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Tyrosine Nitration: Localisation, Quantification, Consequences for Protein Function and Signal Transduction

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The nitration of free tyrosine or protein tyrosine residues generates 3-nitrotyrosine the detection of which has been utilised as a footprint for the *in vivo* formation of peroxynitrite and other reactive nitrogen species. The detection of 3-nitrotyrosine by analytical and immunological techniques has established that tyrosine nitration occurs under physiological conditions and levels increase in most disease states. This review provides an updated, comprehensive and detailed summary of the tissue, cellular and specific protein localisation of 3-nitrotyrosine and its quantification. The potential consequences of nitration to protein function and the pathogenesis of disease are also examined together with the possible effects of protein nitration on signal transduction pathways and on the metabolism of proteins.

Keywords: 3-nitrotyrosine, protein nitration, peroxynitrite, reactive nitrogen species

INTRODUCTION

In spite of its relatively simple structure, the diatomic free radical nitric oxide (nitrogen monoxide) ($NO^{\bullet}$) has been identified as an important messenger molecule with diverse and complex multifunctional actions within biological systems. Generally, direct interactions between $NO^{\bullet}$ and target proteins such as guanylate cyclase account for its physiological properties, whilst its indirect actions via secondary reactions with reactive oxygen species, forming reactive nitrogen species, are likely to account for the participation of $NO^{\bullet}$ in pathology. The specific reactions of $NO^{\bullet}$ and its secondary reaction intermediates with protein

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tyrosyl or tyrosine residues to form nitrated proteins are the subject of this review. We will examine the tissue and cellular localisation of 3-nitrotyrosine, its quantification in normal and diseased tissues and body fluids, and the consequences of protein nitration in relation to specific protein function, metabolism, and modulation of signal transduction mechanisms.

DETECTION OF 3-NITROTYROSINE

Using "nitrotyrosine" as the search term on the National library of Medicine "PubMed" (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) database provided over 650 references dating from 1965 to May 2000 with just over 60% in the last 3 years. Prior to 1992 research related to tyrosine nitration involved chemical modification of protein tyrosines, often using tetranitromethane (TNM), as a tool to investigate the importance of individual tyrosine residues in protein (mainly enzyme) structure/function relationships (lists of proteins modified by TNM can be found in reference[1]). Ohshima and co-workers in 1990 provided the first demonstration that metabolites of 3-nitrotyrosine, 3-nitro-hydroxyphenylacetic acid and 3-nitro-hydroxyphenyllactic acid are present in human urine.[2] However, in the absence of an endogenous source of nitrating agents, this pioneering observation was left unexplored for a further 2 years. The majority of research into protein tyrosine nitration (90% of the cited articles) was initiated by the proposal in 1992 by Joseph Beckman and colleagues that peroxynitrite, the reaction product of $NO^{\bullet}$ and superoxide ($O_2^{\bullet -}$) could nitrate tyrosine residues in proteins such as Cu, Zn superoxide dismutase.[3,4] By 1994 the Beckman laboratory had developed a rabbit polyclonal and mouse monoclonal antibodies that recognised nitrated proteins (see [5] for review) and demonstrated for the first time the presence of nitrated protein in human atherosclerotic lesions[6] and lung tissue from patients with adult respiratory distress syndrome.[7] In that same year Barry Halliwell's group were the first to employ an analytial technique, high performance liquid chromatography with ultraviolet detection (HPLC-UV), to quantify levels of free 3-nitrotyrosine in serum and synovial fluid of patients with rheumatoid arthritis.[8] Since then 3-nitrotyrosine in either the free or protein associated form has been detected in at least 50 human diseases and more than 80 animal models or cell culture systems (Tables I–II).

METHODS FOR MEASURING 3-NITROTYROSINE

There are several methods that can be utilised for the detection of 3-nitrotyrosine. The simplest is spectrophotometric measurement since 3-nitrotyrosine has a peak absorbance at 350–450 nm depending on the pH of the sample. The concentration of 3-nitrotyrosine can be determined by measuring a sample at pH 9 or above (which shifts the peak absorbance to approximately 430 nm wavelength) and using the extinction co-efficient, $E_{430} = 4400\,M^{-1}cm^{-1}$.[345] Spectrophotometric analysis is only reliable for quantifying 3-nitrotyrosine as the free amino acid or protein 3-nitrotyrosine if the sample is relatively pure.[346] Indeed when nitrating a particular protein with micromolar or millimolar concentrations of peroxynitrite or TNM (e.g. to investigate the importance of tyrosine residues in protein structure/function relationships), one can satisfactorily determine the degree of protein nitration using this method. However, for determining levels of 3-nitrotyrosine *in vivo* alternative techniques must be employed, including HPLC with either UV or electrochemical (EC) detection, or gas chromatography-mass spectrometry (GC-MS) or immunological techniques such as immunohistochemistry, immunoprecipitation, western blot or ELISA.

The qualitative techniques using antibodies are confined to the detection of protein 3-nitro-

TABLE I Detection of nitrotyrosine in human disease and animal models of disease (specific nitrated proteins are highlighted in bold)

System	Species	Disease/Condition	Location	Co-localised with or also upregulated	Detection technique	Ref.
CNS	Human	AIDS dementia complex	Frontal cortical neurons, vascular wall	iNOS	Immunohistochemistry	9
		ALS Sporadic & Familial	Spinal cord, **MnSOD** in CSF, **Neurofilament L**, neurofilament aggregates, motor neurons (cell body & axon), astrocytes, vascular wall	nNOS, eNOS 3-nitro-4-hydroxyphenylacetic acid	Immunohistochemistry Immunoblot, HPLC-EC	10–20
		Alzheimer's disease	Hippocampus, neocortex, ventricular CSF Hippocampal neurons (cytosol & nuclei), neurofibillary tangles	Dityrosine	HPLC-EC Immunohistochemistry	21 22–24
			CSF		HPLC-EC	25
		Multiple sclerosis	Monocytes, macrophages, hypertrophic astrocytes, within areas of demyleination	iNOS	Immunohistochemistry	26–28
		Parkinson's disease	*Core of Lewy bodies within* melanized neurons, amorphous deposits associated with intact & degenerating neurons		Immunohistochemistry	29
		Parkinson's disease, Pick's disease, Diffuse Lewy body disease, Alzheimer's disease	Microglia, endothelial cells, degenerating neurons, neuritic sprouts	nNOS	Immunohistochemistry	20
		Progressive supranuclear palsy	Astrocytes, oligodendrocytes, neurons, endothelial cells	MnSOD, Tau, iNOS	Immunohistochemistry	20, 30
		Stroke	Neutrophils & blood vessels of cerebral infarct	iNOS	Immunohistochemistry	31
	Monkey	Ageing	Subcortical white matter	iNOS	Immunohistochemistry	32
	Baboon	MPTP model of Parkinson's disease	Neurons of substantia nigra	nNOS dependent	Immunohistochemistry	33
	Rabbit	Intermittent foetal brain I/R	*Brain homogenates* **(77 kDa protein)**	Lipid peroxidation Protein carbonyls	Immunoprecipitation Immunoblot	34, 35
		Transient spinal cord ischemia	Large pyramidal motor neurons		HPLC-EC Immunohistochemistry	36
	Rat	3-nitropropionic acid model of Huntington's disease	Striatum		HPLC-EC	37, 38
		Ageing	Cerebral cortex	iNOS, nNOS	Immunohistochemistry Immunoblot	39
		Cerebral I/R	Cerebral infarct & peri-infarct regions	iNOS, MPO	Immunohistochemistry HPLC-EC	40–42

TABLE I (*Continued*)

System	Species	Disease/Condition	Location	Co-localised with or also upregulated	Detection technique	Ref.
		Chronic cerebral vasospasm	Perivascular cells, pia		Immunohistochemistry	43
		Carbon monoxide poisoning	Cerebral cortex, perivascular cells		Immunohistochemistry Solid phase ELISA	44
		Cortical ischemia	Cortical blood vessels close to lesion, hippocampus, thalamus	iNOS	Immunohistochemistry	45
		Immobilisation induced stress	Cytosol and nucleus of cortical pyramidal cells	iNOS	Immunohistochemistry	46
		Intrastriatal IFNγ & LPS	Striatal neurons & microglia, perivascular cells	iNOS	Immunohistochemistry	47
		Kainic acid induced excitotoxicity	Cytoplasm of hippocampal pyramidal cells		Immunohistochemistry	48
CNS	Rat	MPTP model of Parkinson's disease	Striatum		HPLC-EC	49
		Neonatal cerebral ischemia	Blood vessels of cortical infarct, T-lymphocytes	iNOS, MPO, PARS	Immunohistochemistry Immunoblot	50, 51
		NMDA, kainic acid & 3-nitroproprionic acid induced neurotoxicity	Striatum	8-OHdG	Immunohistochemistry HPLC-EC	52
		Pneumococcal meningitis	Meningeal blood vessels (at breaches of BBB), inflammatory cells in subarachnoid space		Immunohistochemistry	53
		Sciatic nerve lesion	Spinal motor neurons (cell bodies & axons)	8-OHdG	Immunohistochemistry	54
		Spinal chord injury	Spinal cord	PARS	Immunohistochemistry	55
		Subarachnoid haemorrhage	Vascular tissue with a subarachnoid membrane	iNOS	Immunohistochemistry	56
	Gerbil	Cerebral I/R	Cortical neurons		Immunohistochemistry	57
	Murine	β-Amyloid induced neurotoxicity in cerebral cortex	Cerebral cortex	8-OHdG	Immunohistochemistry	58
		ALS mice (express human mutant Cu/Zn SOD – G37R)	Ventral horn neurons, **Neurofilament L, Glial fibrillary acidic protein** (Schwann cell cytoskeletal protein)	HO-1 Lipid peroxidation	HPLC-EC Immunohistochemistry Immunoblot	59
		ALS mice (express human mutant Cu/Zn SOD – G93A)	Spinal chord astrocytes & motor neurons, cerebral cortex, pyramidal cells of hippocampus	HO-1 Lipid peroxidation	Immunohistochemistry HPLC-EC	60–62
		Apo E-deficient mice	Cerebral cortex, hippocampus, brainstem, cerebellum		HPLC-EC	63
		Cerebral I/R	Vascular wall of pre-infarct region of cerebral cortex		Immunohistochemistry HPLC-EC	64, 65
		Inflammatory demyelination & allergic encephalomyelitis	Spinal chord, macrophages, membrane of T-lymphocytes, cytosol of astrocytes, glial cells	iNOS	Immunohistochemistry	66–68

CNS	Murine	Ischemic stroke	Cerebral neurons and endothelial cells of infarcted region		Immunohistochemistry	69
		Malonate neurotoxicity	Striatum		HPLC-EC	70, 71
		Methamphetamine-induced neurotoxicity	Striatum		HPLC-EC	72, 73
		MPTP model of Parkinson's disease	Striatum Striatum, midbrain Brain homogenates, **Tyrosine hydroxylase**	Dityrosine	HPLC-EC GCMS Immunoblot Immunoprecipitation	74–78 79 80
		NMDA induced excitotoxicity	Striatal neurons		Immunohistochemistry HPLC -EC	81
		Thiamine deficiency in neurodegeneration & BBB breakdown	Axons of thalamic neurons	iNOS, eNOS	Immunohistochemistry	82
		Transgenic Huntington's disease model	Cerebral cortical neurons		Immunohistochemistry	83
		Traumatic brain injury	Cytoplasm and dendritic processes of degenerating cortical neurons		Immunohistochemistry Immunoblot	84, 85
	Monkey	Lyme disease (neuroborreliosis)	Schwann cells	TNFα	Immunohistochemistry	86
PNS		Sciatic nerve I/R	**Glial fibrillary acidic protein**	Protein carbonyls	Immunoblot	87
	Rat	Sciatic nerve injury & hyperalgesia	Schwann cells, macrophages		Immunohistochemistry	88
Ear	Guinea pig	LPS induced ear damage	Nerve fibres, synaptic nerve endings	iNOS	Immunohistochemistry	89
	Human	Glaucoma	Optic nerve heads		Immunohistochemistry	90
Eye	Rat	Autoimmune uveitis	Photoreceptors, ganglion cells, nerve fibres, retinal blood vessels	Lipid peroxidation	Immunohistochemistry	91
CVS	Human	Atherosclerosis	**Plasma & aortic lesion LDL, 30, 180 kDa proteins** necrotic core of plaque, sub-intimal fatty streaks, macrophages, foam cells, smooth muscle cells, endothelial cells	iNOS Oxidised lipoproteins COX-2	Immunohistochemistry Immunoblot GCMS	6, 92–98
		Cardiac allograft rejection	Macrophages & adjacent myocytes from endomyocardium	iNOS	Immunohistochemistry	99
		Coronary bypass graft	Plasma	Lipid peroxidation Protein carbonyls	ELISA	100
		Exercise intolerance in chronic heart failure	Skeletal muscle	iNOS	Immunohistochemistry	101
		Myocarditis	Endocardium, myocardium, coronary vascular endothelial cells, smooth muscle cells		Immunohistochemistry Immunoblot	102

TABLE I (*Continued*)

System	Species	Disease/Condition	Location	Co-localised with or also upregulated	Detection technique	Ref.
CVS	Human	Preeclampsia	Placental villous vessels, villous stroma, cytotrophoblasts, endothelial cells of maternal blood vessels	eNOS	Immunohistochemistry	103–108
		Transplant coronary artery disease	Macrophages, smooth muscle cells	iNOS	Immunohistochemistry	109
	Bovine	Atherosclerosis	***PGI_2 synthase***, coronary artery intima, endothelial cells		Immunohistochemistry Immunoblot Immunoprecipitation	110
	Dog	Cytokine induced myocardial dysfunction	Myocardium		HPLC-UV	111, 112
		Myocardial stunning	Ischemic region of heart	Lipid peroxidation	Immunohistochemistry	113
		Peroxynitrite treated dogs on cardiopulmonary bypass	Left ventricular myocardium	MPO	ELISA	114
	Rabbit	Atherosclerosis & hypercholesterolemia	**β-VLDL**		HPLC-UV	115
	Rat	Angiotensin II induced vascular dysfunction	Endothelial cells of thoracic aorta		Immunohistochemistry	116
		Autoimmune myocarditis	Damaged myocytes, macrophages	iNOS	Immunohistochemistry	117, 118
		Balloon injured arteries	Neointimal smooth muscle & endothelial cells	iNOS	Immunohistochemistry	119
		Cardiac allograft rejection	Transplanted heart, inflammatory cells, myocytes	iNOS	Immunohistochemistry HPLC-EC	120, 121
		Carbon monoxide exposure	Endothelial cells of aorta		Immunohistochemistry Solid phase ELISA	122, 123
		Haemorrhagic shock	Aorta, kidney, liver, lung, gut		Immunohistochemistry	124, 125
		Hypertension (aortic banding induced)	Aorta **(50 kDa protein)**	eNOS	Immunoblot	126
		Hypertension (Lead induced)	Plasma, kidney, heart, liver & brain homogenates		Immunoblot	127
		Hypertension (Renovascular)	Extraglomerular mesangium, wall of glomerular afferent arterioles		Immunohistochemistry	128
		Hypertension (spontaneous)	Renal cortex homogenate **(47, 58, 74, 102 kDa proteins)**		Immunoblot	129
		LPS induced shock	Aortic vessel wall	iNOS	Immunohistochemistry	130, 131
		LPS treatment i.p.	Plasma		HPLC-florescence	132
		Myocardial I/R	Ventricular wall, necrotic myocardium, myocytes, microvascular endothelial cells, lung	MPO, iNOS	Immunohistochemistry	133–135

CVS	Rat	Nitrate tolerance in hypertensive rats	Coronary sinus effluent	Lipid peroxidation	HPLC-UV	136
		TNM treated i.p.	Plasma		HPLC-EC	137
	Murine	Atherosclerosis in LDL receptor-/-mice	Macrophages, foam cells, smooth muscle cells	iNOS	Immunohistochemistry	138
		Auto-immune myocarditis	Myocardiocytes, macrophages	iNOS	Immunohistochemistry	139
Respiratory	Human	Acute lung injury	Lung interstitium, alveolar epithelial cells, proteinaceous alveolar exudate, macrophages, neutrophils, vascular endothelial cells, sub-endothelial tissues		Immunohistochemistry	7
		Acute respiratory distress syndrome with or without NO• inhalation therapy	Plasma **ceruloplasmin, transferrin, α_1-protease inhibitor, α_1-antichymotrypsin, β-chain fibrinogen** Alveolar epithelial cells & capillary endothelial cells, broncholavage fluid	MPO, *o*-tyrosine, 3-chlorotyrosine	HPLC-UV Immunohistochemistry Immunoblot Immunoprecipitation	140–142, 296
		Asthma	Airway epithelial cells, macrophages, neutrophils, eosinophils, vascular endothelial cells & smooth muscle cells, lung parenchyma	iNOS	Immunohistochemistry	143, 144
		Bronchopulmonary dysplasia in premature infants	Plasma		Solid phase ELISA	145
		Cigarette smokers	Plasma		HPLC/GC-TEA	146
		Eotaxin inhalation of patients with allergic rhinitis	Nasal epithelial cells		Immunohistochemistry	147
		Idiopathic pulmonary fibrosis	Airway & alveolar epithelial cells, macrophages, Neutrophils, vascular endothelium & smooth muscle	iNOS	Immunohistochemistry	148
		Infant respiratory failure	Airway biopsy homogenate **(15 kDa protein)**		ELISA, Immunoblot	149
		Neonatal pneumonia	Alveolar exudate	iNOS	Immunohistochemistry	150
		Obliterative bronchiolitis (lung transplant)	Epithelial cells, inflammatory cells, endothelial cells	iNOS	Immunohistochemistry	151–153
		Perennial nasal allergy	Nasal mucosa		HPLC-UV	154
	Dog	LPS induced acute lung injury	Interstitium, alveolar exudate, epithelial cells, macrophages & neutrophils, capillary wall	iNOS	Immunohistochemistry	155
	Guinea pig	Late allergic response	Microvascular endothelial cells, eosinophils	EPO	Immunohistochemistry	156

TABLE I (*Continued*)

System	Species	Disease/Condition	Location	Co-localised with or also upregulated	Detection technique	Ref.
Respiratory	Rat	Asbestos inhalation	Alveolar duct bifurcations, bronchiolar epithelial cells, alveolar macrophages, pleural mesothelium	iNOS	Immunohistochemistry ELISA	157
		Carrageenan induced pleurisy	Lung homogenates **(60, 110, 135 kDa proteins)**, pleural macrophage proteins	MPO, PARS, iNOS, Lipid peroxidation	Immunohistochemistry Immunoblot	158–163
		Heavy metal induced asthma	Neutrophils & macrophages around airways & blood vessels		Immunohistochemistry	164
		Hyperoxic lung injury	**β_1-subunit of Na^+/K^+-adenosine triphosphatase**	HOCl-modified proteins	Immunoprecipitation Immunoblot	165
		Laryngeal injury following intubation & extubation	Laryngeal mucosal & sub mucosal inflammatory cells	MPO	Immunohistochemistry	166
		LPS induced acute lung injury	Alveolar & interstitial macrophages	iNOS	Immunohistochemistry	167
		LPS lung instillation	Bronchial epithelial cells, interstitial inflammatory cells, alveolar macrophages, alveolar capillaries	Aminotyrosine, MPO	Immunohistochemistry HPLC-UV	168
		Lung I/R	Perivascular and diffuse throughout lung	Lipid peroxidation, Protein carbonyls	Immunohistochemistry Amino acid analysis	169
		Obliterative bronchiolitis (lung transplant)	Epithelial cells, fibroblasts	iNOS	Immunohistochemistry	170
		Pulmonary granulomatous inflammation	Pulmonary interstitium alveolar epithelial cells & macrophages, proteinaceous alveolar exudate, neutrophils, endothelial cells, bronchial wall	iNOS	Immunohistochemistry	171
		Radiation induced acute lung injury	Alveolar epithelial cells & macrophages	iNOS, eNOS	Immunohistochemistry HPLC-UV	172
		TNM treatment i.p.	Lung homogenate		HPLC-EC	137
	Murine	Carrageenan induced pleurisy	Alveolar macrophages, airway epithelial cells	Lipid peroxidation, iNOS, COX-2, MPO, PARS, TNFα, IL-1β,	Immunohistochemistry	173
		Herpes simplex virus-1 induced pneumonia	Lung inflammatory cells	iNOS	Immunohistochemistry	174
		Hyperoxia	Lung structural proteins		Immunohistochemistry	175
		Influenza induced pneumonia	Alveolar macrophages, neutrophils & exudate	iNOS	Immunohistochemistry	176
		Interstitial pneumonia	Alveolar macrophages		Immunohistochemistry	177
		LPS induced acute lung injury	Large airway & alveolar epithelial cells, alveolar proteinaceous exudate, macrophages, vascular cells	iNOS	Immunohistochemistry	178
		$NO^\bullet$ inhalation	Lung macrophages		Immunohistochemistry	179

GIT	Human	Celiac disease	Plasma, small intestinal crypt enterocytes	iNOS	Immunohistochemistry Sandwich ELISA	180, 181
		Colon carcinoma	Neutrophils, tissue mononuclear cells, tumour cells, surrounding macrophages & fibroblasts	iNOS, VEGF	Immunohistochemistry HPLC-UV	182–184
		Gastric cancer	Epithelial cells, inflammatory cells, extracellular matrix	iNOS	Immunohistochemistry	185
		Gastric ulcer (associated with Helicobacter pylori)	Active ulcer margins, epithelial cells, lamina propria	iNOS	Immunohistochemistry	186
		Helicobacter pylori gastritis	Epithelial cells, inflammatory cells, extra cellular matrix	iNOS	Immunohistochemistry	187
		Inflammatory bowel disease	Colonic epithelium, lamina propria macrophages & neutrophils	iNOS	Immunohistochemistry	188, 189
		Necrotizing enterocolitis	Enterocytes in apical villi	iNOS	Immunohistochemistry	190
		Oesophageal squamous cell carcinoma	Tumour cells, lymphocytes, macrophages		Immunohistochemistry	191, 192
		Pancreatic carcinoma	Tumour homogenates, **c-Src tyrosine kinase**	iNOS	Immunohistochemistry HPLC-EC, immunoblot Immunoprecipitation	193, 194
		Ulcerative colitis	Epithelial cells, lamina propria	iNOS	Immunohistochemistry	195
	Horse	Small intestine strangulation obstruction	Jejunal mucosal & submucosal leukocytes	iNOS	Immunohistochemistry	196
	Cat	LPS induced ileitis	Epithelial cells of villus tips	iNOS	Immunohistochemistry	197
	Guinea pig	Ileitis	Epithelial cells, neurons	iNOS	Immunohistochemistry	198, 199
	Rat	Acute pancreatitis	Vascular wall	iNOS	Immunohistochemistry	200
		Peritonitis	Peritoneum	iNOS, eNOS	Immunohistochemistry	201
				Plasma, exudate	HPLC-EC	202
		Colitis	Colonic mucosa	iNOS, MPO	Immunohistochemistry	203, 204
		Oesophageal adenocarcinoma	Distal oesophagus, macrophages, epithelial cells	iNOS	Immunohistochemistry	205
		Gastric I/R	Injured gastric mucosa, endothelial cells		Immunohistochemistry	206
		LPS treatment i.p.	Enterocytes at villus tips of intestinal epithelium	iNOS	Immunohistochemistry	207
		Splanchnic artery occlusion shock (I/R)	Mononuclear cells & villus wall of necrotic ileum, aortic vessel wall	Lipid peroxidation, MPO	Immunohistochemistry	208–210
	Murine	Autoimmune diabetes	Pancreatic islet β-cells & macrophages		Immunohistochemistry	211
		Colitis	Inflammatory cells, necrotic epithelial cells, mucosa, submucosa	Lipid peroxidation, iNOS, MPO, ICAM-1	Immunohistochemistry	212, 213
		Splanchnic artery occlusion shock (I/R)	Mononuclear cells of necrotic ileum	Lipid peroxidation, MPO, ICAM-1	Immunohistochemistry	214

TABLE I (*Continued*)

System	Species	Disease/Condition	Location	Co-localised with or also upregulated	Detection technique	Ref.
Hepatic	Human	Cholangiocarcinoma	Malignant bilary epithelial cells	iNOS	Immunohistochemistry	215
		Chronic hepatitis	Hepatocytes, Kupffer cells	iNOS	Immunohistochemistry	216, 217
	Rat	Hepatic allograft rejection	Hepatocytes	iNOS	Immunohistochemistry Immunoblot	218
		Liver carcinoma	Hepatocytes, connective tissue, proteinaceous fluid, sinusoidal endothelial cells	iNOS	Immunohistochemistry	219
		Liver I/R	Centrilobular region	iNOS	Immunohistochemistry	220, 221
		Liver injury during haemorrhagic shock	Kupffer cells, endothelial cells	iNOS	Immunohistochemistry	222
		Liver preservation & transplantation	Liver homogenate		HPLC-EC	223
		Perfused liver at low oxygen tension	Kupffer cells, endothelial cells		Immunohistochemistry	224
	Murine	LPS induced hepatic injury	Hepatocytes around blood vessels	iNOS	Immunohistochemistry	225
		Paracetamol (acetaminophen) toxicity	Centrilobular hepatocytes **(36, 44, 85 kDa proteins)**		Immunoblot Immunohistochemistry	226–228
Renal	Human	Chronic renal failure with septic shock	Plasma		HPLC-UV	229
		Diabetic nephropathy	Proximal & distal tubules, thin limb of loop of Henle, collecting ducts		Immunohistochemistry	230
		Renal allograft rejection	Tubular epithelial cells, **MnSOD**		Immunohistochemistry Immunoblot Immunoprecipitation HPLC-EC	231
		Uremic patients on peritoneal dialysis	Peritoneal biopsies	eNOS, VEGF	Immunohistochemistry Immunoblot	232
	Rat	Glomerulonephritis	Monocytes, neutrophils	iNOS	Immunohistochemistry	233
		LPS induced kidney damage	Endocytic lysosmes in subapical compartment of proximal tubular epithelial cells, macula densa, arterial endothelial cells & smooth muscle cells **(40, 80 kDa proteins)**	nNOS, iNOS	Immunohistochemistry Immunoblot	234, 235
		Nephrosclerosis	Interlobular arteries	Lipid peroxidation	Immunohistochemistry	236
		Renal allograft rejection	Tubular epithelial cells	iNOS	Immunohistochemistry	237
	Murine	Acute renal ischemia in osteopontin KO mice	Kidney homogenate, **(57 kDa protein)**	iNOS	Immunoblot	238
		Sickle cell disease	Tubular epithelial cells, vascular wall, kidney homogenate, **(66, 57, 22 kDa proteins)**	iNOS	Immunohistochemistry Immunoblot	239, 240

Joint	Human	Hip replacement	Pseudosynovial interface membrane, CD68 + macrophages & neighbouring cells	iNOS, COX-2	Immunohistochemistry	241, 242
		Rheumatoid arthritis	Serum, synovial fluid		HPLC-UV	8
	Dog	Experimental oesteoarthritis	Chondrocytes in cartilage, neutrophils	iNOS, IL-1β, COX-2	Immunohistochemistry	243
	Rabbit	Experimental oesteoarthritis	Inner & outer zone of knee menisci		Immunohistochemistry	244, 245
	Rat	Carrageenan induced paw oedema	Inflamed paw	Lipid peroxidation, MPO	Immunohistochemistry	246–249
		Collagen induced arthritis	Inflamed hind paw synovium	Lipid peroxidation, PARS	Immunohistochemistry	250
		Zymosan-activated plasma induced paw oedema	Inflamed paw	MPO	Immunohistochemistry	251, 252
	Murine	Collagen induced arthritis	Inflamed joint, paw extracts, **(10, 66, 80 kDa proteins)**	PARS	Immunohistochemistry Immunoblot	253, 254
Reproductive	Human	Chorioamnionitis & placental abruption	Placenta	iNOS	Immunohistochemistry	255
	Rat	L-NAME induced foetal growth retardation	Uterus at site of implantation	iNOS	Immunohistochemistry	256
		L-NAME induced limb teratogenicity	Foetal limb		Immunohistochemistry	257
Skeletal muscle	Human	Inclusion-body myositis	Vacuolated skeletal muscle fibres, paired helical filaments, vacuoles	nNOS, iNOS	Immunohistochemistry EM	258, 259
	Rabbit	Low-frequency stimulated muscle (muscle fatigue)	**SERCA**	Protein carbonyls	Immunoblot	260
	Rat	Aged rat	**SERCA-2**		Immunoblot Amino acid analysis	261, 262
		Skeletal muscle contractile dysfunction in LPS induced septic shock	Diaphragm muscle fibres, blood vessels **(50, 42, 86, 196 kDa proteins)**	Lipid peroxidation, iNOS, nNOS, eNOS	Immunohistochemistry Immunoblot	263, 264
		Skeletal muscle after I/R	Mast cells, macrophages	iNOS	Immunohistochemistry	265
Skin	Human	Anaphylactoid purpura	Skin lesion neutrophils	iNOS, IL-8	Immunohistochemistry	266
		Malignant melanoma	Melanocytes, small blood vessels	iNOS	Immunohistochemistry	267
		Systemic sclerosis	Skin endothelial cells of superficial microvessels	iNOS	Immunohistochemistry	268
	Rat	Peroxynitrite induced skin inflammation	Skin homogenate, **Albumin (66 kDa)**		ELISA, Immunoblot	269
		Random pattern skin flap & Ischemic skin flaps	Skin homogenate **(66 kDa protein)**		Immunoblot	270, 271
		Thermal injury	Villi of ileum	iNOS	Immunohistochemistry	272
			Skin homogenate		ELISA	273

TABLE I (*Continued*)

System	Species	Disease/Condition	Location	Co-localised with or also upregulated	Detection technique	Ref.
Skin	Murine	Chronic ultraviolet B exposure	Skin homogenate **(66 kDa protein)**	8-OHdG	Immunoblot	274
		Flavone acetic acid treated subcutaneous tumours	Tumour blood vessels & surrounding tumour cells		Immunohistochemistry	275
		Skin paillioma	Dermal tissue	iNOS	Immunohistochemistry	276
		Subcutaneous mutatect murine tumour	Cytoplasmic & nuclear staining throughout tumour	iNOS	Immunohistochemistry	277
Immune	Human	Systemic lupus erythematosus	Serum		Immunoblot	278, 279
		Granuloma	Granuloma epitheloid & multinucleated giant cells, endothelial cells	iNOS	Immunohistochemistry	280
	Murine	Irradiation following bone marrow transplantation	Bronchoalveolar lavage fluid **(30, 66 kDa proteins)**		Immunoblot	281
		Leishmaniasis infection	Macrophage vacuoles & parasites in footpad lesions		Immunohistochemistry	282, 283
		Lymphoma	Spleen & lymph node macrophages & surrounding cells	iNOS	Immunohistochemistry	284
Multiple	Rat	Whole body exposure to radio frequency radiation	Lung, liver, plasma proteins, distal tubule of kidney, villus tips of intestine, blood vessels of intestine		Immunohistochemistry	285
		Zymosan induced shock	Vascular wall, bronchial wall & macrophages of lung, small intestine, liver, aorta	Lipid peroxidation, MPO	Immunohistochemistry	286–292
	Murine	LPS induced shock	Intestine, aorta, lung	iNOS	Immunohistochemistry	293, 294
		Zymosan induced shock	Lung	Lipid peroxidation, MPO	Immunohistochemistry	295

8OHdG = 8-hydroxy-2-deoxyguanosine; AIDS = acquired immunodeficiency syndrome; ALS = amyotrophic lateral sclerosis; BBB = blood brain barrier; CNS = central nervous system; COX-2 = cyclooxygenase-2; CSF = cerebral spinal fluid; CVS = cardiovascular system; ELISA = enzyme linked immunoadsorbent assay; EM = electron microscopy; EPO = eosinophil peroxidase; GCMS = gas chromatography mass spectrometry; GC-TEA = gas chromatography thermal energy analysis; GIT = gastrointestinal tract; HO-1 = hemoxygenase-1; ICAM-1 = intracellular adhesion molecule-1; IFNγ = interferon γ; IL-1β = interleukin-1β; IL-8 = interleukin-8; i.p. = intra-peritoneal; I/R = ischaemia reperfusion; LDL = low density lipoprotein; LPS = lipopolysaccharide; MnSOD = manganese superoxide dismutase; MPO = myeloperoxidase; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; nNOS, eNOS, iNOS = neuronal, endothelial, inducible nitric oxide synthase; NMDA = *N*-Methyl-D-Aspartate; PARS = poly-ADP-ribose synthase; PGI_2 synthase = prostacyclin synthase; PNS = peripheral nervous system; SERCA2 = sarcoplasmic reticulum calcium ATPase-2; TNFα = tumour necrosis factor α; VEGF = vascular endothelial growth factor.

TABLE II Detection of nitrotyrosine in cellular models of disease (specific nitrated proteins are highlighted in bold)

System	Cell type	Exposure to	Protein target/degree of nitration	Detection technique	Ref.
CNS	Astrocytes	Glucose deprivation, SIN-1	Nuclear & cytosolic proteins	Immunocytochemistry	297
		Peroxynitrite	**Glyceraldehyde-3-phosphate dehydrogenase** (inactivated the enzyme)	Immunoprecipitation	298
		IL-1β, IFNγ	Time dependent increase of nitrated proteins up to 7 days	Immunocytochemistry	299, 300
	Hippocampal neurons	$FeSO_4$, Amyloid β peptide	Perinuclear proteins of cell body, neurites	Immunocytochemistry	301
	Mesencephalic cells	Peroxynitrite	30, 70 kDa proteins	Immunoblot	302
	Motor neurons	Brain derived neurotrophic factor deprivation	Cytosolic & nuclear proteins	Immunocytochemistry	303
			Time dependent increase in nitrotyrosine after 8–50 h of deprivation	Immunocytochemistry	304
	Neuroblastoma SH-SY5Y cells	Peroxynitrite	10 cytosolic proteins (25–180 kDa)	Immunoblot	305
		SIN-1	**Focal adhesion protein p130cas** (prevented phosphorylation of protein)	Immunoblot	306
	PC-12 (pheochromocytoma) cells	Methamphetamine	16 fold increase in protein nitration	HPLC-EC	72
		Amyloid β peptide	Cytosolic proteins	Immunocytochemistry	307
CVS	Aortic smooth muscle cells	Peroxynitrite Zymosan activated plasma	60, 110, 135 kDa proteins	Immunoblot	130, 308
	EaHy 926 endothelial cells	Peroxynitrite	**PGI_2 synthase** (inactivated the enzyme)	Immunoprecipitation	309
	HUVEC	Native LDL	50% increase protein nitration from control	ELISA	310
		Peroxynitrite	60 kDa protein	Immunoblot	130
	Platelets	Collagen	**VASP (vasodilator-stimulated phosphoprotein)**	ELISA, Immunoblot	311
		Peroxynitrite, HOCl + nitrite, Nitrogen dioxide	Increase in protein nitration; 3 fold with HOCl + nitrite, 75 fold with Peroxynitrite, 150 fold with nitrogen dioxide exposure	GCMS	312
		Peroxynitrite	**COX** (inactivated the enzyme)	GCMS, Immunoblot	313
Respiratory	A549 alveolar type II epithelial cells	Free 3-nitrotyrosine	**α-tubulin** (changed microtubule organisation, altered cell morphology and increased epithelial permeability)	Immunoblot Immunocytochemistry	314
		SNAP	25, 35 kDa proteins	Immunoblot Immunocytochemistry	315
	Alveolar macrophages	LPS	Cytosolic proteins	Immunocytochemistry	316
	Lung epithelial LA-4 cells	TNFα, IL-1β, IFNγ	Proteins from cell extract and cell culture supernatant	ELISA	317
	Lung fibroblasts	Hyperoxia & $NO^{\bullet}$ exposure	Cell protein extracts	Immunoblot	318
	Neutrophils	TNFα, IL-1β, IFNγ	Proteins from cell extract and cell culture supernatant	ELISA	317
	Pleural macrophages	Carrageenan	60, 110, 135 kDa proteins	Immunoblot	319

TABLE II (*Continued*)

System	Cell type	Exposure to	Protein target/degree of nitration	Detection technique	Ref.
Respiratory	Pleural mesothelial cells	IL-1β, Asbestos fibres	Cytosolic proteins	Immunocytochemistry	320
	Pulmonary artery endothelial cells	Plasma from patients with acute chest syndrome	Perinuclear proteins	Immunocytochemistry	321
		TNFα	Cytoskeletal proteins	Immunocytochemistry	322
		Carbon monoxide	2 fold increase in nitrated proteins	Solid phase ELISA	323, 324
		SIN-1	**80, 90, 100 kDa proteins** (reduced tyrosine phosphorylation of nitrated proteins)	Immunoblot	325
	Pulmonary microvascular smooth muscle cells	IL-1β	Cytoskeletal proteins	Immunocytochemistry	326
GIT	Colon HT-29 epithelial cells	Bile salts	Plasma membrane associated proteins	Immunoblot	327
	Pancreatic carcinoma cells	Peroxynitrite	**c-Src tyrosine kinase** (increased activity)	Immunoblot immunoprecipitation	194
Hepatic	Hepatocytes	SIN-1, SNAP	Perinuclear proteins	Immunocytochemistry	328
	WB-F344 epithelial cells	Peroxynitrite	75, 85 kDa proteins	Immunoblot	329
Renal	Kidney tubular epithelial cells BSC-1	H_2O_2	Cytosolic proteins	Immunocytochemistry	330
	Mesangial cells	IL-1β, SIN-1, Peroxynitrite	**PGI_2 synthase** (inactivation of enzyme)	Immunoblot Immunoprecipitation	331
	nNOS tranfected kidney 293 cells	A23187, L-arginine deficiency	Cytosolic proteins	Immunocytochemistry	332
Immune	Adenocarcinoma cells	Peroxynitrite	Nuclear & cytosolic proteins	Immunocytochemistry Solid phase ELISA	333
	MCF-7 (breast cancer cells)	GSNO	**Tumour suppressor p53 protein**	Immunoprecipitation Immunoblot	334
	Peritoneal macrophages	IFNγ + lipoarainomannan, LPS	3 fold (IFNγ) and 7 fold (LPS) increases in nitrated proteins	ELISA	335
	Phagocytosing Neutrophils	IL-1β, IFNγ, TNFα	Nitration of ingested bacteria	Immunocytochemistry	336
	Polymorphonuclear leukocytes (activated)	Calcium ionophore A23187	2 fold increase in nitrated proteins	HPLC-UV	337
	RAW 264.7 macrophages	GSNO	**Cytochrome *c***	Immunoprecipitation Immunoblot	338
		IFNγ, LPS	10 fold increase in nitrated proteins	Immunocytochemistry Flow cytometry	339
		LPS,IFNγ, L-arginine deficiency	Cytosolic proteins	Immunocytochemistry	340
		Peroxynitrite Spermine NONOate	**p85 regulatory subunit of phosphatidylinositol 3-kinase** (dissociation of the p110 catalytic subunit)	Immunoblot Immunoprecipitation	341
		Peroxynitrite	Cytosolic proteins	Immunoblot	342
		Zymosan	5 fold increase in nitration	HPLC-EC/UV	202
	T-Lymphocytes	Anti-CD3 monoclonal antibody activation	**8 proteins between 40–120 kDa** (inhibition of protein tyrosine phosphorylation)	Immunoblot Immunocytochemistry Flow cytometry	343, 344

$FeSO_4$ = Iron sulfate; HOCl = hypochlorous acid; HUVEC = human umbilical vein endothelial cell; SIN-1 = 3-morpholinosydnonimine; SNAP = S-nitroso-*N*-acetyl-penicillamine-amine; GSNO = S-nitrosoglutathione.

tyrosine, but provide a means to localise nitrated proteins within tissues and also aid the identification of specific nitrated proteins. By comparison ELISA allows simple semi-quantitative measurement of nitrated protein in body fluids or tissue homogenates with a higher throughput than HPLC or GC-MS. The analytical techniques are required to allow true quantitative measurement of nitrated tyrosine in the free or protein form. However, determining levels of protein nitration by HPLC and GC-MS methodologies requires protein hydrolysis, which is generally carried out under acid conditions. This may cause complications as Shigenaga and co-workers (1997) showed that artefactual 3-nitrotyrosine is generated from the acid hydrolysis of proteins when nitrite is present in samples.[202] Since $NO^{\bullet}$ and other RNS are eventually oxidised to nitrite, samples that may contain 3-nitrotyrosine may also be likely to have high levels of nitrite present. To overcome the potential hazards of artificial nitration, alkaline rather than acid hydrolysis can be employed.[347]

Of the 285 references cited in Table I the majority (66%) employed immunohistochemistry to detect 3-nitrotyrosine with 13% using western blot, 3% ELISA, 2% immunoprecipitation followed by western blot, 11% HPLC-EC, 3% HPLC-UV, 2% GC-MS and 4% used a combination of both immunological and analytical techniques. Of the immunological techniques 65% utilised the polyclonal antibody, 25% used the monoclonal and 4% used both, whereas 6% did not specify. Of those studies that employed both analytical and immunological techniques[15,36,52,59,62,64,81,121,168,169,172,231] there were some apparent discrepancies in the detection of nitrated protein by the different methods. For example, Sakurai *et al.* (1998) using HPLC-EC showed that after spinal cord ischaemia there was a transient increase in nitrated protein detected around 8 hours post reperfusion in the ventral part of the spinal cord, whilst immunohistochemistry of the same area of spinal cord showed that nitrated proteins were generated after 8 hours but persisted for as long as 7 days after reperfusion.[36] These apparent conflicts may reflect the differences in the sensitivity and selectivity of the methodologies. Nonetheless, the utilisation of both immunological and analytical techniques may be the best approach as the two methods compliment each other by providing information on the location, specific protein and magnitude of nitration. There have been some problems, both reported and unpublished, with using the monoclonal antibody for some of the immunological techniques[348] since it does not always work well for western blots or immunoprecipitation. This may be due to the fact that the antibody was raised against nitrated keyhole limpet hemocyanin (KLH) with clones screened with nitrated BSA and hence it recognises only few particular nitrotyrosine epitopes on a globular protein very well, but has a narrow binding affinity for other nitrotyrosine epitopes. The polyclonal antibodies, which by their nature recognise several epitopes, have been used with more success, as is demonstrated by their use in the majority of studies that report the detection of nitrated proteins. However, with its broader specificity it may, in addition to nitrated tyrosine, recognise with lesser affinity other similar protein modifications such as *o*-tyrosine. The specificity of the antibodies therefore lies with the use of appropriate controls such as reduction with dithionite (the antibody does not react with aminotyrosine) or competition with 3-nitrotyrosine. Moreover, future approaches will probably include the development of monoclonal antibodies to specific nitrated proteins of interest as has been recently accomplished for α-synuclein.[349]

NITRATION UNDER BASAL CONDITIONS

In addition to the diseases outlined in Table I, 3-nitrotyrosine has been detected during apparently normal physiological conditions. This phenomenon has become apparent, as detection techniques have become more sensitive. Indeed both analytical

TABLE III Detection of nitrated proteins under physiological conditions

System	Species	Location	Detection technique	Ref.
CNS	Human	Spinal cord (**Neurofilament L**)	Immunoblot	14
		Purkinje cells of cerebellum, choroid plexus, cortical neurons	Immunohistochemistry	350
	Rat	Somata and dendrites of interneurons and spiny neurons of caudate-putamen nucleus, outer mitochondrial membranes, near plasma membranes in dendrites and within asymmetric synapses on dendritic spines, globus pallidus, astrocytes, small axons and synaptic vesicles in axon terminals	Immunohistochemistry EM	351
	Murine	Spinal cord (**Neurofilament L, Glial fibrillary acidic protein**)	Immunohistochemistry Immunoblot	59
CVS	Human	Plasma (**Albumin, 58 kDa protein**)	ELISA, Immunoblot	352
	Rat	Mesenteric artery (**60–65 kDa protein**)	Immunoblot	353
GIT	Human	Basal cells of oral mucosa	Immunohistochemistry	191
Immune	Murine	Cortico-medullary junction & medulla of thymus	Immunohistochemistry	354
PNS	Rat	Sciatic nerve (**Glial fibrillary acidic protein**)	Immunoblot	87
Renal	Human	Distal tubules, collecting ducts	Immunohistochemistry	230
	Rat	Proximal & convoluted tubules, endothelial cells of vas recta (**40, 47, 58, 74, 80, 89, 102 kDa proteins**)	Immunohistochemistry Immunoblot	129 234
	Murine	Kidney (**66 kDa protein**)	Immunoblot	239
Reproductive	Quail	Ovarian atretic follicles & post-ovulatory follicles	Immunohistochemistry	355
Respiratory	Rat	**β_1-subunit of Na^+/K^+-adenosine triphosphatase**	Immunoprecipitation Immunoblot	165
Skeletal muscle	Rat	Diaphragm (**50, 42 kDa proteins**)	Immunohistochemistry Immunoblot	263

and immunological techniques show basal levels of nitrated proteins (Tables III–IV) in nervous tissue (brain, spinal cord, peripheral nerve), blood vessels, heart, lung, liver, kidney, pancreas, skeletal muscle, skin, oral mucosa, thymus, ovaries and body fluids such as plasma and CSF.

The quantities of 3-nitrotyrosine measured under normal conditions vary, depending on species, or the type of tissue or body fluid (Table IV). Generally levels of free 3-nitrotyrosine are higher (as a molar percentage of tyrosine) than protein 3-nitrotyrosine levels. This may reflect that protein tyrosine nitration is a more selective process since particular tyrosines are protected from nitration because of the tertiary structure of the protein or the hydrophobicity of particular domains, preventing access to nitrating agents such as peroxynitrite.[360] For example studies using GC-MS and HPLC-EC techniques report plasma levels of free 3-nitrotyrosine at least 30 times higher than protein 3-nitrotyrosine (ranging from 930 to 1000 μmol/mol tyrosine compared to 1–36 μmol/mol tyrosine respectively). In addition to differences between free and protein levels of 3-nitrotyrosine, variations of each form exist between tissues and in the case of the central nervous system between specific regions of brain or spinal cord.

At the cellular level, under basal conditions *in vivo*, nitrated proteins have been localised

TABLE IV Quantification of nitrotyrosine under normal physiological conditions, in human disease and in animal models of disease

System	Species	Disease/Condition	Free or protein	Location	Normal	Diseased	Detection technique	Ref.
CNS	Human	ALS Sporadic & Familial	Free	Lumbar spinal cord	3 ± 1 mmol/mol Tyr	5 ± 1 mmol/mol Tyr	HPLC-EC	15
		Alzheimer's disease	Protein	Hippocampus	0.5 ± 0.2 mmol/mol Tyr	1.7 ± 0.5 mmol/mol Tyr		
				Inferior parietal lobe	0.6 ± 0.2 mmol/mol Tyr	3 ± 0.9 mmol/mol Tyr		
				Superior/middle temporal gyri	3 ± 0.9 mmol/mol Tyr	16 ± 4 mmol/mol Tyr	HPLC-EC	21
				Ventricular CSF	3 ± 1 mmol/mol Tyr	No change		
			Free	CSF	0.3 ± 0.1 mmol/mol Tyr (1.6 ± 0.4 nM)	1.9 ± 0.9 mmol/mol Tyr (11.4 ± 5.4 nM)	HPLC-EC	25
		Normal	Free	Cerebrum	1.6 ± 0.3 mmol/mol Tyr		HPLC-EC	356
				Cerebellum	0.6 ± 0.4 mmol/mol Tyr			
	Murine	ALS mice (express human mutant Cu/Zn SOD-G37R)	Free	Lumbar spinal cord	15 ± 2 mmol/mol Tyr	30 ± 5 mmol/mol Tyr		59
				Cervical spinal cord	17 ± 1 mmol/mol Tyr	28 ± 2 mmol/mol Tyr	HPLC-EC	
				Brain stem	22 ± 1 mmol/mol Tyr	28 ± 3 mmol/mol Tyr		
		ALS mice (express human mutant Cu/Zn SOD-G93A)	Free	Lumbar spinal cord	20 ± 2 mmol/mol Tyr	41 ± 10 mmol/mol Tyr	HPLC-EC	61
		Apo E-deficient mice	Free	Cortex	13 ± 1 mmol/mol Tyr	20 ± 1 mmol/mol Tyr		
				Hippocampus	14 ± 1 mmol/mol Tyr	19 ± 1 mmol/mol Tyr	HPLC-EC	63
				Brain stem	23 ± 2 mmol/mol Tyr	35 ± 2 mmol/mol Tyr		
				Cerebellum	22 ± 1 mmol/mol Tyr	34 ± 1 mmol/mol Tyr		
		Cerebral I/R	Both	Cortex	Not detected	0.4 ± 0.3 mmol/mol Tyr	HPLC-EC	64
		Malonate neurotoxicity	Free	Striatum	0.6 ± 0.1 mmol/mol Tyr	1.3 ± 0.3 mmol/mol Tyr	HPLC-EC	71
					3.3 ± 0.8 mmol/mol Tyr	5.8 ± 1 mol/mol Tyr	HPLC-EC	70
		Methamphetamine neurotoxicity	Protein	Striatum	0.11 ± 0.01 mmol/mol Tyr	0.19 ± 0.02 mmol/mol Tyr	HPLC-EC	72
		MPTP model of Parkinson's disease	Free	Striatum	6 ± 1 mmol/mol Tyr	13 ± 3 mmol/mol Tyr	HPLC-EC	75
					4 ± 1 mmol/mol Tyr	9 ± 2 mmol/mol Tyr	HPLC-EC	76
					4 ± 0.5 mmol/mol Tyr	7 ± 0.5 mmol/mol Tyr	HPLC-EC	77
			Protein	Striatum	0.15 mmol/mol Tyr	0.32 mmol/mol Tyr	GCMS	79
				Midbrain	0.08 mmol/mol Tyr	0.15 mmol/mol Tyr		
		NMDA induced excitotoxicity	Free	Striatum	5 ± 1 mmol/mol Tyr	10 ± 1 mmol/mol Tyr	HPLC-EC	81

TABLE IV (*Continued*)

System	Species	Disease/Condition	Free or protein	Location	Normal	Diseased	Detection technique	Ref.
CNS	Rat	3-nitropropionic model of Huntington's disease	Free	Striatum	2.8 ± 0.5 mmol/mol Tyr	5 ± 0.5 mmol/mol Tyr	HPLC-EC	38
		Cerebral I/R	Both	Cerebrum	Not detected	Peri-infarct 9.5 ± 3.4 mmol/mol Tyr Infarct 5.2 ± 3.4 mmol/mol Tyr	HPLC-EC	40
		Carbon monoxide poisoning	Protein	Brain	7 ± 0.5 pmol/mg protein	44 ± 8 pmol/mg protein	Solid phase ELISA	44
		MPTP model of Parkinson's disease	Free	Striatum	2.3 ± 0.3 mmol/mol Tyr	3.3 ± 0.2 mmol/mol Tyr	HPLC-EC	49
		NMDA induced neurotoxicity	Free	Striatum	1.4 ± 0.6 mmol/mol Tyr	2.9 ± 0.4 mmol/mol Tyr	HPLC-EC	52
GIT	Human	Celiac disease	Protein	Plasma	Not detected	1.27 ± 1.03 μM	ELISA	181
		Pancreatic carcinoma	Protein	Pancreatic tumour	0.5 mmol/mol Tyr	25.5 ± 13 mmol/mol Tyr	HPLC-EC	194
	Rat	Zymosan induced peritonitis	Protein	Plasma Peritoneal exudate	0.4 ± 0.3 μmol/mol Tyr	12.5 ± 3.1 μmol/mol Tyr 14.1 ± 2.3 μmol/mol Tyr	HPLC-EC	202
Joint	Human	Rheumatoid arthritis	Free	Serum Synovial fluid	Not detected Not available	490 ± 270 nM 490 ± 260 nM	HPLC-UV	8
CVS	Human	Atherosclerosis	Protein	Plasma LDL Lesion LDL	9 ± 7 μmol/mol Tyr	840 ± 140 μmol/mol Tyr	GCMS	98
		Coronary bypass graft	Protein	Plasma	3 ± 1 nmol/mg protein	14 ± 5 nmol/mg protein	ELISA	100
				Platelets	6.2 ± 2.6 pmol/mg protein		GC-MS	312
		Normal	Protein	Plasma	7 ± 1.2 mmol/mol Tyr		HPLC-UV	337
					2.3 μmol/mol Tyr		HPLC-EC	223
					1 μmol/mol Tyr		HPLC-EC	202
					5.4 ± 0.9 pmol/mg protein		ELISA	352
					35.4 μmol/mol Tyr (11.9 ± 1.8 pmol/mg protein)		GC-MS	347
			Free	Plasma	930 μmol/mol Tyr (63.4 ± 3.1 nM)		GC-MS	347
					2.8 nM		GC-MS	357
					31 ± 2 nM		HPLC-UV	358
	Dog	Cytokine induced myocardial dysfunction	Both	Myocardium	2.5 ± 1 mmol/mol Tyr	8 ± 2 mmol/mol Tyr	HPLC-UV	111
	Rat	LPS treatment i.p.	Free	Plasma	1 mmol/mol Tyr 105 ± 37 nM	6 mmol/mol Tyr 600 ± 150 nM	HPLC-florescence	132
		TNM treated i.p	Protein Free	Plasma	57 ± 23 μmol/mol Tyr Not detected	336 ± 24 μmol/mol Tyr 1 μM	HPLC-EC	137

Respiratory	Human	Acute respiratory distress syndrome	Protein	Broncholavage fluid	0.29 ± 0.29 nmol/mg protein	2.21 ± 0.65 nmol/mg protein	HPLC-UV	142
		Bronchopulmonary dysplasia in premature infants	Protein	Plasma	0.6 ± 0.1 pmol/mg protein	3.5 ± 0.9 pmol/mg protein	Solid phase ELISA	145
		Cigarette smokers	Protein	Plasma	0.6 ± 0.4 pmol/mg protein	3.9 ± 1.2 pmol/mg protein	HPLC/GC-TEA	146
		$NO^{\bullet}$ inhalation therapy for acute respiratory distress syndrome	Protein	Broncholavage fluid	0.4 ± 0.15 nmol/mg protein	6.76 ± 2.79 nmol/mg protein	HPLC-UV	141
		Perennial nasal allergy	Protein	Nasal mucosa	Not detected	5.8 mmol/mol Tyr	HPLC-UV	154
	Rat	Asbestos inhalation	Protein	Lung	31 ± 3.6 pmol/mg protein	84 ± 12 pmol/mg protein	ELISA	157
		LPS lung instillation	Both	Lung	Not detected	1.6 ± 0.6 mmol/mol Tyr	HPLC-UV	168
		Lung I/R	Protein	Lung	0.6 ± 0.2 mmol/mol Tyr	1 ± 0.1 mmol/mol Tyr	Amino acid analysis	169
		Radiation induced acute lung injury	Both	Lung	Not detected	3.2 ± 0.5 mmol/mol Tyr	HPLC-UV	172
		TNM treatment i.p.	Protein	Lung	22 ± 18 µmol/mol Tyr	79 ± 11 µmol/mol Tyr	HPLC-EC	137
Hepatic	Rat	Liver preservation & transplantation	Protein Free	Liver	9.5 ± 1.1 µmol/mol Tyr 15.7 ± 0.3 µmol/mol Tyr (immediate transplant)	27.5 ± 0.7 µmol/mol Tyr 23.6 ± 2.5 µmol/mol Tyr (6 h preservation before transplant)	HPLC-EC	223
Renal	Human	Chronic renal failure (RF) with septic shock	Free	Plasma	Not detected	16 mmol/mol Tyr (RF) (28 ± 12.3 µM) 55 mmol/mol Tyr (RF + shock) (118.2 ± 22 µM)	HPLC-UV	229
Multiple	Rat	Ageing	Protein	Liver Heart Skeletal muscle	350 ± 100 µmol/mol Tyr 110 ± 10 µmol/mol Tyr 45 ± 5 µmol/mol Tyr	No change during ageing	GC-MS	359

immunologically in neurons,[350,351] astrocytes,[351] endothelial and epithelial cells,[129,230,234] and analytically quantified in platelets.[312] Electron microscopy with immunogold complexes has recently shown the subcellular localisation of nitrated proteins in the cell bodies and axons of neurons from the caudate putamen nucleus and globus pallidus.[351] In this study nitrated proteins were associated with, or close to, organelles including the nuclear membrane, the nucleolus, the outer mitochondria membrane, microtubules, tubovesicles or synaptic vesicles, and at the plasma membrane where synaptic input is received from dendritic processes.[351] The protein nitration appeared to be confined to particular organelles and this varied depending on nerve type suggesting that nitration may play a role in specific organellular functions. These may include modulation of gene expression, axonal transport or synaptic transmission/integration with the degree of nitration correlating with the type or function of the cell. In addition to normal brain function nitration may also be important in ovulation as indicated by the finding of nitrated proteins in atretic and post-ovulatory follicles.[355]

In addition to the tissue, cellular and organellular distribution of nitrated proteins at least three specific nitrated proteins have been identified under normal conditions including neurofilament L,[14,59] glial fibrillary acidic protein[59,87] and the Na^+/K^+ ATPase pump.[165] Another 10 proteins with molecular masses between 40 and 100 kDa have shown to be nitrated but their identities are still to be determined.

TYROSINE NITRATION IN DISEASE

Depending on the disease and tissue, a 2–10 fold increase in the magnitude of protein 3-nitrotyrosine and a quite consistent 1.5–2 fold increase in the nitration of free tyrosine has been reported. Some reports that measure both free and protein 3-nitrotyrosine also suggest that in certain diseases, for example in rheumatoid arthritis,[8] (S. Greenacre and M. Frost, unpublished observations), or a murine model of ALS[59] there are elevations in free levels but no changes in protein levels of 3-nitrotyrosine, again suggesting that protein nitration is a selective process.

There are several disagreements in the literature where some have detected elevated levels of 3-nitrotyrosine in a particular condition whilst others have not. These include diseases or conditions such as atherosclerosis,[361] AIDS dementia complex,[362] aged skeletal muscle,[359] spinal cord ischaemia,[363] hyperoxic lung injury[364] and $NO^\bullet$ inhalation therapy for premature infants.[365,366] Differing sensitivities of detection techniques may account for the differences in some but not necessarily all of these examples.

Elevation of nitrated proteins during disease has been detected in tissues of all the major organs and within most cells types *in vivo* (Table I) including inflammatory cells (neutrophils, eosinophils, mast cells, lymphocytes, macrophages, monocytes, Kupffer cells, astrocytes), vascular cells (endothelial cells, smooth muscle cells) and parenchymal cells (neurons, Schwann cells, myocytes, fibroblasts, chondrocytes, hepatocytes, melanocytes, epithelial cells). Immunohistochemical studies show that during disease processes protein nitration occurs in specific cell types depending on the tissue or type of pathology. In some diseases only parenchymal, or only inflammatory, or only vascular cell proteins are nitrated, yet in other diseases, protein nitration occurs within several cell types. These observations allow us to propose that the site of nitrating agent(s) generation may determine the cell type and specific protein(s) that is modified by nitration. For example we argued that sites of $O_2^{\bullet-}$ production could provide grounds for peroxynitrite formation whereas the presence of inflammatory cells will provide enzymatic catalysts such as peroxidases. The list of specific proteins that are post-translationally modified by nitration is growing and clearly indicate a biological selectivity. Mn superoxide dismut-

ase,[10,231] neurofilament L,[14] α-synuclein,[349] ceruloplasmin, transferrin, α_1-anti-chymotrypsin, α_1-protease inhibitor, β-chain of fibrinogen[296] and c-Src tyrosine kinase[194] have been found nitrated in human pathologies. Furthermore, tyrosine hydroxylase,[80] neurofilament L,[59] glial fibrillary acidic protein,[59] LDL,[98] PGI_2 synthase,[110] sarcoplasmic reticulum calcium ATPase,[260–262] β_1 subunit of Na^+/K^+-adenosine triphosphatase[165] and albumin[269] are proteins found nitrated in animal models of disease.

In cellular models of disease (Table II) nitrated proteins have also been detected when specific cell types were deprived of glucose, trophic factors or L-arginine[297,303,304,332] or exposed to proteins such as cytokines (IL-1β, TNFα, IFNγ),[299,300,317,320,322,326,331,336] collagen,[311] amyloid β-peptide,[301,307] LDL,[310] or drugs such as methamphetamine[72] or the calcium ionophore A23187,[337] or biological extracts from sea weed (carrageenan),[319] yeast (zymosan)[202] or bacteria (LPS).[316,335,339,340] Protein nitration is also induced in certain cells exposed to CO,[323,324] GSNO,[334] hyperoxia,[318] hydrogen peroxide,[330] asbestos fibres[320] or bile salts.[327] Specific nitrated proteins identified within cellular models of disease includes; glyceraldehyde-3-phosphate dehydrogenase (astrocytes),[298] PGI_2 synthase (endothelial[309] and mesangial cells[331]), focal adhesion protein p130cas (neuroblastoma cells),[306] vasodilator-stimulated phosphoprotein[311] and cycloxygenase[313] in platelets, α-tubulin, (epithelial cells),[314] c-Src tyrosine kinase[194] and tumour suppressor p53[334] in tumour cells, and cytochrome *c*[338] and phosphatidylinositol 3-kinase p85[341] in macrophages.

NITRATING AGENTS

Several mechanisms have been proposed for protein nitration *in vivo* and evidence supporting or contradicting the roles of specific reactive nitrogen species has been examined previously.[367] These mechanisms have been investigated mainly *in vitro* with BSA or free tyrosine. It is likely that nitration mechanisms are totally dependent on a source of enzymatic $NO^\bullet$ and its reactions with oxygen or reactive oxygen species (Figure 1).

Nitric oxide ($NO^\bullet$) Nitric oxide can react with the tyrosyl radical to form 3-nitrotyrosine. This reaction has been proposed for the *in vitro* nitration of prostaglandin H synthase-2 (PGH-2 or cyclooxygenase) in the presence of arachidonic acid and the $NO^\bullet$ donor DEA/NO.[368,369] This nitration has been reported during normal activity of the enzyme for eicosanoid generation.

Peroxynitrite ($ONOO^-$) Peroxynitrite is an effective nitrating agent and the yield of peroxynitrite-mediated nitration is increased upon catalysis by transition metals,[370] CO_2[371] and myeloperoxidase.[372] Despite previous reports, it is clear now that the *in situ* generation of peroxynitrite by the simultaneous generation of $NO^\bullet$ and $O_2^{\bullet-}$ generates the same yield of tyrosine nitration as the bolus addition of chemically synthesised peroxynitrite.[373,374]

Nitrite (NO_2^-) Nitrite is the major stable end product of nitric oxide metabolism. Several interactions between NO_2^- and other reactants can lead to the formation of nitrating agents. Acidification of NO_2^- forms HNO_2 (nitrous acid), which has been shown over a 24–48 h period to nitrate ovalbumin and casein.[375] In addition to acidification, NO_2^- can be oxidised by myeloperoxidase (MPO) – derived hypochlorous acid (HOCl) to form nitryl chloride (NO_2Cl), which is also capable of nitrating tyrosine residues in BSA[376] and LDL.[377] However other studies report very low yields or no nitration when HOCl and nitrite are co-incubated with plasma proteins,[378] heart homogenate proteins[379] or pure ribonuclease A.[360] Alternatively, protein nitration by the oxidation of NO_2^- by hydrogen peroxide (H_2O_2) can occur via the formation of peroxynitrous acid, but requiring concentrations of H_2O_2 in excess of that likely to be produced *in vivo*. Finally it has been shown that peroxidase enzymes (myelo-, eosinophil-, horse

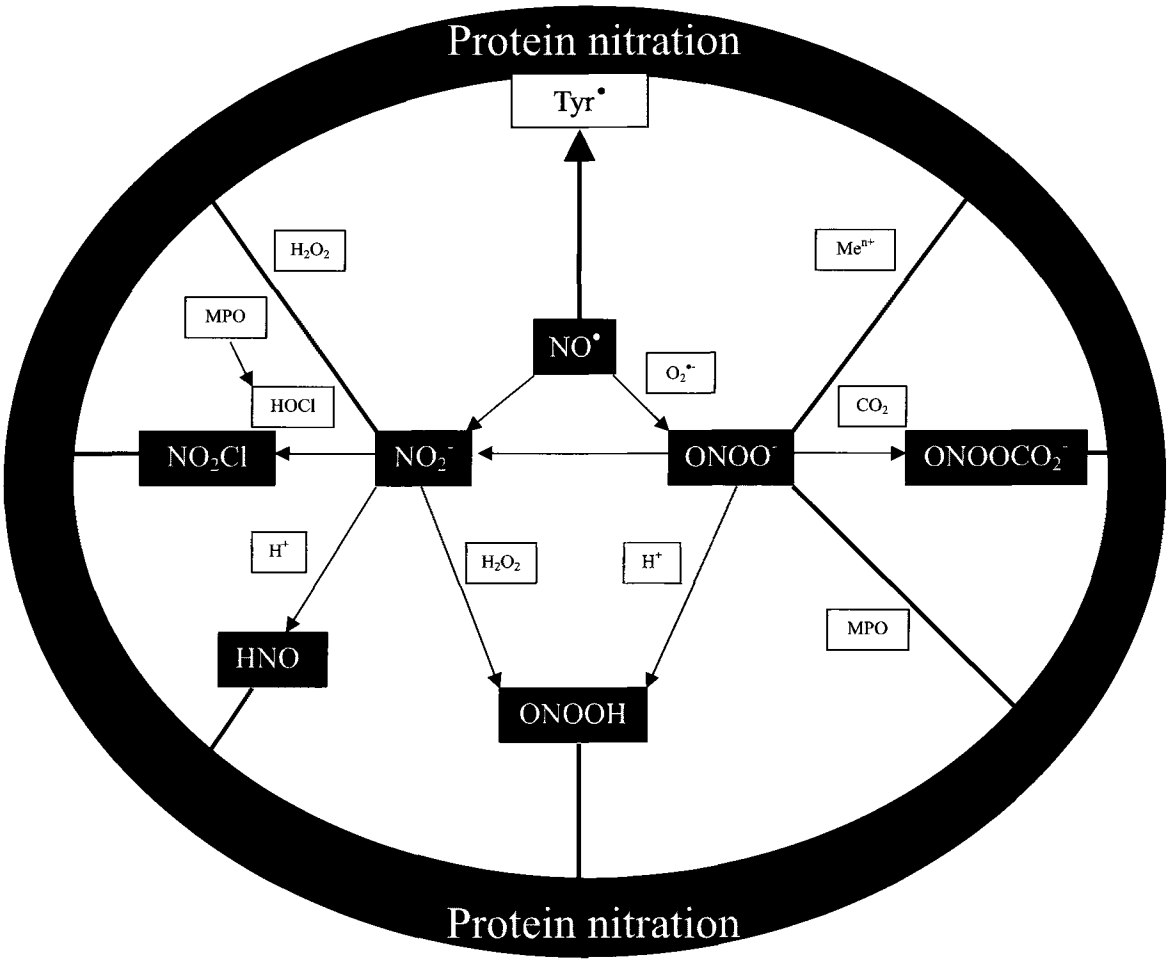

FIGURE 1 Schematic illustration of potential nitrating agents.

radish- peroxidase) in the presence of NO_2^- and H_2O_2 can nitrate proteins in heart homogenates[379] or pure proteins such as BSA,[379–382] ribonuclease A, phospholipase A_2, lysozyme[360] or LDL.[383]

SELECTIVITY OF PROTEIN TYROSINE NITRATION

As previously mentioned several nitrated proteins have been identified *in vivo* both under physiological and pathological conditions. These studies indicate that protein tyrosine nitration is a selective process. The selectivity appears to be a function of the structure of the protein and is independent of the nature of the proximal nitrating agent, the abundance of the protein and the number of tyrosine residues.[360] Berlett *et al.* (1996) first suggested that the surface exposure of certain tyrosine residues is a requirement but not essential for nitration of tyrosine residues in glutamine synthase.[384] Similarly Riordan *et al.* (1967) had indicated the existence of environmentally sensitive tyrosine residues in proteins nitrated by TNM.[385] Following these observations we provided evidence that irrespective of the nature of the nitrating agent, certain tyrosine residues are exquisitely suscep-

TABLE V Post-translational modifications of protein tyrosine residues

Post-translational modification	Enzyme	Consensus Sequence	Reversibility	Function
Phosphorylation	Tyrosine Kinases	[Lys-Arg]-X-X-X-[Glu-Asp]-X-X-X-**Tyr** [387]	Tyrosine Phosphatases	Signal transduction
Sulfation	Tyrosylprotein sulfotransferase	None, but certain structural requirements have been identified [386]	Irreversible, resistant to Chymotrypsin [386]	Protein targeting/ processing
Oxidation Chlorination Bromination	MPO/EPO and non-enzymatic	Not known	Degradation by 20S Proteasome [388]	Covalent cross linking Alterations in function
Nitration	Non-enzymatic MPO/EPO	None, but certain structural requirements have been identified [360, 389]	Potential "denitrase" [390] Degradation by proteasome, Chymotrypsin sensitive [391]	Alterations in function Signal transduction?

tible to nitration. Examination of the factors that may explain this sensitivity revealed similarities between nitration and other post translational modifications of protein tyrosine residues namely sulfation and phosphorylation, which are reviewed below and in Table V.

EFFECTS ON SIGNAL TRANSDUCTION

Tyrosine phosphorylation is one of the most recognized signal transduction events in biology. Tyrosine kinases catalyze the transfer of phosphate to the hydroxyl group of tyrosine residues and phosphatases are responsible for executing the reverse reaction. Sulfation takes place at the same ipso hydroxyl group of tyrosine and is catalyzed by the tyrosyl protein sulfotransferase.[386] In contrast to phosphorylation, sulfation is not reversible and sulfated proteins are resistant to chymotrypsin.[386] The specificity in tyrosine phosphorylation is derived by the recognition of a [Lys or Arg]-X-X-X-[Asp or Glu]-X-X-X-Tyr sequence (X indicates any other amino acid) motif on the target protein.[387] Without this motif and proper folding tyrosine kinases will not phosphorylate tyrosine residues in proteins. The tyrosyl protein sulfotransferase does not require a specific motif but it largely is dependent upon the folding of the protein and the following structural requirements: (1) the presence of a nearby negative charge (usually in position –1 before the tyrosine residue or several acidic residues within 5 residues on either side of the tyrosine), (2) the presence of turn inducing amino acids within the –5 +5 residues of the tyrosine, and (3) absence of steric hindrances.[386] Indeed data suggests that nitration of tyrosine residues may share similar requirements as phosphorylation and sulfation. Our work, as well as Crow *et al.* (1998) suggests that protein tyrosine nitration is effective when acidic residues (mostly glutamate) are in the –1 position.[389] An extensive search and alignment of nitrated sequences of different proteins failed to produce a specific peptide motif. However, in the majority of the cases a negative charge can be found within 2–3 Å of the site of nitration. Similar to sulfation, the folding of the protein, the surface exposure, the paucity of cysteine and methionine residues, and the presence of turn inducing residues appear to constitute requirements for the selectivity of tyrosine nitration.

The magnitude of protein tyrosine nitration appears to be in the same order as tyrosine phosphorylation (0.01–0.1 mole%), which suggests that the levels of 3-nitrotyrosine *in vivo* are sufficient to satisfy a role for signal transduc-

tion. However, before accepting this hypothesis, conclusive evidence that protein tyrosine nitration is physiologically reversible must be obtained. Tantalising and stimulating preliminary reports[390,392,393] of a putative tyrosine nitrase or "denitrase" have been published, opening an area of research that may provide important clues for the potential role of this biological process in signal transduction and in the turnover of nitrated proteins.

TURNOVER AND METABOLISM OF NITRATED PROTEINS

In several animal models of disease the dynamics or half-life of either free or protein 3-nitrotyrosine has been investigated.[36,40,42,50,85,132,269,270,394] For example we have shown that albumin is a major protein target for nitration by peroxynitrite in rat skin and that mechanisms exist for its removal.[269] This was biphasic with a rapid initial loss ($t_{1/2}=2$ h) and a slower loss ($t_{1/2}=22$ h). A similar half-life of 2 h was shown for 3-nitrotyrosine in rat brain during ischemia-reperfusion injury[40] and a half life of 1–2 h has also been reported for free 3-nitrotyrosine in plasma.[132,394] In the majority of animal models the presence of 3-nitrotyrosine is transient and persists for hours rather than days or weeks. Inflammation of skin or joints induced by zymosan causes transient elevations in levels of nitrated proteins within 3–8 h of insult, which return to baselines levels within 24 h (S. Greenacre and S. Brain, unpublished observations). This relatively rapid removal of 3-nitrotyrosine may be significant in modulating the effects of nitration in normal physiology or in disease and may be due to protein degradation, repair or clearance, or a combination of these mechanisms. In addition it has also been shown that 3-nitrotyrosine is lost when nitrated proteins or free 3-nitrotyrosine are exposed to reactive species such as neutrophil-derived hypochlorous acid.[395]

Evidence also exists to suggest that nitration is a reversible process. Published data has suggested the existence of a repair mechanism for nitrated proteins without apparent protein degradation.[390] It is critical to point out that degradative pathways such as the proteasome will accelerate the degradation of some but not all nitrated proteins. Indeed the susceptibility of proteins modified by nitrating agents such as peroxynitrite to degradation by the proteosome varies on the degree of protein modification.[396] While proteins that have been "mildly" modified are more susceptible to degradation by the proteosome, "extensively" modified proteins are poor substrates for proteases being less susceptible to degradation by the proteosome than the unmodified protein. Moreover, in contrast to sulfated tyrosine residues, nitrated residues can be cleaved by chymotrypsin but at a significantly slower rate than tyrosine.[391] Nonetheless the putative tyrosine nitrase appears to be present in a number of tissues and in particular lung and spleen and in one study the activity is induced by endotoxin.[390] This activity appears to be heat and trypsin sensitive, does not utilize free 3-nitrotyrosine as a substrate and exhibits different kinetic profiles towards different nitrated protein substrates. ([390], unpublished observations). Clearly the need for purification and further characterization of this activity is important in order to establish its role in protein nitration. Recently we have devised an alternative ELISA based method in an attempt to validate and facilitate further characterization of this tyrosine nitrase activity.[397]

The metabolic processing of free 3-nitrotyrosine is also unknown. Ohshima *et al.* (1991) has measured nitrated metabolites of nitrotyrosine in human urine, which indicated that deamination and decarboxylation of free 3-nitrotyrosine has taken place.[398] This may be of importance, avoiding the inappropriate utilisation of the covalently modified amino acid in transtyrosinase function as reported for tubulin.[314] Moreover, neither free 3-nitrotyrosine nor protein

nitrotyrosine are reduced by bacterial and other mammalian nitroreductases.[399] These data suggest that the protein tyrosine nitrase and the deamination/decarboxylation of free 3-nitrotyrosine are unique specialised pathways that handle this protein and amino acid modification.

CONCLUSIONS AND FUTURE DIRECTIONS

Certainly there are many unanswered questions and gaps that can benefit from future research. One area is the role of tyrosine nitration in immunological responses. For example, before KLH was utilised to increase immune response to antigens, 2,4 dinitrophenol was coupled to antigens to boost the immune response of otherwise non-antigenic proteins and peptides. Raising antibodies to nitrated proteins has been feasible and relatively easy. Therefore, it is likely that circulating antibodies to nitrated proteins exist, and preliminary data supporting this has been found in our laboratory. A large number of studies cited in Table I (~20%) have reported that macrophages contain large amounts of nitrated proteins. This can be due to generation of nitrating agents by macrophages but more importantly could signify active phagocytosis of nitrated proteins in an effort to remove them from sites of inflammation. Moreover, *in vitro* studies have recently suggested that nitration of cytokines such as IL-5 or IL-8 alter immune responses.[400–403] The discovery and sound demonstration of an enzymatic tyrosine nitrase is needed. Indeed the presence of such enzymatic activity will clearly satisfy the role of nitration in signal transduction events where putative nitrating agents such as peroxynitrite have been shown to play a significant role.[404] A better understanding of the effect of nitration in the function of proteins and its relationship to phenotypic expression is needed. This will require the identification of specific proteins that are modified by nitration and a comprehensive examination of the protein function and turnover rate. Two issues need to be considered when one attempts to associate protein nitration with the expression of a pathological phenotype. Firstly, it is possible that tyrosine nitration will not alter the activity of the protein, as has been shown for transferrin and α_1-anti-chymotrypsin and the trypsin activity of α_1-protease activity.[296] Secondly, care must be taken to investigate the existence of other amino acid modifications that may also participate in altering the function of the protein. The discovery of specific proteins with known crystal structures will also facilitate the precise identification of the structural and other requirements that facilitate the nitration of specific tyrosine residues and specific proteins. Overall, nitration of free tyrosine and protein tyrosine residues is a biological process that is derived from the biological chemistry of nitric oxide and although it is associated mostly with disease states it may also play a significant yet unrecognised role in signal transduction, immune response and protein metabolism.

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