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Functional Implications of Transporters Under Nitrosative Stress Conditions

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ABSTRACT – Nitrosative stress is defined as pathophysiological conditions that are related to covalent modifications of proteins by nitration/nitrosylation by forms of nitrogen oxide (NO_x), leading to DNA damage, ultimately, cell death. This type of stress condition appears to be associated with a number of disease states, including diabetes, inflammation and neurodegenerative diseases. Since these pathological conditions are frequently chronic in nature and, thus, require long-term treatment, changes in pharmacokinetics are likely to affect the therapy. Transporters are membrane proteins that facilitate the movement of substrates, including drugs, across plasma membranes of epithelial / endothelial cells. Since it is now increasingly evident that transporters are pharmacokinetically significant, functional alteration of transporters by this stress condition may have therapeutic relevance. In this review, experimental techniques that are used to study both *in vivo* and *in vitro* nitrosative stress are summarized and discussed, along with available literature information on the functional implication of transporters under conditions of nitrosative stress conditions. In the literature, both functional induction and impairment were apparently present for both drug transporter families [i.e., ATP-binding cassette (ABC) and solute carrier families (SLC)]. Furthermore, a change in the function of a certain transporter appears to have temporal dependency by impairment in the early phase of nitrosative stress and induction thereafter, suggesting that the role of nitrosative stress is complex in terms of functional implications of the transporters. Although the underlying mechanisms for these alterations are not fully understood, protein nitration/nitrosylation appears to be involved in the functional impairment whereas transcript factor(s) activated by nitrosative stress may play a role, at least in part, in functional induction. Interestingly, functional induction under conditions of nitrosative stress has not been observed for SLC transporters while such impairment has been documented for both ABC and SLC transporters. Further investigations appear to be necessary to fully delineate the underlying reasons for these differences on the impact and importance of nitrosative stress conditions.

Key words – Nitrosative stress, NOx, Functional alteration of Transporters, Nitrosative stress models, NOx donors

Nitrosative stress can be defined as pathophysiological conditions that are related to the covalent modification of proteins by nitration/nitrosylation by various forms of nitrogen oxide (NO_x), leading to DNA damage and, ultimately, to cell death (Obrosova et al., 2005). It is generally accepted that nitrosative stress is associated with a number of disease states, including neurodegenerative diseases (Castegna et al., 2002; Markesbery et al., 1998; Mecocci et al., 1994), diabetes (Cai et al., 2005), inflammation (Olsson et al., 1998) and cardiovascular disease (Escobals and Crespo, 2005), primarily by the involvement of inducible nitric oxide synthase (iNOS). In general, these pathological conditions are chronic, thus necessitating pharmacotherapy for the treatment/management of the diseases. Despite the fact that the biochemical mechanism for nitrosative stress has been widely studied (Ridnour et al., 2004), our understanding of the pharmacotherapeutic implications of this type of stress is largely incomplete and additional investigations are clearly called for.

Transporters are membrane bound proteins that are involved in the vectorial transport of solutes across polarized membranes, particularly the plasma membranes of epithelial and endothelial cells (for a review see Kusuhara and Sugiyama, 2004). More importantly, because drugs can be accepted as substrates to transporters, the directional movement of those drugs may be facilitated by transporters that are expressed in these polarized cells. Therefore, carrier-mediated transport processes can be a primary determinant of the pharmacokinetics of certain drugs and any alteration in the function of a given transporter is likely to have pharmacokinetic consequences. For example, the distribution of vinka alkaloids and cyclosporin A (CsA), substrates for the efflux transporter MDR1 (i.e., P-glycoprotein), to the brain was reported to be markedly enhanced in mdr1 knockout mice (Schinkel et al., 1995). In addition, systemic exposure (i.e., area under the plasma concentration - time curve) of pravastatin and pitavastatin, substrates for the OATP1B1 transporter, was significantly higher in individuals having a reduced function allele (e.g., *15 allele) of the transporter (Niemi et al., 2004; Chung

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et al., 2005). Despite the increasingly clear roles of transporters in pharmacokinetics, only handful examples exist in the literature regarding the relevance of nitrosative stress on the function of transporters and/or *in vivo* pharmacokinetics. In this review, the functional implications of transporters both *in vivo* and *in vitro* nitrosative stress conditions are summarized and discussed. It appears evident that functions of solute carrier transporters as well as ATP-binding cassette transporters were affected by nitrosative stress conditions as indicated in sections following this introduction, although the direction of the change was apparently complex (that is, nitrosative stresses lead to functional induction of transporters in some cases, while, in other cases, inhibitory, depending on experimental conditions; see sections 3 and 4). In addition, since experimental conditions could be potential determining factor(s) in the outcome, a brief description of *in vivo*/*in vitro* nitrosative models, along with problems associated with these models, were summarized from the literature and discussed.

Methodological Considerations for *in Vivo* and *in Vitro* Studies of Nitrosative Stress

Nitrosative Stress Model *in Vivo*

Endogenous nitric oxide (NO), generated by the action of nitric oxide synthase, has very distinctive pathological/physiological functions (Moncada et al., 2006). NO, a free radical, is chemically reactive and can readily react with disulhydryl linkages in proteins to form S-nitrosothiols (Chung et al., 2005). While NO may be chemically converted into inorganic nitrite/nitrate, it can also react with superoxide to form peroxynitrite, the most reactive form of NO_x. Peroxynitrite is known to nitrate the hydroxyl groups of tyrosine residues in proteins (Ischiropoulos and al-Mehdi, 1995). In contrast, other forms of NO_x (e.g., inorganic nitrite and nitrates) are relatively inert chemically as they are and, thus, these forms are not generally considered to be involved in nitrostive stresses. These various forms of nitrogen oxide are in equilibrium so that mixtures of the oxide forms are believed to be simulataneously present *in vivo*. In order to study the relationship between nitrosative stress and alterations in the function of a given transporter *in vivo*, it would be reasonable to study the pharmacokinetics of a drug that serves as a substrate of the transport system in disease model(s) of nitrosative stress. A number of experimental nitrosative stress models have been reported in the literature (Table I).

Experimental sepsis model

It is now well established that sepsis, characterized by the production of inflammatory mediators and cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1b), interferon-gamma (IFN-γ), cluster of differentiation 14 (CD14) and intercellular adhesion molecule 1 (ICAM-1) (Essani et al., 1995; Takahashi et al., 1996), is created by microbial infections. Experimental sepsis, which is biochemically similar to clinical sepsis, is typically induced by the administration of a bacterial (e.g., *Escherichia coli*) lipopolysaccharide (LPS; a major component of the outer membrane of Gram negative bacteria) to animals (Türközkan et al., 2001; Unlü et al., 2001). The production of both NO and superoxide are reproducibly increased in animals receiving LPS (e.g., Zhang et al., 2000). Therefore, the administration of LPS to animals, to produce experimental sepsis, could be used as an *in vivo* model of nitrosative stress. Consistent with this conclusion, it was recently demonstrated that an intraperitoneal administration of LPS to guinea pigs led to *in vivo* nitrosative stress in the kidney (Seven et al., 2005). When guinea pigs received *E. coli* LPS at the dose of 12×10^9 colony-forming units/kg, a marked increase in iNOS activity, which is associated with the formation of nitrated tyrosine (nTyr), was noted in the kidneys of LPS treated animals (Seven et al., 2005). These biochemical manifestations are consistent with those found in animals that have been inoculated with bacteria (e.g., *E. coli*) (Shen et al., 2007). In addition, Han et al. (2002) demonstrated that the intracerebral injection of 50 ng LPS was associated with an enhanced level of white blood cells and NO_x levels in cerebrospinal fluid suggesting that the administration lead to nitrosative stress in the cerebrospinal fluid, similar to clinical meningitis. Since LPS-induced nitrosative stress is likely to be relevant to the the development of infections/inflammation (Zhang et al., 2000), this *in vivo* nitrosative stress model may be practically useful in the study of pharmacokinetic alterations in infection/inflammation mediated nitrosative stress. In the application of this *in vivo* model, however, the nature of the pharmacokinetic alteration must be carefully analyzed because the kinetics may be altered without any change in transporter function. For example, it was demonstrated that an experimental inflammation, produced by the administration of a very high dose of bacterial LPS to rats led to the increased brain transport of sucrose, a compound that does not penetrate into the brain, suggesting that the pharmacokinetic change was mediated by an alteration in the blood brain barrier, rather than an alteration in transporter function (Jaworowicz et al., 1998). In addition, it is well known that LPS-treated animals have a reduced glomerular filtration rate, probably as the result of the hypotensive effect of NO_x (Essani et al., 1995), thus suggesting that the renal clearance of drugs is likely to be reduced in ani-

Table I. *Summary of in vivo Nitrosative Models*

Disease model	Method to induce disease model	Identification of nitrosative stress	Method to identify nitrosative stress	Reference(s)
STZ-induced diabetes	Administraion of STZ (*iv/ip*)	Y	Formation of nTyr, Increased level of NO_x, iNOS and NF-κB	Kobayashi et al. (2000), Taguchi et al. (2007), Marfella et al. (2006).
Experimental sepsis	Administration of bacterial lipopolysaccharide (*iv/ip/ic*)	Y	Increased level of NOx and iNOS	Seven et al. (2005), Han et al. (2002) Zhang et al.(2000).
Cyclosporin A (CsA) induced nitrosative stress model	Administration of high dose CsA (25mg/kg) for 21 days	Y	Increased in iNOS and NF-κB activation	Buffoli et al. (2005), Ling et al. (2003), Josephine et al. (2007).
Stress induced nitrosative stress	Immobilization of animals for 6h	Y	Increased in Ca^{++} -independent NOS activity	Madrigal et al. (2006), Sarandol et al. (2007), Pérez-Nievas et al. (2007).
Deoxycholate induced nitrosative stress	Feeding animals in a diet supplemented with 0.2% deoxycholate for 8 months	Y	Formation of nTyr	Bernstein et al. (2006).
Ulcerative colitis model	Feeding animals in dextran sulfate sodium containing fluid (1% dextran sulfate sodium, w/v)	Y	Formation of nTyr	Kimura et al. (1997), Korenaga et al.(2002).
Nitrogen mustard-induced nitrosative stress	Intratracheal administration of nitrogen mustard, 0.5mg/kg	Y	Increased in lipid peroxidation, iNOS and NO_x	Korkmaz et al. (2006), Yaren et al. (2007).
Reperfusion injury	Using in situ isolated rat liver perfusion model, 15 min ischemia, followed by 45 min perfusion	Y	Increased in NO_x, hydroxyl radical and tumor necrosis factor	Goligorsky et al. (2002), Chen et al. (2006).
Cisplatin-induced acute renal failure	Intraperitoneal injection of cisplatin for 6 days	Y	Increased level of NO_x, along with reduced level of glutathione and the activity of superoxide dismutase in the serum	Jiang et al. (2007), Chirino et al. (2004), Kuhad et al. (2006).

mals that have been treated with LPS. These non-specific alterations in pharmacokinetics need to be carefully screened for an adequate assessment of kinetic changes.

Streptozotocin (STZ) induced diabetes

In the literature, the production of superoxide and NO_x is reported to be markedly elevated in aortic rings hyperglycemic rats, created by the administration of STZ (Kobayashi et al., 2000). Consistent with this finding, Taguchi et al. (2007) demonstrated that the formation of nTyr, an index of nitrosative stress, was enhanced in aortas of rats with STZ-induced diabetes (Taguchi et al., 2007; Cai et al., 2005), suggesting that rats with STZ-induced diabetes could be used as an experimental model of nitrosative stress. In relation to this, the formation of nTyr was also noted in carotid plaques in diabetic patients (Marfella et al., 2006), suggesting that experimental diabetes is relevant model of clinical diabetes in terms of nitrosative stress. Similar to the LPS model, however, kinetic properties may be altered without any alteration in transporter function in the diabetes model. For example, it was reported that, in diabetes mellitus, blood flow is reduced (i.e., for the case of brain blood flow, 40% compared with the control value) (Lass and Knudsn, 1990) for certain organs, an alteration that potentially affects the kinetics of distribution/elimination. In addition, the administration of insulin to diabetic animals was associated with a reduced level of nTyr (Cai et al., 2005) in comparison to the nTyr level in diabetic animals without insulin treatment, suggesting that nitrosative stress can be reversed by insulin administration. However, results obtained from insulin treatment experiments should be analyzed with care, since insulin induces the gene expression of a transporter *in vitro* in the absence of nitrosative stress, an effect that may be mediated by NF-κB (Zhou and Kuo, 1997). Therefore, experiments involving insulin treatment should be carefully designed for an accurate assessment to be obtained.

Nitrosative stress created by the administration of CsA

The administration of CsA to animals can also be used as a model of nitrosative stress. Buffoli et al. (2005) reported that the oral administration of CsA led to increased iNOS and NF-κB activation. Previously, NF-κB was proposed as a putative transcription factor for the expression of a number of important transport systems (e.g., mdr1, mrp2) (Thevenod et al., 2000; Kuo et al., 2002). In relation to this observation, it was proposed that CsA nephrotoxicity may be mediated by CsA-induced oxidative/nitrosative stress (Ling et al., 2003). While this model could be useful in studies of the impact of nitrosative stress, the model involves the administration of high doses CsA (25 mg/kg; therapeutic oral dose of 2.5-10 mg/kg for CsA, depending on the indication) for a period of 21 days and, thus, an alteration of the pharmacokinetics by drug-drug interactions may occur, in addition or separate to the functional alteration of transporters. Indeed, Josephine et al. (2007) recently reported that the administration of CsA was associated with a simultaneous increase in the expression of iNOS and xanthine oxidase in renal tissues.

Other models

The above described disease models have been used to study the relationship between nitrosative stress and *in vivo* pharmacokinetics. The following disease models have been reported to have relevance in terms of nitrosative stress, although pharmacokinetic alterations have not been examined for these models.

Stress induced nitrosative stress

It was proposed that stress-related human disorders may be mediated by the accumulation of oxidative/nitrosative stress in tissues (e.g., brain) (Madrigal et al., 2006; Sarandol et al., 2007). To mimic this pathological condition, Pérez-Nievas and co-workers (2007) compared the extent of oxidative/nitrosative stress in the brain of rats from controls vs. immobilization-induced stress. These investigators found that Ca^{++}-independent NOS activity was markedly enhanced in the brain of the disease model. NOS activity was positively correlated with proinflammatory mediators, e.g., PGE(2), whereas the activity was negatively correlated with anti-inflammatory factors, e.g., 15-deoxy-PGJ(2) (Pérez-Nievas et al., 2007). Unfortunately, relationships between stress-induced nitrosative stress and the function of transporters has not been studied extensively.

Deoxycholate-induced nitrosative stress

Bernstein et al. (2006) reported that a prolonged (i.e., 8 month) feeding of a diet supplemented with 0.2% deoxycholate led to inflammation in the colon, which was associated with the formation of nTyr. The nitrosative stress also appeared to lead to angiogenesis, damage to DNA/RNA and proliferation. However, it is not known whether this nitrosative stress condition is related to an alteration in the pharmacokinetics and/or the functions of transporters.

Ulcerative colitis model

It was reported that the expression iNOS is induced in colons of patients with ulcerative colitis, (Kimura et al., 1997; Rachmilewitz et al., 1995; Reynolds et al., 1995). An experimental model for the colitis involved the feeding of dextran sulfate sodium containing fluid (1% dextran sulfate sodium, w/v) to mice (Korenaga et al., 2002). Again, nTyr, an index of nitrosative stress, was detected in inflammatory cells of the colon. Interestingly, however, nTyr formation was also noted in iNOS knockout mice, suggesting that endothelial NO synthase may also be involved in the over-production of NO. Consistent with this observation, immunostaining of the synthase was noted in colonic blood vessels and lamina propria macrophages (Seril et al., 2007). However, no information is available concerning as alteration in the function of the transporter in this model.

Nitrogen mustard-induced nitrosative stress

Literature reports indicate that NO may be involved in nitrogen mustard-induced toxicity (Korkmaz et al., 2006). Related to this possibility, an intratracheal administration of nitrogen mustard to animals led to the development of both oxidative and nitrosative stress, as evidenced by an increased level of lipid peroxidation, iNOS induction and an increased excretion of NO_x (Yaren et al., 2007). Despite the potential use of this experimental system as a model of *in vivo* nitrosative stress, however, the model has not been tested for alterations in pharmacokinetics.

Nitrosative stress by reperfusion injury

It was reported that ischemia, followed by reperfusion, led to a nitrosative stress condition (Goligorsky et al., 2002). Chen and co-workers (2006) reported that, using an in situ isolated rat liver perfusion model, 15 min of ischemia, followed by 45 min of reperfusion, resulted in a marked increase in the levels of NO, hydroxyl radicals and tumor necrosis factor. This nitrosative stress model is yet to be used in the study of alteration in pharmacokinetics by nitrosative stress.

Cisplatin-induced acute renal failure

Renal dysfunction has been reported in patients receiving

cisplatin (Masuda et al., 1994). The drug is reported to enhance the production of hydrogen peroxide and hydroxyl radicals, suggesting that oxidative stress is involved in the renal damage (Jiang et al., 2007). The formation of peroxynitrite is also increased (Chirino et al., 2004) in cisplatin-induced renal dysfunction and, thus, treatment of experimental animals with cisplatin may lead to nitrosative stress conditions *in vivo*. Indeed, Kuhad et al. (2006) reported that intraperitoneal injection of cisplatin to rats led to the development of oxidative/nitrosative stress and, ultimately, reduced renal function. In particular, serum levels of NO_x were significantly elevated, along with reduced levels of glutathione and superoxide dismutatse activity in the serum. Despite the potential of this model, altered pharmacokinetics have yet to be studied.

Nitrosative Stress Model *in Vitro*

Under pathological and physiologically conditions, NO is produced from L-arginine by the action of the nitric oxide synthases (i.e., iNOS, neuronal NOS and/or endothelial NOS) (Radi, 2004) and is subsequently converted to other forms of NO_x (e.g., peroxynitrite or inorganic nitrite). Therefore, to induce *in vitro* nitrosative stress for studying alterations in the function of transporters, the exposure of an *in vitro* system that expresses the target transporter with NO_x donors appears to be a reasonable approach. In the literature, various exogenous NO_x donors are available for experimental use. Similar to *in vivo* nitrosative stress models, however, a careful examination of the results is necessary, since a number of different forms of NO_x are simultaneously present. Therefore, a direct link between a particular NO_x form and the functional alteration of a transporter would be extremely difficult, if not impossible, to verify. In addition, the mechanisms for the formation of NO_x and the chemical reactivities differ significantly among the various NO_x donor classes. For example, certain donors require enzyme(s) for the generation of NO_x while other donors are able to produce NO_x nonenzymatically. Even for the spontaneously NO_x-generating donors, multiple mechanisms (e.g., reaction with exo-/endogenous thiols, reduction and/or oxidation) are involved for the formation of NO_x, depending of the nature of the donor (Romero et al., 2006). Frequently used NO_x donors are summarized (Table II) as follows.

Nitrovasodilators

Nitrovasodilators represent the oldest class of NO donors (Chung et al., 1992). Representative organic nitrates include nitroglycerin (GTN) and isosorbide dinitrate (ISDN). NO can be generated from GTN both enzymatically as well as nonezymatically (Chung et al., 1992), where a three-electron reduction is involved. Organic nitrites (i.e., isobutyl nitrite) are nitrous acid esters formed by the reaction between an alcohol and nitrous acid. Organic nitrites can also spontaneously generate NO as well as enzymatically (Kowaluk and Fung, 1991) via reduction involving an one-electron transfer. Despite the fact that nitrovasodilators are converted to NO_x, the level of NO is reported to be in nM range (Chung et al., 1992). NO_x levels found in *in vivo* nitrsoative models are typically in the μM range (Han et al., 2002; Maeng et al 2007), suggesting that the use of certain nitrovasodilators for producing an *in vitro* nitrosative stress model may be inadequate in terms of the amount of NO_x produced.

Table II. *Summary of in vitro Nitrosative Models*

NO_x donor compound (Abbreviations in the parenthesis)	Class	Predominant NO_x form released	Mechanism of NO_x generation	Reference
Nitroglycerin (GTN) Isosorbide dinitrite (ISDN)	Organic nitrates	NO·	Enzymatic and nonenzymatic	Chung et al. (1992).
Isobutyl nitrite	Organic nitrites	NO·	Spontaneous and enzymatic	Kowaluk et al. (1991).
3-Morpholinosydnomine (SIN-1)	Heterocyclic NO-releasing compounds	$·ONOO^-$	Spontaneous	Wang et al. (2004).
Sodium nitroprusside (SNP)	Transition metal nitrosyls	NO·	Nonenzymatic	Wang et al. (2004).
Diethylamine(DEA/NO) Diethyltriamine (DETA/NO)	NONOates	NO·	Spontaneous	Vodovotz et al. (1999).
S-Nitroso-N-acetyl-D,L penicillamine (SNAP)	S-Nitrosothiol	NO^+	Spontaneous	Wang et al. (2004).
Angeli's salt	Nitroxyl-generationg compounds	NO^-	Spontaneous	Dutton et al. (2004).

Other NOx donors

In contrast to the organic nitrates/nitrites, certain NO donors are available that spontaneously release NO_x to the level found in *in vivo* nitrosative stress (i.e., approximately 10~200 μM). These donors typically contain either nitroso- or nitrosyl-functional groups. 3-Morpholinosydnonimine (SIN-1), a metabolite of molsidomine from an enzymatic reaction in the liver, produces NO_x. This zwitterion, which is formed by combining morphine and sydnonimine, spontaneously decomposes at physiological pH to NO· and a superoxide anion. Since NO is known to react readily (that is, the reaction rate is primarily dependent on the diffusion rate of the reactants) with superoxide, the formation of peroxinitrite is highly likely (Huie and Padmaja 1993), suggesting that this NO_x donor can be regarded as primarily a peroxynitrite donor. Sodium nitroprusside (SNP) is a complex comprised of a ferrous ion and five anionic cyanide groups (CN^-) and nitrosonium ion (NO^+). The donor is known to produce NO by an one electron transfer, via photolysis, and subsequent reduction in the presence of thiols, a reducing agent (Yamamoto and Bing, 2000). Diethylamine (DEA)/NO and diethylenetriamine (DETA)/NO, also known as member of the NONOate class, are diazeniumdiolate derivatives, in which NO is covalently linked to DEA and DETA, respectively. These compounds spontaneously release NO·. S-nitrosothiols (e.g., S-nitroso-N-acetyl-penicillamine or SNAP) are a class of naturally occurring NO-donating compounds. These compounds are reported to spontaneously release NO^+ from a nitroso-thiol moiety of the donor. Angeli's salt is an agent that spontaneously generates NO^- under physiological conditions. Amongst the NO_x donors described above, it is noteworthy that there are significant differences between the primary NO_x forms generated from the donors. For example, NONOates and SNP primarily generate NO· while NO^+ and NO^- are primarily released from SNAP and Angleli's salt, respectively. Therefore, it would be necessary to test more than one NO_x donor for a complete delineation of the relationship between nitrosative stress and functional alterations of transporters. In addition to the primary NO_x form generated from the donors, the rate of release of NO could affect the primary form of NO_x present in the incubation medium. For example, the reaction of NO with oxygen could be described as the second order of the NO concentration (Chung et al., 1992). Therefore, the reaction rate would be hyperbolically increased with NO concentration. Thus, the rapid NO releasing donors (viz, comparatively more NO at a given time; e.g., SNAP) are likely to be more prone to form inorganic nitrite. Other experimental artifacts for NO_x donors include the possibility of the formation of a byproduct of NO_x donors, which could potentially affect the function of proteins. For example, cyanide, ferrocyanide, or ferricyanide ions (i.e., byproducts of SNP) have been reported to affect protein function (Dulak et al., 2000).

Other in vitro nitrosative model

Some reports (e.g., Gharavi and El-Kadi., 2007) proposed that, by exposing cells to LPS/TNF-a, the treatment may trigger the intracellular formation of peroxynitrite and that this experimental system may be applicable to studies of the functional roles of NO/ peroxynitrite in cells. Although the generation of NO appeared to be induced by TNF-α in a concentration-dependent manner, the production of NO by stimulation with LPS was not dependent on LPS concentration, suggesting that the combination of LPS/TNF-a could be an useful tool in the study of NO_x mediated effects *in vitro*. Unfortunately, however, the impact on functional activities of transporters has not been studied in this nitrosative stress model.

Quantification of NO_x

A number of analytical procedures are available for the determination of NO_x (for a review, see Taha, 2003). Traditional methods to quantify NO_x have been achieved through a bioassay that utilizes the vasodilating properties of NO (e.g., Palmer et al., 1988), through a spectrophotometric assay that measures the spectral change of oxyhemoglobin on reaction with NO (e.g., Feelisch and Noack, 1987) or through a colorimeteric assay that exploits the reaction of NO_x with the Griess reagent (Green et al., 1982). More recent analytical methods for the detection of NO_x utilized redox-chemiluminescence detection, in which a chemiluminescence reaction of NO and ozone is quantified by a photomultiplier. However, in certain reports (e.g., Palmer et al., 1988), the chemiluminescence assay involved a reflux step with glacial acetic acid and potassium iodide, which may result in the conversion of NO from inorganic nitrite/nitrate, nitrosothiols and nitrosamines. In other reports (e.g., Chung and Fung, 1990), headspace sampling, followed by the direct introduction of the sample into the chemiluminescence detection system, was applied to eliminate the artifactual conversion to NO. It was reported that, using a cell permeable probe, a strong fluorescence could be produced by the reaction between the probe and peroxynitrite (Setsukinai et al., 2003). Therefore, it appears possible that this fluorescence assay would be applicable to the determination of intracellular concentrations of peroxynitrite.

In general, technical challenges in the quantification of different forms of NO_x are typically due to a lack of specificity in

the NO_x assay. Even in cases of assays using chemical detection (e.g., redox-chemiluminescence assay), inaccurate quantification of the nitrogen oxide form is possible, depending on the experimental procedures used (e.g., reflux). Furthermore, in a more recent analytical procedure (e.g., the use of a fluorescence probe), other free radicals in the system (e.g., hydroxyl radical) react readily with the probe, potentially resulting in artifactual values. Therefore, there is no reliable assay for the accurate determination of the concentration of a given nitrogen oxide form at the present time.

Nitrosative Stress as a Mechanism for Induction of Function and Expression of Transporters

In the literature, a number of studies have shown that nitrosative stress is associated with an enhanced function and/or expression of transporters, thus suggesting that the stress condition has important regulatory implications in the function of transporters. Bridges et al. (2001) showed that the incubation of ARPE-19 cells, retinal pigment epithelial cells, with SIN-1, an NO_x donor that preferentially forms peroxynitrite, for 2 h led to the formation of nTyr in ARPE-19 cells as determined by a radio-immunohistochemical method. Furthermore, these authors found that a number of transporters, including the cysteine/glutamate transporter and taurine transporter, were up-regulated in their *in vitro* model. Similar up-regulation was also noted with SNP, another NO_x donor, in retinal epithelial cells (Bridges et al., 2001), consistent with the findings for SIN-1. Uchiyama et al. (2005) reported that the function and expression of the Na^+-dependent neutral amino acid transporter (ASCT2) was enhanced when Caco-2 cells, a frequently used model for the intestinal epithelial barrier, were pretreated with S-nitrosothiol class NO donors, particularly with SNAP. Paik et al. (2005) demonstrated that long-term exposure (i.e., 24 h) of NO donors including SNP in human endothelial cells isolated from umbilical veins led to the up-regulation of the function and expression plasma membrane glucose transporter 1 (GLUT1). Similarly, Schwartz et al. (2006) reported that peroxynitrite induced a significant increase in arginine uptake and cationic amino acid transporter-2 (CAT-2) mRNA expression in rat mesangial cells.

In addition to transporters that facilitate the transport of endogenous compounds, the function/expression of drug transporters appeared to be affected by NO_x exposure. For example, pretreatment of Caco-2 cells with SNAP resulted in a concentration dependent decrease in intracellular accumulation of CsA (Dixit et al., 2005), a substrate for P-glycoprotein, an efflux transporter, probably by an IFN-γ dependent mechanism. In relation to this alteration, these authors found that the decreased transport by enhanced functional activity of the efflux transporter was correlated with an increased expression of efflux transport (Dixit et al., 2005). Consistent with these observations, pretreatment with SNP also increased the function and protein expression of the P-glycoprotein (Bauer et al., 2007) in rat brain capillaries when the isolated tissue was subjected to continuous exposure to a low level of TNF-α, suggesting that this alteration may have implications in the long-term consequences on the pharmacokinetics of P-glycoprotein substrates.

This literature information concerning *in vitro* experimental systems demonstrates that multiple transport systems (e.g., P-glycoprotein, ASCT2, b-amino acid transporter and CAT-2), regardless of the type (i.e., transporters for endogenous substances or drugs), are functionally induced in diverse endo-/epithelial cell systems (e.g., retinal epithelial cells, colon carcinoma cells, brain capillary endothelial cells and kidney mesangial cells) by pretreatment with NO_x donors. These multiplicities, i.e., transport systems, barrier systems and NO_x donors, suggest that functional induction may be prevalent in other transport systems throughout the body, presumably by a common mechanism. Furthermore, it appears reasonable to hypothesize that pathological conditions with nitrosative stress conditions have kinetic consequences *in vivo* for compounds that are subjected to the transporters.

Consistent with this statement, the pharmacokinetics of certain drugs appear to be significantly affected in disease models that are known to be under nitrosative stress. For example, in an experimental diabetes model, a nitrosative stress model, Van waarde et al. (2002) reported that mRNA and protein expression of multidrug resistance P-glycoprotein type 2 (MDR2) in the liver were increased in association with increased billiary phospholipid output, a putative function of MDR2 *in vivo*. Furthermore, a similar experimental diabetes model was also reported to be associated with a decreased brain distribution of fluorescein, a substrate for an organic anion transporter (OAT3) and a multidrug resistance protein (MRP2), as determined by an in situ brain perfusion technique (Hawkins et al., 2007). These authors found that the existence of diabetes led to an increased expression of MRP2, but not OAT3, in the blood brain barrier, suggesting a certain specificity exists for the case of nitrosative stress dependent induction of transporters. The expression of other transporters (e.g., Na^+-K^+ ATPase) was also enhanced under conditions of nitrosative stress induced by experimental diabetes (Egleton et al., 2003). Concerning nitrosative stress induced by other disease models, Heemskerk et al. (2007) recently demonstrated

that experimental endotoxemia, created by the administration of LPS, led to the up-regulation of a number of important ATP-binding cassette transporters, including ABCB1 (P-glycoprotein), ABCB11 (Bsep) and ABCB2 (MRP2). However, it is noteworthy that conflicting observations also exist in the literature so that a similar nitrosative model is also known to reduce the function and expression of transporters (see the following section 'Nitrosative stress as a mechanism for impairing the function of transporters'). Therefore, the underlying mechanisms of nitrosative stress on the function of transporters are obviously complex and remain to be delineated.

Literature information suggest that a certain transcription factor, which is activated during the nitrosative stress, is involved in disease models associated with nitrosative stress conditions. For example, in a study reported by Dixit et al. (2005), pretreatment of Caco-2 cells with IFN-γ and SNAP increased the binding of DNA to NF-κB, a transcription factor, which may ultimately lead to an enhanced transcription of the gene encoding the P-glycoprotein. Consistent with this finding, Bauer et al. (2007) also demonstrated that, after a relatively long-term exposure (i.e., 6 hr), with TNF-a and SNP signaled via NF-κB, thereby leading to an increased expression and function of P-glycoprotein. In the literature, it appears that NF-κB is a transcription factor that is activated by a number of stress-related signals (e.g., cytokines, hypoxia, reactive oxygen species, heat shock and heavy metals), including nitrosative stress conditions (Shen et al., 2005). While it was proposed that other transcription factors (e.g., AP-1 and Nrf2) that may be separately involved or in concert with NF-κB, in respond to stress-related signals, it remains to be clarified whether these additional factors are responsible for regulating the expression of transporters during nitrosative stress. This aspect of the regulatory mechanism warrants further investigation.

Recently, Maeng et al. (2007) demonstrated that nitrosative stress led to the up-regulation of the P-glycoprotein transporter in the brain of STZ-induced diabetic rats and MBEC4 cells, an *in vitro* model of the blood brain barrier. Using a number of NO_x donors with varying rates of NO release, we identified a reaction product between superoxide and NO, most likely peroxynitrite, which appeared to be involved in the induction of function and expression. In addition, both the level of nuclear translocation of NF-κB as well as the DNA binding activity of a nuclear extract to the NF-κB consensus oligonucleotide were increased in MBEC4 cells that had been pretreated with SNP (Maeng et al*.,* 2007). Therefore, our data suggest that nitrosative stress, regardless of whether it is *in vivo* and *in vitro* nitrosative stress, may induce the expression and function of the efflux transporter via a NF-κB dependent mechanism (Fig. 1).

Nitrosative Stress as a Mechanism for Impairing the Function of Transporters

Although there are examples in the literature that suggest a relationship between nitrosative stress and the induced function/expression of transporters, this stress condition is also known to be involved in the impairment of transporter functions. The underlying mechanism(s) for the impairment has not been clearly delineated, although the nitration or nitrosylation of target proteins and/or reduced expression are believed to be involved.

In our study, the function of the P-glycoprotein was impaired in MBEC4 cells that had been pretreated with SNP for a short-term (i.e. 20 min). The percent of daumonycin (i.e., a well-known substrate for P-glycoprotein) remaining in the brain, a pharmacokinetic variable representing drug efflux out from the

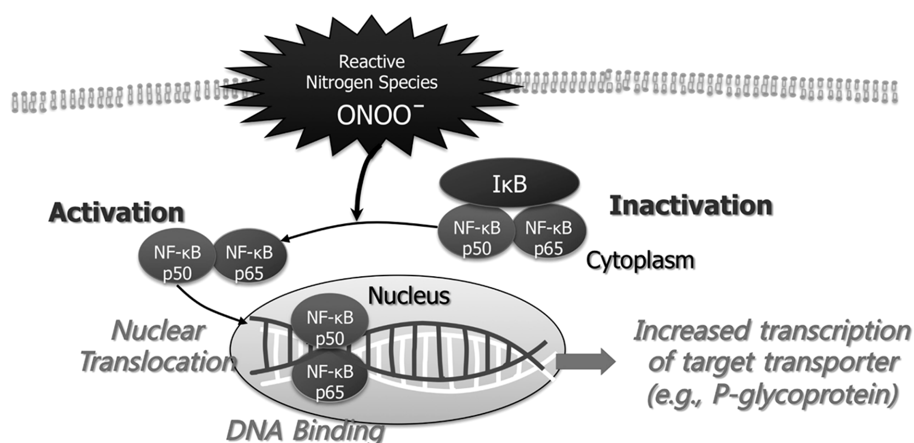

Figure 1. Schematic representation of the role of NF-κB on the induction of a target transporter.

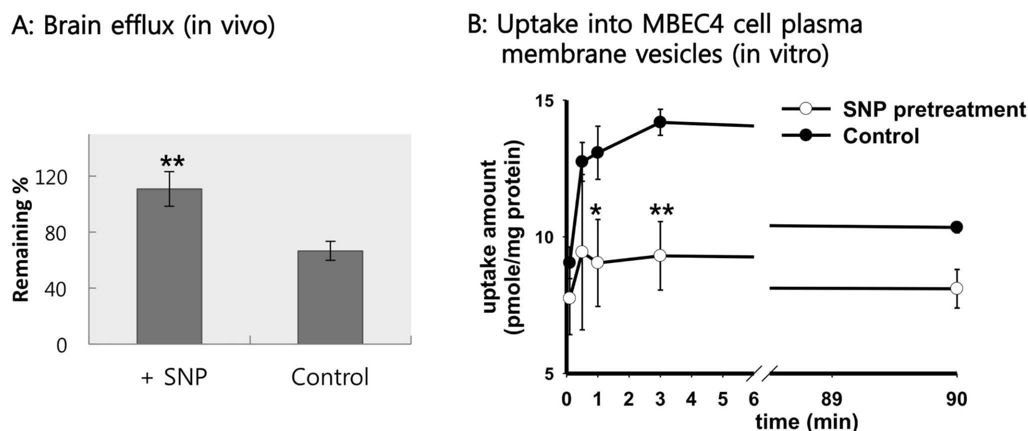

Figure 2. Functional impairment of the P-glycoprotein by sodium nitroprusside (SNP), an NO_x donor. Panel A, rats were microinjected (5 μL, 20 mM aqueous solution) in the caudate putamen region of the brain and allowed to equilibrate for 30 min. *In vivo* daunomycin efflux (dose, 12.5 pmole) was examined by microinjection to the brain region. Key: +SNP, SNP pretreated rats; Control, vehicle injected rats. Data are expressed in the mean±standard deviation of triplicate runs. Panel B, 10^8 MBEC4 cells per batch were harvested, homogenated and differentially centrifuged. N_2 cavitation, followed by sucrose density gradient centrifugation, was used to obtain plasma membrane vesicles. After typical characterization (i.e., marker enzyme, functionality assessment with Na^+-dependent glucose transport), the vesicle preparation was subjected to *in vitro* nitrosative stress by pretreatment with 1 mM SNP for 20 min. P-glycoprotein activity in vesicles was measured using daunomycin as a substrate. Key: SNP pretreatment, vesicles pretreated with SNP; Control, vesicles without the pretreatment. Double slash (//) in the figure represents an interval omission. Data are expressed in the mean±standard deviation of triplicate runs. For both panels, * and ** represent statistical difference by $p<0.05$ and $p<0.01$ in comparison with corresponding controls, respectively, by the student's t-test.

brain, after a direct injection to the brain was enhanced in SNP pretreated rats compared with that in controls (Fig. 2-A). Furthermore, when plasma membrane vesicles of MBEC4 cells (i.e., an *in vitro* model of the blood brain barrier) were pretreated with SNP, the P-glycoprotein mediated ATP dependent uptake of daunomycin was depressed (Fig. 2-B), suggesting that P-glycoprotein function was impaired by NO_x both *in vivo* and *in vitro*. Consistent with these observations, Yamauchi et al. (2007) recently demonstrated that the cellular accumulation of rhodamine 123, a substrate for the P-glycoprotein, in MBEC4 cells was increased (i.e., reduced efflux) dose-dependently by the addition of NO solutions (14 and 28 ìM) at 10 min intervals during a 30-min period. The study demonstrated that NO or NO_x inhibited the function of the P-glycoprotein efflux pump in brain capillary endothelial cells. Since we found that P-glycoprotein function was depressed in membrane vesicles that had been pretreated with SNP, the inhibitory effect of NO or NO_x appeared to be mediated by a direct interaction with the transporter. In our previous studies, we also investigated the roles of NO_x on the sinusoidal uptake of triethylmethylammonium (TEMA) and tributylmethylammonnium (TBuMA), representative substrates for OCT, and taurocholate, a representative substrate for NTCP, in isolated hepatocytes (Song et al., 2002). Na^+-dependent taurocholate uptake was found to be decreased, while Na^+-independent TEMA and TBuMA uptake remained unchanged by the SNP pretreatment (10 mM for 20 min). A kinetic analysis revealed that the V_{max} for taurocholate transport was decreased by 43% (1137±101 vs 651.8±153 pmol/min/10^6 cells, control vs SNP-pretreated cells) by the SNP pretreatment and that K_m remained unchanged As a result, a 34% decrease in the value of CL_{int} (28.98±5.10 vs 19.13±6.78 μL/min/10^6 cells) for taurocholate transport was found in hepatocytes that had been exposed to SNP.

Evidence for a direct relationship between the functional impairment of transporters and post-translational modifications (i.e., nitration or nitrosylation of target proteins) also have been reported. Park and co-workers (2002) reported the chemical modification of cystein residue at 342 by peroxynitrite led to the inhibition of the activity of the human dopamine transporter (hDAT) in EM4 cells that stably expressed the transporter. In addition, Shao et al. (2005) reported that the nitration of tyrosine 192 by peroxynitrite was associated with an impaired ABCA1-dependent cholesterol transport. Celedon and co-workers (2007) also reported that treatment with peroxynitrite resulted in a decrease in an anion transport that was partially due to the modification of cystein residues. Therefore, these chemical modifications of amino acid residues may be related to an impairment in function. However, further studies appear necessary for complete delineation since it is not known whether a specific chemical modification is directly linked to the function.

Consistent with functional inhibition in *in vitro* nitrosative stress, indications of the functional impairment of transporters has been reported in various *in vivo* animal models of nitrosative stress (e.g., diabetes, inflammation). In our previous study, we showed that the elimination clearance of benzylpenicillin (BPC) and taurine from cerebrospinal fluid (CSF) was reduced as the result of the induction of experimental inflammation in the CSF (Han et al., 2002). The uptake of BPC and taurine was found to be suppressed in isolated choroid plexi (i.e., the blood-CSF barrier, CP) obtained from LPS-induced inflammation rats and control CP pretreated SNP, indicating that more than one transport system is functionally impaired in the presence of pathological levels of NO_x. In addition, since BPC is transported with a high affinity by OAT3 (Tahara et al., 2005), the alteration in the kinetics *in vivo* and *in vitro* may be a manifestation of an impaired function of the anion transporter by form of NO_x. Furthermore, the mRNA level of OAT3 was found to be dramatically decreased by LPS administration (Cherrington et al., 2004), suggesting that the functional impairment and the reduced expression of OAT3 by NO_x may be cooperatively involved in the reduced anion transport reported in our previous studies.

For the case of LPS-induced systemic inflammation models, a number of studies consistently reported an impaired function of the P-glycoprotein in the liver, brain and intestine. For example, the P-glycoprotein mediated efflux of digoxin (i.e., a well-known P-glycoprotein substrate) was reduced to 56% of the control values in jejunum samples obtained from control or LPS-treated rats (Kalitsky et al., 2004). Wang et al. (2005) reported that LPS-induced systemic inflammation caused an increased retention (i.e., reduced efflux) of ^{99m}Tc-sestamibi (a substrate for the P-glycoprotein) in the brain, heart, liver and fetal tissues, consistent with a reduction in *mdr1a* mRNA levels in these organs. In LPS-treated mice, LPS-acute inflammation resulted in a significant decrease (50%) in the biliary clearance of doxorubicin (a substrate of the P-glycoprotein) in association with a significant reduction (i.e., 30% of the control) in P-glycoprotein protein levels (Hartmann et al., 2005).

Functional impairments in a number of transporters have been also noted in experimental diabetes models. Grover and co-workers (2004) documented that in STZ-induced diabetes, TEA (an OCT substrate) uptake in isolated proximal tubule cells was imparied. TEA accumulation was significantly lower for all concentrations compared with the values for control cells. As a result, V_{max} for TEA transport in kidney cells from diabetic animals was significantly lower (i.e., by 46%) than that in cells from control animals without any measurable alteration in K_m. These authors reported that the hyperglycemic condition was associated with a decreased expression rOCT1 and rOCT2 in kidneys, thereby leading to a reduced function, consistent with the impaired function. Minamizono et al. (2006) reported that blood-to-retina as well as the blood-brain transport of dehydroascorbic acid (DHA), a substrate for the facilitative glucose transporter (GLUT1), was reduced by 65.5% and 84.1%, respectively, in diabetic rats compared with those in control rats. In addition, Mooradian (1987) reported that the brain uptake index (BUI) for choline in diabetic rats (13.9%) was significantly lower than that in control rats (22.6 %). These observations, therefore, suggest that the functions of multiple transport systems (e.g., OCTs, GLUT1, choline transporter) are affected by diabetes, although further studies are needed to link these pharmacokinetic changes to alterations in transporter function. For example, diabetes is known to alter the blood flow, thereby affecting tissue distribution without any alteration in transporter function. Other factors (e.g., plasma protein binding, distribution to red blood cells) that are determinants of distribution kinetics may also be considered in these disease models, although this aspect of kinetic alteration has not been systemically studied in relation to nitrosative stress conditions. A careful interpretation of the results is obviously warranted before attempting to report a correlation between the role of NO_x with transporter function and pharmacokinetics,

Considerations of Experimental Designs

In the literature related to transporter functions (and/or pharmacokinetics) under conditions of nitrosative stress, considerable conflicting results among reports exist. That is, in certain cases, nitrosative stress appeared to induce the function of a particular transporter whereas similar stress conditions were associated with an impaired function of the same transporter. Even our own results indicated a impairment in P-glycoprotein function (Fig. 2) in *in vivo*/*in vitro* nitrosative stress while diabetes mediated nitrosative stress appears to induce the function of the efflux transporter (Maeng et al., 2007). This problem may be partly due to differences in the duration of NO_x exposure. According to the literature, one of the principle mechanisms for the functional impairment of transporters is likely to be their covalent modification [i.e., the nitration/nitrosylation of certain amino acid residue(s)]. Since the outcome would be the result of a direct interaction between NO_x and a transporter, a relatively short duration of NO_x exposure (e.g., less than 1 h) would be sufficient for functionally impairing a transporter. In contrast, an increased level of expression/function of a transporter would require a series of events (e.g.,

nuclear translocation of NF-κB – DNA binding – increase in target mRNA – increase in protein synthesis – increase in transporter function), thereby necessitating a significant delay for activation to be observed. Indeed, in our *in vitro* nitrosative stress model using MBEC4 cells (Maeng et al., 2007), we found a transient inhibition in the function of the P-glycoprotein by 1 h of stress and a functional induction of the efflux transporter thereafter. Therefore, depending on the duration of NO_x exposure in a given experimental system, conflicting results (that is, impairment vs. induction) are a distinct possibility.

In addition to the duration NO_x, the level of NO_x exposure appears to be important. In our diabetes model, the total concentration of NO_x, as determined by the colorimetric method of Green et al. (1982), was approximately 0.12 mM. In comparison, the total concentration of NO_x in an incubation solution of 1 mM SNP (i.e., an *in vitro* model of nitrosative stress) was approximately 0.16 mM. Functional alteration of the anion transporter was evident when the total NO_x concentration was down to approximately 0.014 mM in an experimental meningitis model (Han et al., 2002), suggesting that approximately 0.01-0.2 mM is a pathological relevant concentration range for disease related NO_x exposure. Therefore, *in vitro* experimental conditions with significantly different NO_x concentrations may lack pathological/physiological relevance. This over- or under-exposure may, in turn, cause an experimental artifact considering the fact that NO_x (e.g., NO) has a series of physiological functions (e.g., vasodilation thereby affecting blood flow and distribution).

As indicated in a previous section, the types of NO_x donors used may also affect the experimental outcome, since the predominant NO_x species can be expected to differ, depending on the donor used (Table II). It is recommended that additional NO_x donors be included in experiments to confirm the experimental outcome. In addition, an experimental condition (e.g., the addition of superoxide dismutase to decrease the level of peroxynitrite) that changes the concentration of the predominant NO_x species is likely to facilitate the interpretation of results.

Conclusions

Nitrosative stress is known to be associated with a number of disease states, including diabetes, inflammation and neurodegenerative diseases. In relation to stress conditions on transporter function, studies of the functional implication of transporters under conditions of nitrosative stress are currently incomplete. Available data in the literature indicate that both functional induction and inhibition are possible, and that the alteration may have a temporal dependency (i.e., inhibition at an early phase of nitrosative stress and induction thereafter). Regarding this temporal dependency, the origin of these complications appears to be the underlying mechanisms responsible for the functional alterations. In the case of drug transporters, typically ATP-binding cassette (ABC) transporters, but not solute carrier transporters (i.e., SLC 21 and 22 families), appear to be affected by functional induction; In contrast, functional inhibition was observed for both ABC and SLC transporters. Since the literature information regarding the underlying reasons for the lack of functional induction of SLC transporters in nitrosative stress is unclear, further studies of the regulatory mechanism(s) of SLC transporters in nitrosative stress is obviously necessary, if we are to fully appreciate the role of nitrosative stress on transporter function.

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