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Invited Review

The Mechanisms for Nitration and Nitrotyrosine Formation *in vitro* and *in vivo*: Impact of Diet

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The detection of 3-nitro-L-tyrosine residues associated with many disease states, including gastric cancer, has implicated a role for peroxynitrite *in vivo*, and thus endogenously produced nitric oxide and superoxide. Additionally, dietary nitrate has been suggested to be involved in the pathogenesis of gastric cancer through a mechanism involving reduction to nitrite and subsequent formation of potentially mutagenic nitroso-compounds. Studies have now demonstrated that a multitude of reactive nitrogen species other than peroxynitrite are capable of producing nitrotyrosine. Thus, we have reviewed the evidence that dietary nitrate, amongst other reactive nitrogen species, may contribute to the body burden of nitrotyrosine.

Keywords: Nitrotyrosine, nitrate, nitrite, peroxynitrite, nitric oxide, nitration, reactive nitrogen species

Abbreviations: Nitrotyrosine, 3-nitro-L-tyrosine; RNS, reactive nitrogen species; BSA, bovine serum albumin; LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein; EGC, epigallocatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; HNO_2, nitrous acid; Cl-NO_2, nitryl chloride; SIN-1, 3-morpholino-syndominine; $NO_2^{\bullet}$, nitrogen dioxide; NO-, Angeli's salt; NO_2BF_4, nitryl salt; iNOS, inducible nitric oxide synthase; NFL, low molecular weight neurofilament; MnSOD, managanese superoxide dismutase; SERCA, sarcoplasmic reticulum calcium ATPase; ALS, amyotrophic lateral sclerosis; SP-A, surfactant protein A; i.v., intravenous; NHPA, 3-nitro-4-hydroxyphenylacetic acid; NHPL, 3-nitro-4-hydroxyphenyllactic acid; NO_2^-, nitrite; NO_3^-, nitrate; N_2O_3, dinitrogen trioxide; $NO^{\bullet}$, nitric oxide; HOCl, hypochlorous acid; EDRF, endothelium derived relaxing factor; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNos, neuronal nitric oxide synthase; HbO_2, oxyhaemoglobin; Hb^{3+}, methaemoglobin; NH_3, ammonia; $O_2^{\bullet-}$, superoxide; XOD, xanthine oxidase; Mϕ, macrophage; N, nitrogen; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; HRPT, hypoxanthine-guanine phosphoribosyl transferase; DEN, diethylnitrosamine; DEA, diethylamine

INTRODUCTION

The presence of 3-nitro-L-tyrosine (Figure 1) has often been considered to be a "fingerprint" of peroxynitrite formation *in vivo*. However, it

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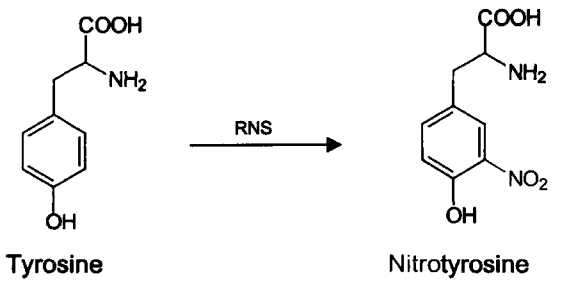

FIGURE 1 The structures of tyrosine and its nitrated product, 3-nitro-L-tyrosine.

has been demonstrated that, at physiological pH, a multitude of reactive nitrogen species (RNS) that may occur *in vivo* can nitrate both free and protein-bound tyrosine on the *ortho*-position *in vitro* (Table I). In addition, nitration of tyrosine residues by tetranitromethane has been used to elucidate the role of specific tyrosine residues in protein function.[24,25] Inhibition nitrotyrosine formation by RNS has been demonstrated *in vitro* by many compounds including dietary phenolics, such as the catechine polyphenols[3] and to a lesser extent hydroxycinnamates[2] (Table I). *In vivo*, nitrotyrosine formation has been prevented in humans by supplementation with ascorbic acid (1 g, twice daily)[26] and in a guinea pig model of ileitis by genistein.[27]

The formation of nitrotyrosine has been implicated in many inflammatory conditions, neurodegenerative diseases and cancer due to elevated levels observed both in humans (Table II) and in animal models of gut inflammation[27,30] and skin cancer[60] amongst others. However, there are some conflicting studies in which nitrotyrosine levels do not appear to change in disease.[61,62] The nitrotyrosine detected *in vivo* appeared to be localised in a variety of cells: epithelial cells,[26,31,33,57] polymorphonuclear leukocytes,[26] macrophages,[29–31,50] smooth muscle cells,[29,31] extracellular matrix,[26,57] neurons[40,42,48,49] and microglia.[42,51] Within cells it is often located close to inducible nitric oxide synthase (iNOS).[29,30,51,54] Nitric oxide itself is unable to nitrate tyrosine,[6,63–65] thus indicating the involvement of higher oxides of nitrogen.

Several purified proteins (Table I) have been nitrated *in vitro*, and some nitrated proteins have been identified in cell studies. These include prostacyclin synthase from rat mesangial cells treated with peroxynitrite or SIN-1,[66] the focal adhesion protein $p130^{cas}$ in human neuroblastomas SH-SY5Y cells treated with SIN-1[67] and multiple nitrated proteins in heart homogenates treated with peroxynitrite, or a mixture of myeloperoxidase, nitrite and hydrogen peroxide.[68] At present, however, nitrotyrosine residues have only been isolated from human serum albumin,[7] human low density lipoprotein,[7,9] low molecular weight neurofilament (NFL),[62] manganese superoxide dismutase (MnSOD),[69] rabbit β-VLDL apoproteins[70] and murine sarcoplasmic reticulum calcium-ATPase 2a (SERCA2a).[65]

Nitration of tyrosine residues in proteins induces the change of tyrosine into a negatively charged hydrophilic nitrotyrosine moiety that may alter enzyme activity. This could have deleterious effects due to mitochondrial respiratory dysfunction,[71] impaired lung function following inactivation of surfactant protein A (SP-A),[17] disrupted microtubule organisation that leads to altered cell morphology and epithelial-barrier dysfunction,[72] reduced antioxidant defence due to diminished activity of superoxide dismutase,[12,64,13] impaired cellular function due to reduced GTP binding,[15] inactivation of tyrosine kinases resulting in decreased phosphorylation[73] and thus affecting signalling cascades and inhibited neurofilament assembly weakens cell structure[74] and may reduce motor neuron survival. However, nitration of tyrosine may play a protective role by attenuating the thrombotic properties of tissue factor,[18] elimination of foreign bodies engulfed by phagocytes,[75] thymocyte negative-selection via apoptosis[76] and targeting proteins for degradation and elimination.[73] The majority of proteins appear to be unable to incorporate free nitrotyrosine upon their assembly,[72,77] the exception being α-tubulin that subsequently cannot function correctly.[72]

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TABLE I Reactive nitrogen species involved in tyrosine nitration *in vitro*

Nitrating species	Substrate	Inhibitors
Peroxynitrite ($OONO^-$)	Tyrosine[1–5]	Ascorbyl palmitate,[1] ascorbic acid,[5] caffeic acid,[2] catechin,[3] chlorogenic acid,[2] *m*-coumaric acid,[2] *o*-coumaric acid,[2] *p*-coumaric acid,[2] epicatechin,[3] EGC,[3] ECG,[3] ferulic acid,[2] gallic acid,[3] EGCG,[3] glutathione,[5] α-tocopherol,[3] trolox,[2,3] uric acid[5]
	Bovine serum albumin (BSA)[6–8]	Ascorbate,[6] uric acid,[6] sulfhydryls[6]
	Human serum albumin[7]	
	Low density lipoprotein (LDL)[7,9,10]	
	α_1-antiproteinase[7]	
	Angiotensin II[11]	
	Fibrinogen[7]	
	Manganese superoxide dismutase[12,13]	
	Monocyte chemoattractant Protein-1 (MCP-1)[14]	
	Pepsinogen[7]	
	Rac2[15]	
	Ribonucleotide reductase[16]	
	Surfactant protein A[17]	
	Tissue factor[18]	
Nitrous acid (HNO_2)	Tyrosine[19,20]	Catechin,[20] epicatechin,[20] EGC,[20] ECG,[20] EGCG,[20] ferulic acid,[20] rutin[20]
	BSA[8,19]	
	Angiotensin II[11]	
Nitryl chloride ($Cl\text{-}NO_2$) produced from: nitrite + HOCl	BSA[8]	
	LDL[10]	
3-Morpholino-syndominine (SIN-1): produces both $O_2^{\bullet-}$ and $NO^{\bullet}$	Tyrosine[4]	
	Bovine serum albumin[8]	
	Surfactant protein A[21]	Urate[21]
SpermineNONOate produces $NO^{\bullet}$	Tyrosine[4]	
	BSA[8]	
	Surfactant protein A[21]	
Oxidation products of nitrite produced by: peroxide + H_2O_2 + $NaNO_2$	Tyrosine[22]	
	BSA[22]	
Nitrogen dioxide ($NO_2^{\bullet}$)	Tyrosine[1,23]	
	BSA[23]	
	Bovine eye lens α-crystallin[23]	
Angeli's salt (NO^-)	BSA[8]	
Nitryl salt (NO_2BF_4): produces NO_2^+	Tyrosine[22]	

Proteins capable of removing the nitro-group from nitrotyrosine in proteins, in the absence of protein degradation, have been isolated from canine prostate[78] and and there appears to b ted proteins from rat

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TABLE II Evidence of *in vivo* formation of nitrotyrosine, nitrate and nitrite in human disease. A selection of the diseases and the biological samples in which nitric oxide has been implicated due to the detection of elevated levels of nitrotyrosine, nitrate and nitrite

	Disease	Site of elevated nitrotyrosine	Site of elevated nitrate/nitrite
Inflammation	Atherosclerosis	Atherosclerotic lesions[9,28,29] Atherosclerotic plaques[30,31]	
	Gastritis	Gastric antral biopsy[26] Gastric fundus biopsy[31]	Gastric juice[30]
	Gastric ulcer	Ulcer margins[31]	Gastric juice[32]
	Ulcerative colitis	Colonic mucosa[33,34]	Urine[35]
	Crohn's disease	Colonic mucosa[34]	Urine[35]
	Celiac disease	Plasma[36] Duodenal biopsy[37]	Plasma[36] Urine[38]
	Septic shock	Plasma[39]	Plasma[39]
Neurodegenerative	Alzheimer's disease	Hippocampal tissue[40–42] Cerebrospinal fluid[41,44]	Cerebrospinal fluid[43]
	Amyotrophic lateral sclerosis (ALS)	Cerebrospinal fluid[45] Spinal cord[48,49]	Cerebrospinal fluid[46,47] Serum[46]
	Multiple sclerosis	Central nervous system tissue[50] Brain tissue[51]	Cerebrospinal fluid[50]
	Parkinson's disease	Subtantia nigra[40]	Cerebral spinal fluid[52] Neutrophils[53]
Cancer	Colon cancer	Tumour tissue[54,55]	Plasma[56]
	Gastric cancer	Gastric biopsy[57]	Serum[58] Gastric juice[59]

effect of nitrotyrosine. However, nitrotyrosine can be transported into cells[72] and absorbed from the diet by rats.[81] Intravenous (i.v.) administration of nitrotyrosine in rats inhibited the vasoconstrictive and hemodynamic responses to angiotensin II[82] and attenuated the hemodynamic responses produced by α- and β-adrenoceptor agonists (norepinephrine, epinephrine, phenylephrine and isoproterenol).[83] It has also been demonstrated that nitrotyrosine administered by i.v. injection to rats has a half-life in the plasma of 1.67 hours[84] and an oral dose (100 μg) was converted into two metabolites; 3-nitro-4-hydroxyphenylacetic acid (NHPA) and 3-nitro-4-hydroxyphenyllactic acid (NHPL) that were excreted in the urine (44% and 5% of oral dose, respectively).[81] These metabolites have been proposed as biomarkers of human exposure to nitrosating agents[81] such as nitrate and nitrite. This has been further corroborated as NHPA appears to be excreted in the urine of human volunteers, with a profile similar to that of nitrate, following consumption of a nitrate-rich meal.[85]

Thus, the origin of free nitrotyrosine detected *in vivo* probably arises from the nitration of free tyrosine, the breakdown of nitrated proteins and dietary intake and absorption. Uncertainty still remains as to whether nitrotyrosine formation initiates the onset of disease or is a consequence of the progression of disease.

NITRATE/NITRITE

Nitrate (NO_3^-) and nitrite (NO_2^-) are naturally occurring, water soluble anions. The nitrate ion is the conjugate base of the strong acid nitric acid ($pK_a = -1.37$). The nitrite ion is the conjugate base of the weak acid nitrous acid ($pK_a = 3.37$) which decomposes readily to give water and dinitrogen trioxide (N_2O_3) or nitric acid, nitric oxide ($NO^\bullet$) and water. Salts of both acids are readily soluble in water with nitrites being much more stable than the acid itself.[86]

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I. The Fate of Nitrate and Nitrite in the Human Body

The fate of nitrate in the human body is represented schematically in Figure 2. Ingested nitrate first encounters specialised symbiotic nitrate-reducing bacteria, that reside on the dorsal surface of the tongue and which reduce some (5%) of the nitrate to nitrite.[87–91] The nitrite thus formed is then chemically reduced to nitric oxide ($NO^{\bullet}$) under the acidic conditions of the stomach (Figure 3A).[89] Nitrate is absorbed from the stomach into the blood stream where it rapidly enters erythrocytes.[88] Salivary glands concentrate and actively secrete nitrate from the circulation into the oral cavity,[87–89] 25% of dietary nitrate undergoes enterosalivary recirculation in this way.[87,90,91] The majority of nitrate (60–70%) is excreted unchanged in the urine within 24 hours of ingestion[85,87,92] and appears to be predominantly tubular.[88] Maximal urinary excretion occurs 4–6 hours following challenge with potassium nitrate[87] or as it occurs naturally as part of a meal.[85] A minor amount is also excreted unchanged in the sweat[87] and faeces (0.1–0.5 %).[87,92] In radiolabel tracer studies in humans using ^{15}N-labeled nitrate, the nitrogen from administered $^{15}NO_3^-$ was recovered as ammonia and urea in the urine (3%) and faeces (0.2%).[92] The half-life of nitrate in the body has been determined to be approximately 5 hours.[92]

After administration of nitrite in meat, urinary excretion of nitrate but not nitrite was elevated[93] indicating that nitrite is oxidised during its passage through the body possibly in the stomach (Figure 3A). Oxidation of nitrite to nitrate can also occur via reactions with oxyhaemoglobin,[94] HOCl[10,95] and peroxidases.[22,96]

II. Endogenous Sources

Nitrate balance studies conducted in humans consistently conclude that a greater amount of nitrate is excreted than can be accounted for by

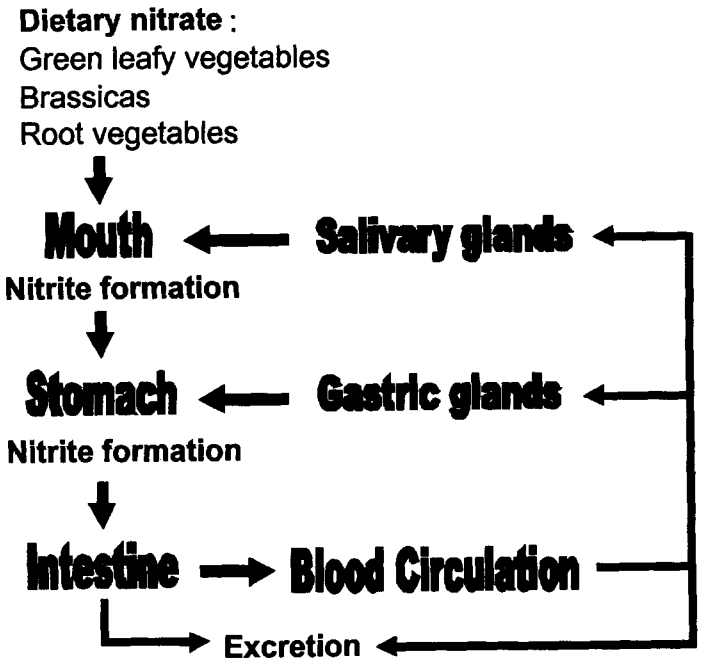

FIGURE 2 Diagrammatic scheme representing the fate of nitrate following ingestion by humans.

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