Inducible Nitric Oxide Synthase (iNOS) Inhibitors from Plants

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Nitric Oxide (NO), a gas at temperatures below -152°C has long since been known as a toxic smog pollutant. Present reports furnish the fact that nitric oxide is synthesized in many cells endogenously. While the endogenous production of NO may significantly benefit an organism, its excessive production has been implicated in the pathogenesis of many diseases involving the cardiovascular, immune and nervous system. Inhibition of NO formation may have therapeutic benefit in patients with septic shock or inflammatory diseases. Phytochemicals isolated from various medicinally important plants provide valuable agents that suppress the expression of inducible nitric oxide synthase (iNOS) enzyme which are hence useful for the prevention of various diseases. Systematic work performed during the last decade has been reviewed and tabulated in this article.

Nitric oxide (NO) was honored as the molecule of the year in 1992¹. Nitric oxide which was earlier considered as an environmental pollutant and a highly toxic gaseous free radical, is now known to play a crucial bioregulatory role as an intercellular messenger in a number of physiological processes such as vasodilatation, neurotransmission, platelet aggregation, in the cytostatic and cytotoxic action of macrophage and neutrophils²³. An important property of NO is that it rapidly and spontaneously reacts with a superoxide anion (O₂⁻) to form a peroxynitrite anion (ONOO⁻), and its conjugate acid, peroxynitrous acid (ONOOH) which is more toxic to biological system than O₂⁻ or NO alone⁴.

Biosynthesis of NO:

Nitric oxide (NO) is biosynthesized in living organisms by the oxidation of L-arginine to NO and citrulline via an intermediate N⁶-hydroxy-L-arginine (NHA). The entire process is catalyzed by a remarkable family of enzymes, the nitric oxide synthases (NOS). The reaction is overall a five-electron oxidation of L-arginine using nicotinamide adenine dinucleotide phosphate (NADPH) as the source of electrons. The NO thus generated is rapidly converted to stable products of nitrogen oxide, nitrite and nitrate⁵.

Assay of nitric oxide:

Nitric oxide analysis in activated macrophages is done with the help of Griess reaction. NO released from the cells is detected and quantified photometrically as its stable product nitrite by a simple colorimetric reaction. (Griess reaction)⁶. In this reaction the cellular production of NO is determined by measuring its stable product nitrite in cell culture supernatants. Griess reagent [sulfanilamide/ N-(1-naphthyl)-ethylenediamine dihydrochloride] added to various cell culture supernatants converts nitrite into a purple azo dye which can be quantified photometrically and thus used as a parameter for the NO synthesis of cultured cells⁷.

Nitric oxide synthases (NOS):

NOS is a heme-containing enzyme with a sequence similar to cytochrome P-450 reductase⁸. Several isoforms of NOS are now known to exist which are either constitutive NOS (cNOS) or inducible (iNOS). The cNOS include the endothelial (eNOS) or neuronal (nNOS) isoforms that are calcium-dependant and release small and regulated amount of NO required for various physiological functions. On the
other hand, the iNOS is Ca^{2+} independent and produces large amounts of NO continuously for longer periods of time and is responsible for the cytotoxicity of NO.

Inducible Nitric oxide synthase (iNOS):

Inducible nitric oxide synthase (iNOS) is an inflammation-induced enzyme that catalyzes the production of nitric oxide (NO), a molecule that may lead to carcinogenesis. Macrophages and some other cells have a transcriptionally inducible form of iNOS (iNOS) that remains undetectable until these cells are activated. Interferon-γ and bacterial lipopolysaccharides (LPS) are the most potent activators of the iNOS gene in murine macrophages. Tumor necrosis factor-α (TNFα), originally discovered by its anti-tumor activity, is one of the most pleiotropic cytokines acting as a host defense factor in immunologic and inflammatory responses. However, high production of NO by iNOS may induce host cell death and inflammation.

Inhibitors of iNOS:

Dirsch et al. stated that in the field of inflammation research the inducible nitric oxide synthase (iNOS) has become an important pharmacological target, since over-production of nitric oxide (NO) after induction of this enzyme seems to be associated with numerous pathological conditions. Over expression of iNOS by various stimuli, resulting in over-production of NO, contributes to the pathogenesis of septic shock and some proinflammatory effects including vasodilatation, edema, cytotoxicity and autoimmune diseases. Therefore, it is valuable to develop inhibitors of iNOS for potential therapeutic use. Thus, agents that suppress the expression of iNOS mRNA or enzyme protein will be useful for the prevention of various diseases. These agents have therapeutic potential in treating the hyperfunctioning of the NO pathway. Such agents should inhibit iNOS selectively without inhibiting the constitutive NO release.

Among the most widely used drugs in antiinflammatory therapies, synthetic glucocorticoid, dexamethasone is highly effective in controlling inflammation and this may be in part, due to its ability to inhibit iNOS expression. Glucocorticoid inhibition of NO production was described in cytokine-stimulated mesangial cells. Di Rosa et al. first demonstrated that dexamethasone and hydrocortisone also inhibit the production of NO in the lipopolysaccharides and IFN-γ stimulated macrophage cell line J 774. Walker et al. studied the mechanisms by which these synthetic glucocorticoids suppress IFN-γ stimulated iNOS expression in RAW 264.7 cells. Several analogues of L-arginine are now known to act as synthetic iNOS inhibitors.

One potential source for novel iNOS inhibitors is the diverse area of natural products. Compounds isolated from plants that are iNOS inhibitors showed inhibition of nitric oxide (NO) synthesis in a dose-dependent manner in murine macrophage-like RAW 264.7 cells stimulated with interferon-γ plus lipopolysaccharides. Murine macrophage-like cell line, RAW 264.7, is a suitable cell model to perform in vitro studies of the iNOS system. Since the systematic work on the isolation of compounds inhibiting the excess NO production has been reported during the last few years and there is no such compilation of NOS inhibitory compounds. Hence, we have reported the iNOS inhibitory compounds isolated from different plant parts of various families described in Table 1 and their structures, in fig. 1. This review summarizes the work reported till 2001.

NATURAL INHIBITORS

Alkaloids:

Two quinazoline alkaloids dehydroevodiamine (1) and evodiamine (2) isolated from Evodia rutacearapa inhibited NO production in IFN-γ/LPS-stimulated RAW macrophages in concentration-dependent manner. Compound 1 inhibits NO production in almost equipotent manner, whether added before IFN-γ or before or after LPS application, indicating that the compound suppresses the activity of iNOS at multiple levels where as compound 2 affects only the IFN-γ related actions. These compounds account for the antiinflammatory property of this plant.

Coumarins:

Till now eight coumarins have been tested for NO inhibitory activity. Of these, the three coumarins 5-[(6′,7′-dihydroxy-3′,7′-dimethyl-2-octenyl)oxy]psoralen (4), 5-geranyloxypсорalen (5) and oxypeucedanin (9) isolated from Citrus hystrix act as inhibitors of LPS, IFN-γ induced NO generation in RAW 264.7 cells. Compound 5 was found to be highly active (IC_{50} = 14 μM) where as other coumarins (4,9) bearing isoprenyl (IP) or geranyl (GR) chains with hydroxyl groups were drastically less active (IC_{50} values as 130 μM and 310 μM, respectively). The isolated compounds showed no detectable cytotoxicity at every concentration tested. The structural difference among these coumarins were found only in the side chains. Thus, these compounds can further be used in chemo-preventive activity in rodents and humans. 5-Geranyloxypсорalen (5), 8-geranyloxypsoralen (6) and 5-geranyloxy-7-methoxycoumarin (7) isolated from the 80 % methanol-wa-
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<td>37</td>
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<td>39</td>
<td>6 Rhododendrol</td>
<td><em>Tinospora tuberculata</em></td>
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*Compound structure number is given in parenthesis.

The portion of the lemon peel extract exhibited inhibitory activity toward tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus (EBV) activation at a concentration of 10 μM in Raji cells. Compound 6 and 7 inhibit the LPS/IFN-γ triggered iNOS expression pathway or iNOS enzyme activity. In all, compound 6 was indicated to have higher inhibitory activity towards EBV activation, O₂⁻ and NO generation than compounds 5 and compound 7. Furanocoumarins, imperatorin (8) and deltoin (3) had been suggested to be the major components of *S. divaricata* to inhibit NO production in RAW 264.7 cells. The IC₅₀ values of compounds 8 and 3 (64 μM and 35 μM, respectively) are much higher than for the polyacetylene, falcarnidine (17) and panaxyol (20)³. Scopoletin (10) a coumarin, isolated from
Fig. 1: Chemical structures of the naturally occurring iNOS inhibitors.
A. feddei inhibits the production of NO-induced by IFN-γ plus LPS in RAW 264.7 macrophages. The mechanism for the inhibition of NO production was due to suppression of the expression of iNOS mRNA as well as enzyme protein. Scoleopterin has also been isolated from the dried bark of Fraxinus rhynchophylla. The compound inhibited NO production by IFN-γ plus LPS-stimulated RAW 264.7 cells with IC_{50} value of 52 μM. Compound did not show cytotoxicity within concentration of 1-50 μg/mL. Cnidin (11), a coumarin isolated from the root extract of Angelica koreana inhibited the expression of nitric oxide synthases in RAW 264.7 cells with IC_{50} of 7.5 μM.

Diarylethpanoids:

Two diarylethpanoids, hirsutanol (12) and oregarin (13) isolated from fresh leaves of Alnus hirsuta, an indigenous species growing in Korea, were found to be potent iNOS inhibitors. They showed inhibition of NO production in interferon-γ (IFN-γ) and LPS-activated RAW 264.7 cells in a dose-dependent manner. Their IC_{50} values were 3.8 and 14.3 μM, respectively. This activity was due to the suppression of expression of iNOS mRNA.

Diterpenes:

Andrographis paniculata utilized in traditional system of medicine is used for the treatment of bacterial infection and inflammatory diseases (e.g. Rheumatoid arthritis). Andrographolide, (14) a bicyclic diterpenoid lactone, isolated from this plant display NO synthesis inhibitory effect. The inhibitory effect was due to the inhibition of iNOS protein induction and this inhibition of iNOS synthesis may contribute to the beneficial haemodynamic effects of andrographolide in endotoxic shock. Andalusiol (15) was isolated from the acetone extract of Sideritis foetens (aerial parts). Compound 15 did not cause direct inhibition of iNOS activity, but rather affected the expression of the enzyme during the initial 0-6 h after LPS stimulation. This shows the anti-inflammatory property of this compound. Four known kaurane diterpenes (16-19) were isolated by activity-guided fractionation from the plant Isodon japonicus with IC_{50} values of 0.02 (0.06), 0.21 (0.58), 0.05 (0.15) and 0.12 mg/ml (0.35 mM), respectively.

Flavonoids:

5,4'-Dihydroxy-6,7,8,3',5'-pentamethoxyflavone (20) and 5,4'-dihydroxy-6,7,8,3'-pentamethoxyflavone (21) isolated from methanol extract of Cleome droserifolia suppressed the NO production in dose-dependent manner. IC_{50} values for the suppression of NO production by 20 and 21 were 50.5 and 85.5 mM, respectively. The activity of compound 21 was weaker than that of compound 20. The compounds might be immunosuppressive constituent of C. droserifolia and have a potential to be antiinflammatory and immunomodulatory agents.

Polycyctenes:

Five polycyctenes, falcarinol (22), falcarinone (23), panaxadol (24), panaxynol (25) and panaxotriol (26) isolated from the ethylacetate extract of roots of S. divaricata inhibited nitrite production by iNOS. The IC_{50} value of falcarinol, falcarinone, panaxadol, panaxynol and panaxotriol were 1.98, > 20, 6.58, 2.23 and 9.58 mM, respectively. Among these, falcarinone showed a marginal effect on NO production suggesting that the hydroxy group at C-3 plays a critical role in their inhibitory effect.

Sesquiterpenes:

Ethyl acetate soluble fraction of Saussurea lappa yielded compound dehydrocostuslactone (27), a sesquiterpene lactone that reduced the level of NO production in LPS-activated macrophages cell culture systems by inhibiting iNOS expression. The IC_{50} value was 3.0 μM. This compound may have potential in the treatment of endotoxemia. Yomogin (28), a eudesmane sesquiterpene isolated from dichloromethane soluble fraction of Artemisia princeps was tested for NO production in LPS-activated murine macrophages. It exhibited potent inhibition on NO production with IC_{50} value calculated as 3±0.14 μM. This result suggested an anti-inflammatory activity of compound 28. Sesquiterpene lactones, costunolide (29) and parthenolide (30) were isolated from Magnolia grandiflora. Comparable activities for both the compounds in inhibition of NO production was with an IC_{50} value of 0.43 μM for compound 29 and 0.56 μM for the compound 30.

Tannins:

A catechin, which is a polyphenolic phytochemical, epigallocatechin-3-gallate (EGCG) (31) isolated from green tea is the most potent in terms of antioxidative capacity and has been ascribed to have the predominant role in cancer chemoprevention. It has been found that it also inhibits the NO production and iNOS gene expression.

Miscellaneous:

Curcumin (32), a dietary polyphenolic, isolated from Curcuma zanthorrhiza inhibits the NO production in a concentration-dependent manner with an IC_{50} of 6 μM. Curcumin decreases the activity and protein levels of iNOS by reduc-
ing the expression of iNOS mRNA. Exact mechanism for
inhibition of iNOS induction by curcumin is not known\textsuperscript{7,31,32}. Rhododendrol (37) and its glucoside, epi-rhododendrin (33) isola
ted as active principle from ethylacetate soluble and n-
butanol soluble fractions of \textit{Acer nikoense} also suppressed
the NO production. Compound 37 significantly reduced the
maximal level of NO release in the LPS-stimulated macrophage
when given p.o. at a dose of 50 mg/kg/day and did not show
any cytotoxic effect toward the macrophage, where as its
glucoside (33) suppressed the NO production by 35%
when p.o. administered at a dose of 30 mg/kg/day and the
activity was weaker than that of compound 37. Thus, the
compounds 33 and 37 have a potential to be anti-inflamma-
atory drugs\textsuperscript{33}. Ferulaldehyde (34) was isolated from dried bark
of \textit{Fraxinus rhynchophylla}, which was screened for NO inhi-
bition activity. The compound inhibited NO production by
interferon-\(\gamma\) (IFN-\(\gamma\)) plus LPS-stimulated RAW 264.7 cells with
\(IC_{50}\) of 90 \(\mu\)M. The compound did not show cytotoxicity in
the concentration range of 1-50 \(\mu\)g/mL. The inhibition of NO
production of 34 was due to suppression of the expression of
iNOS protein\textsuperscript{35}. Two inhibitors of NO were isolated from
the methanol extract of stem bark of \textit{Magnolia obovata}. Their
structures were elucidated as honokiol (35) and magnolol
(36) with \(IC_{50}\) value of 16.8 and 6.4 \(\mu\)M, respectively. Both
reduced the inducible level of iNOS and TNF-\(\alpha\) in the LPS-
activated macrophage cell culture system. Thus, these com-
ounds may be used in treatment of endotoxemia and in-
flammation accompanied by the overproduction of NO and
TNF-\(\alpha\)\textsuperscript{34}. The aqueous extract of \textit{Tinospora cordifolia}
exerts inhibitory effects on enhanced NO formation in both cell
and cell-free systems. Thus, the compounds present in this
extract may be responsible for anti-inflammatory and
antiinfective activity of this plant\textsuperscript{36}.

CONCLUSIONS

Till now thirty compounds of different classes have been
isolated from various plants of different families for NOS inhi-
bitory activity. They all inhibited either the expression of
iNOS enzyme or inhibition of NO production by IFN-\(\gamma\) plus
LPS-stimulated RAW 264.7 cells. Among these the
coumarins are the major group of compounds isolated as
NOS inhibitors. Till now eight coumarins have been isolated
from five different plants. The coumarins that bear a
geranyloxy group appear to be potent NOS inhibitors. These
naturally occurring NOS inhibitors may be used as antiin-
flammatory drugs or in the treatment of endotoxemia. There-
fore, it would be worth while to isolate naturally occurring
compounds, which may act as potent and selective inhibi-
tors of iNOS for potential therapeutic use.

ACKNOWLEDGEMENTS

Authors thank the Director, CIMP for constant encour-
agement and for the award of Senior Research Fellowship
for Ms. Neerja Pant.

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