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Neuroprotection Against Ischemic Brain Injury Conferred by a Novel Nitrate Ester

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Abstract—Nitrates exhibit a selectivity of action in different tissue types not fully recognized: in particular, the neuromodulatory and cardiovascular properties can be dissociated. A novel nitrate showed relatively weak systemic effects, but in the middle cerebral artery occlusion rat model of focal ischemia, reduced the cerebral infarct by 60–70% when administered 4 h after the onset of ischemia.

One of the initiating events in ischemia-induced neuronal cell death is excessive release of the excitatory amino acid glutamate, with a consequent excitotoxic activation of glutamate receptors. In particular, prolonged or excessive activation of the *N*-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors has long been associated with ischemic brain injury.[1] NMDA receptor activation allows the influx of calcium into the postsynaptic neuron. Calcium overload initiates multiple processes that contribute to cellular injury and death, including the activation of proteases, and inhibition of mitochondrial respiration leading to failure of cellular energy stores and activation of cell death programs.

The increase in intracellular calcium also results in activation of a number of calcium/calmodulin-dependent enzymes, including nitric oxide synthase (NOS). NO generated by NOS is a membrane permeant, gaseous free radical that acts as an intra- and intercellular messenger via activation of soluble guanylyl cyclase (sGC) and production of cyclic GMP (cGMP), and through cGMP-independent mechanisms. Excessive production of NO, via excitotoxic activation of NMDA receptors, may lead to generation of cytotoxic peroxynitrite, and is a contributing factor in ischemic injury and cell death as a consequence of inhibition of mitochondrial energy production.[2,3] However, NO generation also may have neuroprotective effects: via the action of NO as an antioxidant; via NO-mediated inhibition of caspases; via NO-mediated reduction of NMDA receptor activity; or via cGMP-dependent pathways, that inhibit programmed cell death.[4—6] Thus sGC activation and cGMP formation in the brain represents an effective neuroprotective strategy in cerebral ischemia.

Classical nitrates such as nitroglycerin (glyceryl trinitrate, GTN), are nitrovasodilators that activate sGC, in vivo, increasing tissue cGMP levels, leading to smooth muscle relaxation.[7–9] GTN and other classical nitrates have been used in the clinical treatment of angina for over 120 years, and have found application in other cardiovascular disorders. The high therapeutic utility of GTN is due, in part, to fortuitous tissue selectivity resulting in enhanced venodilator activity. The development of novel nitrates, that exploit tissue selectivity to target therapeutic applications beyond angina, may lead to new therapeutic agents. However, the very potent hypotensive effects of GTN limit expanded therapeutic applications for GTN itself. A novel nitrate is reported herein that (1) dissociates the neuromodulatory and cardiovascular properties of nitrates, and (2) represents the first nitrate that is neuroprotective in an animal model of ischemic stroke when administered post-ischemia.

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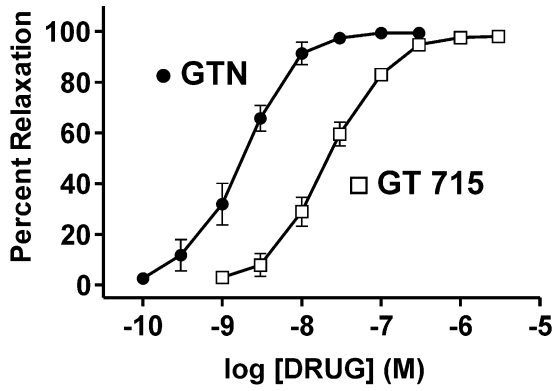

Figure 1. Concentration–response curves for relaxation by GTN and GT715 of isolated rat aortic strips, submaximally contracted with phenylephrine (0.1 μM).[12] Data are expressed as the mean ± S.E.M. EC_{50} values for relaxation were 1.9 ± 0.7 and 23.8 ± 9.6 nM for GTN ($n = 6$) and GT715 ($n = 6$), respectively ($P < 0.001$, Students t-test).

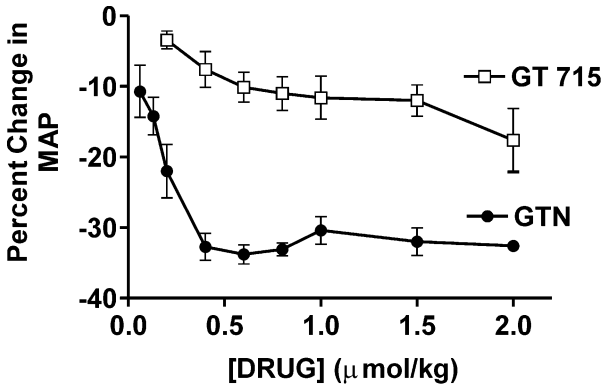

Figure 2. Mean arterial blood pressure (MAP) measured continuously for Inactin®-anaesthetized rats treated with GTN or GT715. Data are expressed as the mean ± S.E.M. Data were obtained from 6 different animals. Measurements were made by connecting an aortic catheter to a pressure transducer for the recording of blood pressure and heart rate. Baseline MAP measurements were collected for 30 min, after which the responses to GTN (0.06–2.0 μmol/kg) or GT715 (0.2–2 μmol/kg) were obtained. Each dose was administered iv over 1 min, with an interval sufficient to allow blood pressure to stabilize to pre-infusion level.

The SS-nitrate, GT715, ($[O_2NOCH_2CHONO_2CH_2S]_2$) is a member of a family of disulfide-containing nitrate esters that have been shown to release a readily measurable flux of NO, in contrast to GTN, on reaction with thiols.[7,10] SS-nitrates cannot, in general, be obtained from nitration of hydroxyalkyl disulfides because of the strong oxidizing conditions of nitration media.[11] We have explored a number of routes to GT715 (and other SS-nitrates), including: oxidation of $O_2NOCH_2CHONO_2CH_2SH$; reaction of samarium salts with $O_2NOCH_2CHONO_2CH_2SCN$; and nitration of the precursor diallyl disulfide with $I_2/AgNO_3$ or $Tl(NO_3)$. However, oxidation of the precursor Bunte salt is preferred, with a yield of $\geq 60\%$ obtained from minor modifications of our previously published procedure.[11]

The ability of GTN and GT715 to induce relaxation of vascular smooth muscle was compared in rat thoracic aortic strips, precontracted submaximally with phenylephrine.[12] The cumulative concentration–response curves obtained demonstrated that GT715 differed from GTN by over an order of magnitude in its ability to relax isolated rat aorta in vitro (Fig. 1).

The effects of GTN and GT715 on blood pressure, in vivo, were compared using continuous blood pressure measurements made on anaesthetized rats. Intravenous administration of GTN produced substantial reductions in mean arterial pressure. In contrast, intravenous administration of GT715 over the same dosage range produced minimal effects on blood pressure (Fig. 2). The difference in potency for vascular relaxation in vitro correlated well with the relatively weak effect of GT715 on systemic blood pressure in the whole animal.

The temporary middle cerebral artery occlusion (MCAO) model was used to measure the influence of GT715 on infarct volume (Table 1). Cerebral ischemia was induced in rats under halothane anesthesia by occlusion of the origin of the right middle cerebral artery with a blunted, silicone coated nylon thread. The thread was removed after 2 h, following re-anesthesia. The rats were sacrificed after 24 h under light halothane anesthesia and the brains sectioned. Each 2-mm thick coronal section was stained with 2% triphenyltetrazolium and fixed in 4% paraformaldehyde, to allow imaging and quantification of the total infarct volume by a technician blinded to the treatment protocol. The areas of total and cerebral cortical infarct were scored in each section to obtain infarct volumes.

GT715 in DMSO, or DMSO vehicle was administered by subcutaneous injection 2 h or 4 h post-ischemia (Table 1; Fig. 3). Analysis of brain sections indicated a

Table 1. Brain infarct volumes (mm^3) in rats treated with GT715 or vehicle 2 or 4 h delay post-ischemia

Delay	Total	Cortical
2 h (vehicle)	221 (35)	156 (30)
2 h (GT715)	86 (20)	50 (23)
4 h (vehicle)	232 (37)	160 (32)
4 h (GT715)	97 (25)	45 (21)

Means ± (SEM), $n = 10$.

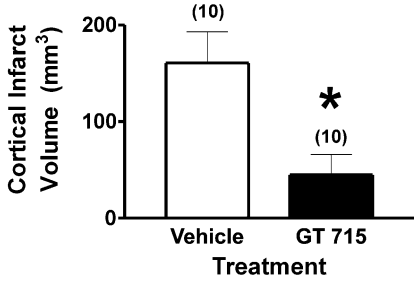

Figure 3. Effect of GT715, administered 4 h after the onset of ischemia, on the outcome of ischemic brain injury. The volume of the total and cerebral infarct in the brains of GT715-treated animals was significantly decreased compared to vehicle-treated animals (*, $P < 0.01$, Student's t-test). GT715 in DMSO, or DMSO vehicle was administered by subcutaneous injection: (a) 2 h post-ischemia (in five divided doses of 200 μmol/kg body weight at 2, 4, 5, 6, 8, and 10 h after the onset of ischemia), see Table 1; or (b) 4 h post-ischemia (in five divided doses of 200 μmol/kg body weight 4, 5, 6, 8, and 10 h after the onset of ischemia.

significant rescue of ischemic brain tissue, especially in the cerebral cortex, mediated by GT715.

The hypothesis that GTN and other classical nitrates, such as isosorbide dinitrate (ISDN), must undergo biotransformation in vivo to give NO, was developed hand-in-hand with the elucidation of the identity of Endothelium Derived Relaxing Factor as NO.[13,14] Although the mechanism of nitrate biotransformation is yet to be defined, there is ample evidence that nitrates can act as NO-mimetics in vivo.[9] Other nitrovasodilators are known, such as nitroprusside, which act as NO-donors, but these are of limited clinical utility. Similarly, various classes of NO-donors, such as diazeniumdiolates and nitrosothiols are under intense study, but are not presently in clinical use, their utility being limited to applications as biological probes.[15,16] Indeed, the prevalent use of NO-donors, to determine the biological functions of NO itself, has sometimes led to perplexing biological activity data. It is likely that some of the observed disparities can be explained by differences in the biological chemistry of the NO-donors themselves, including rates of NO release, tissue selectivity for NO release, competition with both nitrosylation and nitration reactions, and biological activity inherent to the intact NO-donor. These attributes may be exploited to develop selective NO-mimetic small molecules that have potential as therapeutic agents.[15,16] Nitrates represent persuasive targets because as a class they are clinically proven, and because they are not spontaneous, uncontrolled NO-donors.[17]

Lipton's seminal work on the interaction of NO with NMDA receptors showed that some nitrovasodilators are neuroprotective in models of NMDA receptor-mediated excitotoxic neuronal injury.[2] In the temporary MCAO rat model of focal ischemic stroke, GTN (25 mg/kg) when administered iv during the 2 h ischemic period, was shown to reduce total infarct volume by 20% relative to controls in animals sacrificed at 24 h.[18] The potent hypotensive activity of GTN required continuous co-administration of the pressor agent, pheneylephrine, and resulted in only a modest increase in cerebral blood flow in the penumbra.

ISDN has also been reported to provide neuroprotection in an MCAO model, but in this case, pretreatment 1 h before ischemia, with subsequent continuous administration was required to demonstrate a neuroprotective effect.[19] The use of potent vasodilators such as GTN is contraindicated in conditions of cerebral ischemia, since they may potentially worsen cerebral blood flow to infarcted areas due to their hypotensive effects. This limitation might be overcome by dissociation of (1) the neuroprotective effects of sGC activation and elevation of tissue cGMP levels in the brain, from (2) the systemic effects of sGC activation and elevation of tissue cGMP levels in vascular smooth muscle, leading to vasorelaxation and decreases in systemic blood pressure.

GTN and GT715 were shown to exert differential effects on cardiovascular function, with the novel nitrate, GT715, being a weaker vasodilator with minimal effects on mean arterial pressure in the whole animal compared to GTN. We have previously shown that GT715 is both more potent and more effective as an activator of sGC in the brain, and more effective in elevating cGMP levels in hippocampal brain slices, compared to GTN.[20] In contrast, GTN produces a much greater accumulation of cGMP in vascular tissue compared to GT715 (data not shown), which is consistent with the relatively weak vascular effect of GT715. Thus, the neuromodulatory and systemic hypotensive effects of nitrates can clearly be dissociated.

In order to examine the neuroprotective activity of GT715, the temporary MCAO rat model of focal ischemic stroke was used. The post-ischemic neuroprotective effects of this novel nitrate were studied, because post-ischemic administration is a clinically relevant paradigm. GT715 reduced the total infarct volume by 60–70% relative to vehicle controls when administered post-ischemia (Table 1).

There is likely more than one neuroprotective mechanism for GT715 in cerebral ischemia. The NO-sGC-cGMP signal transduction system has been linked to several potential neuroprotective pathways in the brain. NO possesses neuroprotective properties related to activation of sGC and the production of cGMP, since cGMP has been found to protect neurons against excitotoxic injury,[21] and to promote neuronal survival and inhibit apoptotic cell death in a number of neuronal cell types.[22] Furthermore, cyclic nucleotides (cAMP and cGMP) attenuate lipid peroxidation-mediated neuronal injury,[23] and cGMP decreases both resting intracellular Ca^{2+} levels and the elevations in intracellular Ca^{2+} concentrations that follow exposure to glutamate.[24] Moreover, the neuroprotective properties of the soluble β-amyloid precursor protein have been attributed to selective elevation of intracellular cGMP levels and activation of a cGMP-dependent protein kinase.[25] Recently, it also was demonstrated that elevating cellular levels of cGMP depresses excitatory synaptic transmission in the hippocampus, possibly via a direct, PKG-independent interaction between cGMP and the α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) subtype of excitatory amino acid receptors.[26] Thus, activation of sGC and elevations in intracellular cGMP levels may act via multiple biochemical pathways, to ensure the survival of neurons subjected to ischemic injury, Ca^{2+} overload, or oxidative stress.

Despite the nearly ubiquitous nature of NO-sGC-cGMP signaling in the body, the molecular characteristics of sGC are relatively poorly understood. sGC is a hemoprotein that is assembled as a heterodimer consisting of α and β subunits, and is activated endogenously by the binding of NO to the heme iron.[27] There are at least two subunit subtypes within each of the α and β subunit families (α1, α2, β1, β2), and recent data suggests that there may also be splice variants within the subunit subtypes that could add to the diversity of possible enzyme assemblies.[28] Nitrates function as NO-mimetics capable of activating sGC.[7] However, differences in

the molecular structure of sGC, in regulation of sGC, and in the tissue localization of sGC will lead to differences in the efficacy of activation of the enzyme by different nitrates.

The known biological actions of NO can be classified as either sGC/cGMP dependent or independent. Amongst the sGC/cGMP-independent properties associated with NO are (a) protein nitrosylation and (b) antioxidant activity. Lipton has argued that inhibition of NMDA receptor mediated responses by NO, probably through protein nitrosylation, affords neuroprotection.[2] Whilst NO and some NO-donors may be potent inhibitors of NMDA stimulated respones, rather high IC_{50} values of 1.2 and 1.7 mM have been reported for GTN and ISDN, respectively.[29] There is good evidence that NO is a potent chain-breaking antioxidant.[30] However, we have observed that GTN possesses no antioxidant activity in a variety of lipid peroxidation models, including Fe(II)-induced peroxidation in cerebrocortical homogenates.[31] In contrast, we observed that GT715 did provide modest inhibition of lipid peroxidation in the presence of added thiols.

The novel nitrate, GT715, effectively allows dissociation of the neuromodulatory and peripheral hypotensive effects of nitrates, providing substantial neuroprotection in an animal model of focal ischemic stroke when administered 4 h post-ischemia. Interestingly, we have also observed that GT715 reverses the cognitive impairment caused by scopolamine in a model of Alzheimer's dementia, whereas GTN is ineffective.[20]

Acknowledgements

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