanthine and xanthine to uric acid, utilizing either NAD^+ or O_2 [29]. Importantly, XO plays a dual role as a key cellular provider of intra-mitochondrial NO derived from nitrite stores. Furthermore, molybdenum is an essential cofactor in XO and must be contained within an enzymatically synthesized molybdopterin cofactor whose synthesis requires four distinct enzymatic activities [30]. The biological importance of molybdenum cofactor (MOCO)-dependent nitrite reductases in realized in the case of genetically determined MOCO deficiencies representing a severe autosomal recessive neonatal metabolic disease that causes seizures and death or severe brain damage [31]. It has also been established that inorganic nitrate which is converted to inorganic nitrite via the action of nitrate reductases will provide additional synergistic production of physiologically desirable intra-mitochondrial NO [32,33].

In sum, inorganic nitrite, previously thought to represent an inert metabolite of cellular nitrogen metabolism, has been established as an essential precursor to dynamic production of NO in response to physiological demands [28,34,35]. Given the pluripotent role of NO as a selective, temporally-defined chemical regulator of mitochondrial respiration and cellular bioenergetics, the expansion of prokaryotic denitrification systems into mitochondrial NO/nitrite cycling complexes represents a series of evolutionary modifications of existential proportions.

Evolution of Morphine from a Plant Alkaloid into Pluripotent Signaling Molecule: Coordinate Development of Cognate

Typical alkaloids expressed by prokaryotic and eukaryotic cells are small heterocyclic compounds containing weakly basic nitrogen groups that are critically important for mediating

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biological activities via receptor-mediated signaling pathways or stereo-selective effects on enzymatic activities [36–39]. In many cases, the protonated nitrogen groups contained within the alkaloid structure are contributed by one or more primary amino acids incorporated as biosynthetic intermediates. Accordingly, the great diversity in alkaloid structure may be selectively realized by biosynthetic pathways employing standardized stereo-chemical reactions in conjunction with cellular reservoirs of relatively simple nitrogen-containing precursors. Based these criteria, the wealth and diversity of alkaloid structure are predictive measures of the expansive evolutionary headroom required for stereo-selective matching of related classes of these small heterocyclic compounds with receptive protein signaling complexes throughout prokaryotic and eukaryotic phyla [40–43].

Morphine, the prototype opiate alkaloid, is synthesized by Papaver somniferum as a major phytoalexin devoted to antimicrobial host defense. Interestingly, the prototype catecholamine dopamine (DA) derived from the amino acid L-tyrosine serves as an essential precursor in the morphine biosynthetic pathway in Papaver and in the biosynthetic pathways of approximately 2500 chemically distinct benzylisoquinoline (BIQ) alkaloids expressed by plant orders Ranunculales, Eumagnoliids, Rutaceae, Lauraceae, Cornaceae and Nelumbonaceae [44,45]. With this in mind, concerted scientific effort can only provide potentially validating empirical and bioinformatics data bases to provide evolutionary justification of the exponentially expanded role of morphine, outside of the restricted realm of pain and analgesia, as a pluripotent chemical regulator of diverse physiological processes involved in normative maintenance of cellular bioenergetics. Additionally, these contentions are elegantly supported by concerted empirical findings that have demonstrated 1) the presence of low, sub-analgesic concentrations, chemically authentic morphine in eukaryotic cells and organ systems, 2) the presence of a *de novo* biosynthetic pathway responsible for eukaryotic morphine expression, with striking similarities to the extensively characterized multi-enzyme plant pathway, and 3) the presence of a cognate 6-transmembrane helical (TMH) domain opiate receptor expressed from the μ opioid receptor (MOR) gene that is exclusively responsive to morphine alkaloid, as reviewed [46,47].

In light of the above, potential validation of the primordial role of morphine as a secreted plant alkaloid with host-defense recognition capabilities has been provided by early studies demonstrating marked inhibition of growth parameters in cultures of *E. coli* following administration of the morphine-related opiate alkaloid levorphanol [48–50]. Subsequently, a provocative biochemical study demonstrated that the envelope protein expressed by *E. coli* envY gene represents a stereospecific, saturable, and high affinity morphine binding site [51]. A later study observed that administration of the morphine analogue, levorphanol, markedly diminished *E. coli* chemotaxis to serine, aspartic acid and galactose, although pharmacological characterization and attribution of opiate alkaloid responsiveness to the E. coli envy envelope protein was inconclusive [52].

We have previously hypothesized that the evolution of the morphine as a multi-faceted signaling molecule is functionally dependent on the diversity of expression of cellular receptors capable of stereo-selective, high affinity, binding of the opiate alkaloid based on strict molecular parameters that include its rigid heterocyclic backbone and protonated amine and hydroxylated recognition sites [46,47]. The successful transition and exponential adaptation of a primordial prokaryotic "morphinergic" signaling pathway to accommodate complex regulatory activities in cellular and organ systems of higher animals are clearly dependent on the retention and further development of specialized receptor complexes that selectively mediate transductive effects of morphine and related opiate alkaloids. Collected work from our group has provided important molecular, biochemical, and pharmacological characterization of cognate μ3 opiate receptors that are selectively tailored to mediate the cellular regulatory effects of morphine and related morphinan alkaloids via stimulation of NO production and release [47,53,54]. The μ3 opiate receptor is a Class A rhodopsin-like member of the superfamily of G-protein coupled receptors (GPCRs) that has been genetically modified to eliminate an N-terminal amino acid sequence of approximately 90 amino acids that constitute the extracellular and TMH1 domains and part of the first intracellular loop (IL) of the classic μ1 opioid receptor. Importantly, the μ3 opiate receptor retain the empirically defined ligand-binding pocket distributed across conserved TMH2, TMH3, and TMH7 domains of the μ1 opioid receptor that provides a recognition profile that is restricted to rigid BIQ/morphinan alkaloids typified by morphine and its extended family of chemical congeners. Mechanistically, elimination of the extended extracellular N-terminal protein domain within the primary sequence of the μ3 opiate receptor confers strict selectivity for morphine and related opiate alkaloids by removing secondary extracellular binding and conformational stabilization required for recognition of endogenous opioid peptides.

Regulatory Coupling of Morphine and Nitric Oxide in Mediating Mitochondrial Function and Cellular Bioenergetics: Functional Relevance to the Human Microbiome

Relatively recent lines of investigation have introduced an additional level of regulatory complexity into a developing model of opiate action whereby "morphinergic" stimulation of NO expression is mediated by activation of L-arginine-dependent constitutive NOS (cNOS) [55–61]. Additionally, collected empirical

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data indicate that the potent pharmacological effects of morphine on mitochondrial respiration and O2 consumption are mediated via functional activation of NO production and intra-mitochondrial release [28,62]. Interestingly, biochemical studies published over 30 years ago reported the presence of functionally active opiate binding sites on rat liver mitochondrial outer membranes that putatively mediated potent inhibitory actions on oxidative phosphorylation [63,64]. These prior observations relating to mitochondrial opiate receptors were functionally confirmed by a recent publication from our laboratory demonstrating morphine/μ3 opiate receptor mediated NO release from isolated human mitochondria [65]. As discussed in depth above, we propose the regulatory effects of "morphinergic" coupling to cNOS activation on mitochondrial function and cellular bioenergetics are synergistically amplified by coordinate activation of cellular and mitochondrial nitrite reductases [27,28,35,66].

The evolutionary development and functional presence of a morphine, opiate alkaloid-selective, μ3 opiate receptor across animal species, its functional coupling to cNOS and mitochondrial nitrite reductases, raises provocative questions relating to the existence of a similar regulatory pathway within classes of human gut microbiota [67]. Along these lines, we may speculate that primordial opiate-alkaloid effects on bacterial growth and energy production have been evolutionary modified in concert with NO production and release to mediate regulatory activities within classes of human gut microbiota. Accordingly, dynamic interactions within communities of gut modulate GI motility which is functionally associated with bacterial survival [40,41,68]. Interestingly, the COOH-terminal domain of μ3 opiate receptor resembles a primitive chemokine-like receptor, including functional clustering of cysteine residues indicative of NO involvement [54]. If so, vertical mu opiate gene evolution may have occurred from its chemokine origin. This conclusion is now supported by the empirical evidence from other related studies, demonstrating a chemokine presence and involvement with communication between bacteria and with their host [40,41,43].

Conclusions

It appears opiate alkaloid selectivity, as noted earlier, was maintained in evolution because so many other biochemical molecules are really alkaloid in nature, thus representing a branching from the common origin [36,39,69]. In this regard, amino acids, dopamine, cocaine, nicotine, nucleic acids, etc, may also be considered to be alkaloid in nature [36–39]. Morphine may have fit into its dynamic life sustaining role because it is an alkaloid and has a relatively long half-life, just one of many such compounds incorporated into the nitrogen rich framework of molecular "information" [36–39]. Given this, one can better understand and predict the inter-phyla level of chemical communication that arises especially with alkaloid compounds since some of this information may have arisen from horizontal gene transfer and much latter in evolution [40–43]. The chance occurrence of NO association with the nitrogenous alkaloids, especially morphine, probably enhanced the significance of NO coupling in cellular processes involving its purposeful generation, e.g., via nitrite reductase. This level of insight allows one to suggest that newer variations of the common chemical alkaloid theme will emerge and be both commercially and functionally efficacious.

Conflict of interest

None.

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