UPEI-100, A CONJUGATE OF LIPOIC ACID AND APOCYNIN, MEDIATES NEUROPROTECTION IN A RAT MODEL OF ISCHEMIA/REPERFUSION

Barry J. Connell, Monique C. Saleh, Bobby V Khan, Desikan Rajagopal and Tarek M. Saleh*

Department of Biomedical Science, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada, C1A 4P3 and InVasc Therapeutics, Atlanta, Georgia USA

Running head: UPEI-100 mediates neuroprotection following I/R

*Address for Correspondence:

Tarek M. Saleh, PhD,
Professor and Chair
Dept. Of Biomedical Sciences
Atlantic Veterinary College
University of Prince Edward Island
Charlottetown, P.E.I., Canada
C1A 4P3
phone : (902) 566-0819
fax : (902) 566-0832
email : tsaleh@upei.ca
ABSTRACT

Previous work in our laboratory has provided evidence that pre-administration of apocynin and lipoic acid at subthreshold levels for neuroprotection, enhanced the neuroprotective capacity when injected in combination. Therefore, the present investigation was designed to determine if a co-drug consisting of lipoic acid and apocynin functional groups bound by a covalent bond, named UPEI-100, is capable of similar efficacy using a rodent model of stroke. Male rats were anaesthetized with Inactin (100 mg/kg, iv) and the middle cerebral artery was occluded for 6 hours (MCAO), or allowed to reperfuse for 5.5 hrs following a 30 min occlusion (ischemia/reperfusion; I/R). Pre-administration of UPEI-100 dose-dependently decreased infarct volume in the I/R model (p<0.05), but not in the MCAO model of stroke. Using the optimal dose, UPEI-100 was then injected during the stroke or at several time points during reperfusion and significant neuroprotection was observed when UPEI-100 was administered up to 90 mins following the start of reperfusion (p<0.05). A time course for this neuroprotective effect showed that UPEI-100 resulted in a decrease in infarct volume following 2 hours of reperfusion compared to vehicle. The time course of this neuroprotective effect was also used to study several mediators along the antioxidant pathway and showed that UPEI-100 increased the level of mitochondrial superoxide dismutase (SOD2) and oxidized glutathione (GSSG) and decreased a marker of lipid peroxidation due to oxidative stress (HNE-His adduct formation). Taken together, the data suggest that UPEI-100 may utilize similar pathways to those observed for the two parent compounds; however, it may also act through different mechanism of action.

Keywords: Co-drug, stroke, autonomic function, oxidative stress.
INTRODUCTION

Ischemic stroke due to occlusion of cerebral vasculature, results in hypoxia and hypoglycemia, failure of ATP-dependent pumps, disruption of ionic equilibrium and calcium homeostasis, excitotoxicity and eventual cell death (for review, see 24). Reintroduction of blood flow (ie. with thrombolytic therapy) can arrest and/or reverse these adverse events. However, if recanalization is delayed beyond 4 to 6 hours, further neuronal death, known as reperfusion injury, will occur due to the formation of reactive oxygen species (ROS; 19). Several laboratories have demonstrated that NADPH oxidase (NOX) is a major source of superoxide generation and that NOX is involved in mediating ischemia-reperfusion (I/R)-induced neuronal death (3, 9). The pathophysiological importance of NOX in hypoxia has led many researchers to attempt to modify the activity of NOX in both permanent and transient (I/R) animal models of stroke.

Apocynin is regarded as a powerful antioxidant and anti-inflammatory agent, primarily by interfering with the assembly of the cytosolic and membrane-bound subunits to inhibit NOX activation (3, 39). For this reason, apocynin has been tested and shown to have promise as a neuroprotectant in many rat models of stroke through its ability to attenuate ischemic damage following cerebral I/R (14, 22, 41, 42). However, the enthusiasm over the potential clinical benefit of apocynin as a neuroprotectant has been dampened due to the narrow therapeutic dose-range in which apocynin is effective (22, 40). This is likely due to the high dose of apocynin required to measure neuroprotective effects (39), as well as the fact that apocynin has been shown, under certain circumstances, to stimulate ROS production and thereby increase cellular oxidative stress (7).

Lipoic acid (LA) is an antioxidant with strong free radical scavenging abilities (4) and several researchers have shown in different rat models of stroke, that administration of LA
produced significant neuroprotection by decreasing infarct size (15, 31, 33, 43). Further, several laboratories have demonstrated that administration of LA in combination with another compound can produce significant neuroprotective effects exceeding the effect of either drug alone (1, 2, 18, 20). Our laboratory has provided further support for this hypothesis and demonstrated that when using the same stroke modes as is being used in this publication, co-administration of non-protective doses of apocynin with non-protective doses of LA produced significant neuroprotection compared to either drug injected alone (13).

There has been recent interest in the use of co-drugs as a therapeutic approach in various pathologies (16). In several different animal models of pathology, the development and administration of a co-drug, containing LA covalently linked to another therapeutic drug, has been shown to have a greater positive effect compared to the injection of a solution containing a mixture of the two drugs (16, 38). However, to the best of our knowledge, no research has been published examining the use of co-drug as a neuroprotectant following I/R injury. Based on our previous observation demonstrating the beneficial effects of co-administration of a solution containing apocynin with LA (13), our laboratory has developed a co-drug that is a covalent conjugate between LA and apocynin (named UPEI-100).

There is a limited window of opportunity following cerebral vascular occlusion for thrombolytic therapy to protect against further I/R-induced cell death (4 hours following the onset of clinical signs in humans). This represents a critical time frame within which to study the efficacy of neuroprotectants. Also, most cardiovascular consequences following stroke occur within the first 4-6 hrs post-stroke in humans (27). Therefore, it is our intention to examine the acute, neuroprotective effects of a co-drug, UPEI-100, using a rodent model of I/R recently developed and validated in our laboratory (12). We examined tissue harvested from the ischemic
cortex in animals pretreated with UPEI-100 to determine the effect of UPEI-100 on various cellular antioxidant pathways.
MATERIALS AND METHODS

All experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Prince Edward Island Animal Care Committee.

General surgical procedures

All experiments were conducted on male Sprague-Dawley rats (total of 229 rats; 250-350 g; Charles Rivers; Montreal, PQ, CAN). For all animals, food and tap water were available ad libitum. Rats were anaesthetized with sodium thiobutabarbitral (Inactin; Sigma-Aldridge; St. Louis, MO, USA; 100 mg/kg; ip), which provided a stable plane of anesthesia for the full duration of the experimental time period. To monitor blood pressure and heart rate, a polyethylene catheter (PE-50; Clay Adams, Parsippany, NJ, USA) was inserted into the right femoral artery. For intravenous administration of drugs, a second polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, USA) was inserted into the right femoral vein. Arterial blood pressure was measured with a pressure transducer (Gould P23 ID, Cleveland, OH) connected to a Gould model 2200S polygraph. Heart rate was determined from the pulse pressure using a Gould tachograph (Biotach). These parameters were displayed and analyzed using PolyviewPro/32 data-acquisition and analysis software (Grass; Warwick, RI, USA). An endotracheal tube was inserted to facilitate breathing. Body temperature was monitored and maintained at 37 ± 1°C using a Physitemp feedback system (Physitemp Instruments; Clifton, NJ, USA).
**Middle cerebral artery occlusions (MCAO)**

We have previously published the detailed methodology for permanent (MCAO) and transient (I/R) occlusion of the middle cerebral artery (12). Briefly, animals were placed in a David Kopf stereotaxic frame (Tujunga, CA, USA) and the right middle cerebral artery (MCA) approached through a rostra-caudal incision of the skin and frontalis muscle at the approximate level of bregma. Blood flow through the MCA was impeded by the placement of surgical suture behind the MCA at 3 designated positions along the exposed vessel. The sutures were positioned so that the middle of each suture applied pressure to the underside of the MCA and impeded blood flow as confirmed using laser Doppler flowmetry (OxyFlo, Oxford-Optronix, Oxford, UK; 12). This 3-point placement of surgical sutures produced a highly reproducible and consistent focal ischemic lesion (permanent ischemia) restricted to the prefrontal cerebral cortex. For transient, ischemia-reperfusion (I/R), the sutures were left in place for 30 mins and then removed (reperfusion) to allow blood flow to return to the area for an additional 5.5 hours (209 rats received I/R). In 12 rats, sutures were left in place for 6 hours as a model of permanent ischemia.

**UPEI-100 synthesis**

To synthesize UPEI-100, LA and apocynin were linked via a by covalent bond between the phenol hydroxy group of apocynin and the carboxylic acid group of LA. Apocynin (0.01M) was taken in a well dried 100 mL round bottom flask followed by (0.01M) of LA, 0.01M of dimethylaminopyridine (DMAP) in 50 mL anhydrous dichloromethane (CH₂Cl₂). The dicyclohexylcarbodiimide (DCC; 0.01M) was added in small quantities over a period of 45 minutes. The entire reaction was performed under nitrogen atmosphere. After stirring overnight, the pure compound was purified on a silica column chromatography after an aqueous work up.
The pure compound was characterized by proton nuclear magnetic resonance spectroscopy and mass spectrometry.

Apocynin  Lipoic Acid     UPEI-100

**Effect of UPEI-100 on infarct volume following both transient (I/R) and permanent MCAO**

To examine the effect of UPEI-100 on I/R-induced cell death, injections of UPEI-100 (0.01, 0.05, 0.1 or 0.5 mg/kg; 1 ml/kg; iv; n= 4 per group) or vehicle (25% EtOH; 1 ml/kg; iv; n=6) were made 30 minutes prior to the onset of MCAO. The sutures were left in place for 30 minutes and removed. Reperfusion was allowed for 5.5 hours and the experiment was terminated.

To examine the effect of UPEI-100 on ischemia-induced cell death only, injections of UPEI-100 (0.1 or 1.0 mg/kg; 1 ml/kg; iv; n= 4 per group) or vehicle (25% EtOH; 1 ml/kg; iv; n=4) were made 30 minutes prior to 6 hours of permanent MCAO (sutures left in place for 6 hours). The concentration of 0.1 mg/kg represents the optimal dose of UPEI-100 as determined above and 1.0 mg/kg represents a 10-fold higher concentration of the optimal dose, as the optimal dose of 0.1 mg/kg was not effective in producing neuroprotection. The experiments were terminated at the end of the 6 hours of occlusion.

To determine if UPEI-100 was neuroprotective when administered during the 30 minute period of occlusion, injections of UPEI-100 at a dose which produced optimal neuroprotection based on the dose-response-curve described above (0.1 mg/kg; 1 ml/kg; iv; n=4) or vehicle (25%
EtOH; 1 ml/kg; iv; n=4) were made 15 minutes following the onset of MCAO. After a further 15 minutes, the sutures were removed to allow for an additional 5.5 hours of reperfusion.

To examine the effect of UPEI-100 on reperfusion injury alone, injections of the optimal dose of UPEI-100 (0.1 mg/kg; 1 ml/kg; iv; n=4 per group) or vehicle (1 ml/kg; iv; n=4 or 5 per group) were made following 30 minutes of MCAO, immediately prior to the removal of the sutures, or 30, 60, 90, 120 or 180 minutes following suture removal (start of reperfusion). In all cases, the experiments were terminated following 5.5 hours of reperfusion.

To determine if UPEI-100 required bioactivation and/or metabolism to an active intermediate in order to produce neuroprotection, we investigated the effect of direct intracortical injections of UPEI-100 on infarct volume in our model of transient occlusion (I/R). UPEI-100 (0.01, 0.1, 0.5 or 1.0 µM in 200 nl; n=4 per group) or the same volume of vehicle (0.0125% EtOH; 200 nl; n=6) was injected into the ipsilateral hemisphere (bregma -0.3 mm, lateral -5.5 mm and depth of -3.0 mm from the dorsal surface of the brain; 32) 10 minutes prior to suture placement followed by 30 minutes of MCAO and 5.5 hours of reperfusion.

**Effect of UPEI-100 on baseline blood pressure and heart rate**

To determine the effect of UPEI-100 (0.1 mg/kg; 1 ml/kg; n=4) or vehicle (25% EtOH; 1 ml/kg; n=4) administration on baseline blood pressure and heart rate, continuous recordings of blood pressure and heart rate were taken prior to and following drug or vehicle administration. Further recordings of the parameters were made at 15, 30, 45, 60, 90 and 120 minutes following drug administration, at which time the experiment was terminated.
Effect of UPEI-100 on cardiac baroreceptor reflex sensitivity, blood pressure and heart rate following I/R

To determine the effect of drug administration on autonomic reflex activation, the baroreceptor reflex was evoked using a bolus intravenous injection of the α-adrenergic receptor agonist, phenylephrine-hydrochloride (Sigma-Aldridge; St.Louis, MO, USA; 0.1 ml; 2.5 μg/ml; iv). The ratio of the peak change in the magnitude of the reflex bradycardia to the magnitude of the phenylephrine-induced pressor response (ΔHR/ΔMAP) was used as a measure of baroreceptor reflex sensitivity (BRS; 51). BRS was tested at 10 minutes and immediately prior to UPEI-100 (0.1 mg/kg; 1 ml/kg; n=3) or vehicle (25% EtOH; 1 ml/kg; n=3) administration. BRS was then tested 15 and 30 minutes following drug administration (prior to MCAO), and then 5, 10, 20 and 30 minutes during MCAO (30 minute MCAO), as well as 10, 20, 30, 60, 90, 120, 150, 210, 270 and, 330 minutes following suture removal. The experiments were terminated after 5.5 hours of reperfusion.

Time course of the effect of UPEI-100 on infarct volume following I/R

To examine the effect of UPEI-100 on the change in infarct volume over the 5.5 hours of reperfusion, injections of the optimal dose of UPEI-100 (0.1 mg/kg; 1 ml/kg; iv; n=4 per group) or vehicle (25% EtOH; 1 ml/kg; iv; n=4) were made 30 minutes prior to the onset of MCAO. The sutures were left in place for 30 minutes. Brains were removed and infarct volume was measured at the end of 30 minutes of MCAO, or at 30 minutes, 1, 2, 4 or 5.5 hours following reperfusion (n = 4-5 per group).
Histological Procedures

At the end of each experiment where infarct volume was measured, animals were transcardially perfused with phosphate buffered saline (PBS; 0.1 M; 200 mls), the brains removed and sliced into 1 mm coronal sections using a rat brain matrix (Harvard Apparatus; Holliston, MA, USA). Sections were incubated in a 2% solution of 2,3,5-triphenol tetrazolium chloride (TTC; Sigma-Aldrich; St. Louis; MO, USA) for 5 minutes. Infarct volumes were calculated with the use of scanned digital images of each brain section. The infarct area for both sides of each brain section was calculated using a computer-assisted imaging system (Scion Corporation; Frederick, MD, USA). The infarct areas for each side for each individual section were averaged and multiplied by the width of each section (1mm) to give the infarct volume for each section. The sum total of all the individual infarct volumes provided the infarct volume for each rat.

Effect of UPEI-100 on markers of oxidative stress

To examine the effect of UPEI-100 on various markers of oxidative stress throughout the 5.5 hours of reperfusion, injections of the optimal dose of UPEI-100 (0.1 mg/kg; 1 ml/kg; iv; n=5 per group at each time point) or vehicle (25% EtOH; 1 ml/kg; iv; n=5 per group at each time point) were made 30 minutes prior to the onset of MCAO. The sutures were left in place for 30 minutes. Brains were removed and cortical tissue was collected at the end of the 30 mins of occlusion (no reperfusion), or following 30 minutes, 1, 2, 4 or 5.5 hours of reperfusion. At the end of each time point, animals were perfused transcardially with 200 mL of 100 mM phosphate buffered saline (pH 7.4) and the brains removed. The ipsilateral cerebral cortex was isolated and an 8 mm diameter biopsy punch was used to obtain tissue from the ischemic core. The tissue
was weighed and homogenized (40% w/v) in ice cold PBS. Aliquots of whole homogenate were frozen -80°C immediately for measurements of hydrogen peroxide (H₂O₂). The remaining sample was diluted 1:2 with ice-cold PBS and centrifuged 12,000 X g for 15 min at 4°C. Aliquots of the supernatant were stored at -80°C until assayed for protein levels, superoxide dismutase (SOD1 and SOD2) activity, 4-hydroxynonenal-histidine (HNE-His) adducts (OxiSelect Kits, Cell Biolabs Inc; San Diego, CA, USA), reduced glutathione (GSH), oxidized glutathione (GSSG; Cayman Chemical Company, Ann Arbor MI, USA), and cytoplasmic enrichment of DNA fragments (apoptosis) (Roche Diagnostics, Montreal, QC, CAN) using selective assay kits according to manufacturer’s specifications.

Statistical analysis

Data were analyzed using a statistical software package (SigmaStat and SigmaPlot; Jandel Scientific, Tujunga, CA). All data are presented as a mean ± standard error of the mean (S.E.M). Differences were considered statistically significant if p ≤ 0.05 by an analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis. When only two groups were being compared the Student’s t-test was used.
RESULTS

The effect of pre-administration of UPEI-100 on infarct volume

This experiment was designed to determine the effect of UPEI-100 pre-administration on infarct volume following I/R. Pre-administration of UPEI-100 resulted in a dose-dependent neuroprotection with a dose of 0.1 and 0.5 mg/kg resulting in a significant decrease in infarct volume compared to the administration of vehicle (p≤0.05; Fig. 1A).

To examine the effect of UPEI-100 on ischemia-induced cell death only, injections of UPEI-100 (0.1 or 1.0 mg/kg) or vehicle were made 30 minutes prior to 6 hours of MCAO (sutures left in place for 6 hours). Neither concentration of UPEI-100 (the optimal dose of 0.1 mg/kg as determined above or 1.0 mg/kg, a 10-fold increase in the optimal dose) produced significant neuroprotection when infarct volume was measured 6 hours following the start of MCAO (p≥0.05; Fig. 1B).

The effect of systemic UPEI-100 injection on blood pressure and heart rate

The following experiment was designed to determine the effect of UPEI-100 on arterial pressure and heart rate for a period of 2 hours following administration. Mean baseline arterial pressure and mean heart rate prior to drug administration was 112 ± 11 mm/Hg and 388 ± 26 bpm. Administration of UPEI-100 (0.1 mg/kg; i.v.) did not significantly alter mean arterial blood pressure or mean heart rate at any time point during the 2 hour continuous recording compared to vehicle (p≥0.05; data not shown).
The effect of UPEI-100 pre-administration on cardiovascular parameters following I/R

The following experiment was designed to determine the effect of pre-administration of UPEI-100 on mean arterial pressure, mean heart rate, and the mean cardiac baroreceptor reflex sensitivity (BRS) before, during and following 30 minutes of MCAO. Mean arterial pressure, heart rate and BRS prior to UPEI-100 administration were 119 ± 15 mmHg, 402 ± 22 bpm, and 0.55 ± 0.05 bpm/mmHg respectively and prior to vehicle administration were 110 ± 9 mmHg, 398 ± 19 bpm, and 0.52 ± 0.05 bpm/mmHg respectively. These values did not change following UPEI-100 or vehicle administration prior to MCAO (p \geq 0.05 for all comparisons; Fig. 2A, 2B and 2C). During 30 minutes of MCAO and during 5.5 hours of reperfusion, there were no significant differences in the mean arterial pressure or mean heart rate compared to pre-MCAO values (p \geq 0.05 for both UPEI-100 and vehicle; Fig. 2A and 2B). However, mean BRS values in both the UPEI-100 and vehicle groups were equally significantly decreased within 5 minutes of the beginning of MCAO (0.23 ± 0.05 bpm/mmHg and 0.28 ± 0.08 bpm/mmHg respectively; p \leq 0.05 for both groups compared to pre-MCAO values; Fig 2C) and remained significantly depressed throughout the 30 minutes of MCAO (p \leq 0.05 for both groups compared to pre-MCAO values; Fig 2C). The mean BRS for both groups remained significantly decreased compared to pre-MCAO values throughout the 5.5 hours of reperfusion (p \leq 0.05 at all time points measured for both groups compared to pre-MCAO values; Fig 2C).

The effect of UPEI-100 on infarct volume when administered either during MCAO or following the start of reperfusion

UPEI-100 or vehicle was injected (iv) at various time points during MCAO or following the start of reperfusion. There were no significant differences in the mean infarct volumes when
vehicle was injected during MCAO or at any time point during reperfusion (p≥0.05), therefore, the vehicle data for all time points was pooled (n=29) in Figure 3. All statistical comparisons were made between the infarct volumes measured following vehicle and UPEI-100 administration at each time point.

Administration of UPEI-100 (0.1 mg/kg; iv) 15 minutes into a 30 minute period of MCAO (15 minutes prior to the start of reperfusion) produced significant neuroprotection compared to vehicle when infarct volume was measured following 5.5 hours of reperfusion (p≤0.05; Fig. 3).

We determined the effect of UPEI-100 on reperfusion injury only, by measuring the infarct volume following drug administration immediately prior to suture removal, or 30, 60, 90, 120, or 180 minutes following the start of reperfusion. Administration of UPEI-100 (0.1 mg/kg) at time 0 (start of reperfusion), and 30, 60 and 90 minutes following the start of reperfusion resulted in significant decreases in infarct volume compared to vehicle administration (p≤0.05 at each time point; Fig. 3). Administration of UPEI-100 (0.1 mg/kg) 120 and 180 minutes following suture removal did not result in a significant difference in infarct volume compared to the administration of vehicle at those time points (p≥0.05; Fig. 3).

The effect of intracortical injections of UPEI-100 on infarct volume following I/R

This experiment was designed to determine the effect of direct cortical pre-injection with UPEI-100 on infarct volume following I/R. UPEI-100 pre-injection resulted in a dose-dependent neuroprotection with a dose of 1.0 µM resulting in a significant decrease in infarct volume compared to the intracortical pre-injection of vehicle (p≤0.05; Fig.4).
**Time course for the effect of UPEI-100 on infarct volume**

In animals pre-treated with UPEI-100 (0.1 mg/kg) or vehicle and undergoing MCAO for 30 minutes, we observed a rapid increase in infarct volume from 30 minutes into the reperfusion period until 2 hours of reperfusion (Fig. 5). After 2 hours of reperfusion, the mean infarct volume continued to increase significantly in the vehicle group. In contrast to the vehicle pre-treated group, the mean infarct volume in the UPEI-100 group did not increase further during the remaining reperfusion period (Fig. 5). In addition, the mean infarct volume in the UPEI-100 group was significantly smaller than the vehicle pre-treated group when measured at 4 and 6 hours ($p \leq 0.05$ at each time point; Fig. 5). By the end of the 5.5 hours of reperfusion, the mean infarct volume of the UPEI-100 group was approximately 53 % smaller than the vehicle group.

**Effect of UPEI-100 on SOD activity**

In animals pre-treated with UPEI-100 or vehicle and undergoing MCAO for 30 minutes, we did not observe any significant differences in cytoplasmic SOD1 activity levels between these two groups at any time point during 5.5 hours of reperfusion ($p \geq 0.05$ for each time point measured; Fig. 6A).

In contrast, mitochondrial SOD2 activity levels at the 4 hour time point were significantly higher in the UPEI-100 group compared to vehicle ($p \leq 0.05$; Fig. 6B). There were no other significant differences measured at any time point, however, the SOD2 activity levels were consistently higher in the UPEI-100 group compared to those in the vehicle pre-treated group at each time point (Fig. 6B).
**Effect of UPEI-100 on glutathione (GSH) levels**

We did not measure any significant differences in the total amount of GSH (includes both oxidized and reduced forms) in brain samples taken from either UPEI-100 or vehicle treated rats at any time point (p ≥ 0.05 for each comparison; Fig. 6C). In contrast, the amount of the oxidized form of GSH (GSSG) was significantly greater in the UPEI-100 treated rats than the amount of GSSG measured in vehicle treated rats after 5.5 hours of reperfusion (p ≤ 0.05; Fig. 6D). There was no difference in the level of GSSG between the two groups at any other time point (p ≥ 0.05 at each time point; Fig. 6D).

**Effect of UPEI-100 on hydrogen peroxide levels**

In UPEI-100 pre-treated rats, the level of hydrogen peroxide was not significantly greater than the level of hydrogen peroxide in vehicle treated rats at any time point over the 5.5 hours of reperfusion (p > 0.05; Fig. 6E). However, in both UPEI-100 and vehicle pre-treated groups, the level of hydrogen peroxide increased significantly over time (p < 0.05; Fig. 6E).

**Effect of UPEI-100 on protein (HNE-His) adduct levels**

The following experiment was designed to determine whether UPEI-100 mediated neuroprotection resulted in a change in HNE-His adduct levels. HNE-His adduct levels following UPEI-100 administration 30 minutes prior to occlusion were significantly attenuated compared to vehicle administered following 2 hours of reperfusion (p ≤ 0.05; Fig. 6F). HNE-His adduct levels were not significantly different in the two groups at any other time point (p ≥ 0.05 for each time point measured; Fig. 6F).
Effect of UPEI-100 on DNA fragmentation (apoptotic cell death)

The following experiment was designed to determine whether the UPEI-100 mediated neuroprotection observed was mediated by an alteration in the extent of apoptotic cell death. DNA fragmentation was quantified as an indicator of apoptotic cell death. No significant changes in the extent of DNA fragmentation in animals pre-treated with either UPEI-100 or vehicle throughout the experimental time course were observed (p≥0.05 for each time point measured; Fig. 6G).
DISCUSSION

Oxidative stress associated with excessive production of reactive oxygen species (ROS) is a fundamental mechanism of brain damage in reperfusion injury following ischemic stroke. The multiplicity of mechanisms involved in ischemia- and reperfusion-induced neuronal damage following an occlusive stroke remains an obstacle in providing treatment in clinical settings (26). Drugs targeting more than one mechanism of action could potentially overcome this dilemma. The synthesis and development of co-drugs using simple yet biologically relevant molecules as building blocks provides the ability to simultaneously target multiple pathways involved in the pathogenesis of neurological diseases, specifically, pathways involved in the initiation of oxidative stress-induced neuronal damage following reperfusion. Chemical combinations of LA with other compounds have previously been demonstrated to provide neuroprotective effects greater than the two compounds administered on their own. Covalent linkage of LA with ibuprofen has been demonstrated to be neuroprotective in rodent models of Alzheimer’s disease where administration of the co-drug decreased the oxidative damage due to the infusion of Aβ (1-40) (26). In addition, a co-drug produced by chemically linking LA with L-dopa or dopamine decreased neuronal oxidative damage associated with the administration of L-dopa or dopamine alone (17). To the best of our knowledge, there have been no other reports where apocynin has been chemically linked to other compounds and no other reports where administration of a co-drug was used to attenuate damage due to oxidative stress following ischemia or ischemia/reperfusion.

In this study, we determined that UPEI-100, a chemical combination of two naturally occurring antioxidants, LA and apocynin, produced dose-dependent neuroprotection against neuronal cell death as observed in a previously validated, novel model of ischemia-reperfusion (I/R) injury (12). The results demonstrated that UPEI-100 produced dose-dependent, short-term
neuroprotection (within 5.5 hours of reperfusion) in a model of focal ischemia which is restricted
to the cerebral cortex. Further, the dose of UPEI-100 required to produce significant
neuroprotection (0.1 mg/kg) was many fold less compared to the doses required for either
apocynin or LA on their own (14, 15). Also, this optimal dose of UPEI-100 produced significant
neuroprotection when administered 15 minutes prior to the start of reperfusion, just prior to the
induction of reperfusion, and 30, 60 and 90 minutes following the onset of reperfusion. The
reason for administering UPEI-100 during the occlusion was to mimic the clinical situation
where a patient would present during a stroke. This result does not indicate if UPEI-100 or an
intermediate metabolite produced as a result of hepatic biotransformation was responsible for
mediating the neuroprotective effect. We were able to reproduce the dose-dependent
neuroprotection with direct cortical injections into the ischemic area which suggests that the
parent compound UPEI-100, and not a metabolite(s), was responsible for producing the
neuroprotection observed.

There have been other reports which demonstrated an attenuation of infarct volume when
apocynin was administered during an occlusion of the MCA, but these benefits were lost when
administration of apocynin was delayed following the onset of reperfusion (11, 14, 21, 40, 41).
However, one laboratory demonstrated that administration of apocynin in gerbils 5 minutes
following 5 minutes of global ischemia, decreased neuronal degeneration and delayed neuronal
death and microglial activation when assessed 4 days later (42). It is not known if these
apocynin-induced beneficial effects translated to a decrease of infarct size as it was not measured
in that study. We have also demonstrated that administration of the most effective dose of LA (5
mg/kg) did not result in significant neuroprotection when administered just prior to the beginning
of reperfusion, and only produced neuroprotection when administered prior to both occlusion
and reperfusion (15). It therefore appears that UPEI-100, a chemical combination of apocynin and LA is far superior then either compound alone in its ability to provide neuroprotection when administered during reperfusion. Interestingly, UPEI-100 did not produce neuroprotection when administered prior to a 6 hour permanent occlusive stroke (no reperfusion). These results support the suggestion that UPEI-100 produced neuroprotection against reperfusion injury alone, perhaps via decreasing ROS production, as ROS-induced growth of ischemic volume due to reperfusion injury has been demonstrated by many labs (23), and/or possibly via free radical scavenging.

Clinically, elevated sympathetic tone (sympathoexcitation) and abnormal electrocardiograms have been observed within 1 to 2 hours following thrombolytic or hemorrhagic stroke involving the MCA (27, 28). Such autonomic dysfunction increases the risk of sudden cardiac death (27, 28) and can be mimicked in rat models of MCAO (8). Arrhythmogenesis and sudden cardiac death, which can occur following MCAO in humans, is associated with depressed baroreflex sensitivity (BRS; 5). Our results have demonstrated that during the 30 minute occlusion, BRS was significantly depressed and administration of UPEI-100 did not alter the level of BRS depression despite the UPEI-100-induced neuroprotection. The increase in neuronal survival represented by an attenuated ischemic volume was not associated with recovery of autonomic function. Our laboratory has demonstrated a similar dissociation between changes in infarct volume and autonomic function following drug intervention prior to MCAO (34). We suggest that for an agent injected systemically, it may be required to have multiple sites of action within the CNS as well as in the periphery (such as the myocardium) to decrease or reverse the sympathoexcitation and subsequent autonomic function observed following stroke. Many studies have demonstrated significant neurochemical and
electrophysiological alterations in extracortical autonomic and cardiovascular regulatory nuclei shortly following MCAO (35). This suggests that any drug which demonstrates functional cardiovascular protection may be required to act extracortically to prevent an abnormal sympathetic outflow and to restore or prevent changes in autonomic function following MCAO.

Neuronal hypoxia and the ensuing mitochondrial response are involved in both the initiation of both necrotic and apoptotic pathways leading to cell death (10). Severe cerebral ischemia causes neuronal mitochondria to be unable to produce adenosine triphosphate leading to necrotic cell death (10). The mitochondrial antioxidant enzyme, manganese superoxide dismutase (SOD2) is the primary cellular defense enzyme involved protecting cells from oxidative stress (10) and has been shown to reduce oxidative stress following cerebral I/R (10) as SOD2-deficient mice have enhanced infarct size following cerebral ischemia (11) and overexpression of SOD2 provided neuroprotection following cerebral ischemia (11). We observed enhanced SOD2 activity throughout the 5.5 hours of reperfusion (reaching significance following 4 hours of reperfusion) when UPEI-100 was administered 30 minutes prior to I/R. We have previously demonstrated using the same model of I/R that LA administered 30 minutes prior to I/R also increased SOD2 activity (15) while apocynin was ineffective (14) in altering SOD2 activity. UPEI-100 did not alter cytosolic copper-zinc SOD (CuZnSOD or SOD1) activity which is consistent with previous results from our laboratory where neither of the two parent compounds, apocynin or lipoic acid, altered SOD1 activity (14, 15). There appears to be support in the literature for a selective effect of LA on mitochondrial function as LA is a protein bound cofactor for mitochondrial α-ketoacid dehydrogenase and serves a critical role in mitochondrial energy metabolism (37). In addition, exogenous LA is reduced to dihydro-lipoic acid (DHLA) within the mitochondria (37) and both lipoic acid and DHLA have been shown to
have powerful antioxidant activity and ROS scavenger abilities (29). Therefore, we conclude that at least part of UPEI-100-induced neuroprotection may be due to the actions of the LA functional group of UPEI-100 on SOD2 activity.

Glutathione is another key intracellular antioxidant and protects cells by scavenging free radicals (for review, see 36). Glutathione is involved with the breakdown of peroxides, regulating the nitric acid cycle, DNA synthesis and repair, and maintenance of protein disulfide bonds. In addition to its role in the prevention of oxidative stress, glutathione also helps maintain exogenous antioxidants such as vitamins C and E. Within cells, glutathione exists as reduced (GSH) and oxidized states (GSSG). In healthy cells, more than 90% of the total glutathione pool is in the reduced form while less than 10% exists in the oxidized or disulfide form. An increased level of GSSG is generally indicative of enhanced oxidative stress. Interestingly, we have reported above that UPEI-100 administration resulted in a significant increase in the amount of GSSG at the 5.5 hours of reperfusion time interval. It is possible that the UPEI-100-induced enhancement in the activity of SOD2 during the 5.5 hours of reperfusion resulted in higher levels of hydrogen peroxide production. However, the measured levels of hydrogen peroxide were not different between vehicle and UPEI-100 groups indicating that the neurons were able to adequately deal with this excess hydrogen peroxide. Hydrogen peroxide may be fully reduced to water, but may also form hydroxyl radicals in the presence of ferrous or cuprous ions (30). A slow increase in hydroxyl radical levels over the 5.5 hours of reperfusion following UPEI-100 administration could have attenuated the activity of glutathione reductase, the enzyme responsible for reducing GSSG into GSH, resulting in enhanced levels of GSSG as measured following 5.5 hours of reperfusion.

Lipid peroxidation in models of I/R occurs very quickly (6, 42) and byproducts of lipid
peroxidation can form adducts with proteins and DNA and thus may play an important role in the underlying mechanism for oxidative stress-induced neuronal apoptosis (9). Our data suggest that UPEI-100 pretreatment resulted in a decrease in I/R-induced lipid and protein peroxidation (HNE-HIS adducts) at 2 hours of reperfusion, suggesting that UPEI-100 was effective in preventing oxidative stress at this time point. Interestingly, the 2 hour time point represents the time when the infarct volume of the UPEI-100 and vehicle treated groups diverge (Fig. 5). Since the level of apoptotic death, measured as the amount of DNA fragmentation, remained the same at all time points during reperfusion, this would suggest that the growth in infarct volume in the vehicle-treated group may have been primarily due to necrotic cell death. We speculate that the increase in neuronal survival measured when rats were pretreated with UPEI-100 was due to the ability of UPEI-100 to attenuate reperfusion-induced oxidative stress and subsequent necrotic cell death.

We therefore conclude that during the initial period following stroke and reperfusion, UPEI-100-induced neuroprotection was primarily due to increased SOD2 activity, thereby causing an increase in the neurons ability to immediately deal with reperfusion-induced mitochondrial superoxide production. This, along with reduced peroxidation of lipids and proteins as a result of oxidative stress, combined to produce our observed neuroprotective capacity of UPEI-100. We should mention that although we conducted time-dependent measures of molecular mediators of cellular stress pathways, these changes do not necessarily suggest a cause-and-effect relationship between UPEI-100 and these proteins. This is particularly true since the temporal changes in these proteins do not correspond to all time points in which neuroprotection was observed. Therefore, studies are currently underway utilizing siRNA technology to establish this relationship.
Perspectives and Significance

At present, there are no pharmacological treatments available other than thrombolytic therapy such as tissue plasminogen activator (t-PA), and this is used in only about 4% of patients presenting to a hospital following an acute ischemic stroke (25). In addition, patients must present themselves to a hospital within approximately 4 hours of the stroke onset to be eligible for tPA therapy (19). Providing effective pharmacological treatment immediately following an acute stroke to lessen the cerebral damage remains an elusive goal. UPEI-100 is a potential therapeutic candidate to protect against the negative outcomes associated with reperfusion-induced ischemia.
ACKNOWLEDGEMENTS

The authors wish to thank Ms. Ashley Patriquen for her technical assistance in performing the ELISAs.
GRANTS

The work was funded by a grant from the Atlantic Canada Opportunities Agency (ACOA) Atlantic Initiatives Fund (AIF).
AUTHOR CONTRIBUTIONS

Barry J. Connell and Monique Saleh were responsible for performing all of the in vivo experimentation and statistical design of the studies respectively. Dr. Rajagopal was responsible for the synthesis of UPEI-100 and providing us with milligram quantities. He also wrote the section dealing with compound synthesis and is co-inventor on the patent. Drs. Tarek M. Saleh and Bobby V. Khan are the co-principle investigators and senior scientist on the grant from ACOA-AIF.
REFERENCES


14. Connell BJ, Saleh M, Khan BV, Saleh TM. Apocynin may limit total cell death


20. Gonzalez-Perez O, Gonzalez-Castaneda RE, Heurta M, Luquin S, Gomez-Pinedo U, Sanchez-Almaraz E, Navarro-Ruiz A, Garcia-Estrada J. Beneficial effects of α-lipoic acid plus vitamin E on neurological deficit, reactive gliosis and neuronal...

21. Jackman KA, Miller AA, DeSilva TM, Crack PJ, Drummond GR, Sobey CG.


27. Myers MG, Norris JW, Hachinski VC, Sole MJ. Cardiac sequlae of acute stroke.


40. Tang XN, Cairns B, Cairns N, Yenari MA. Apocynin improves outcome in


FIGURE LEGENDS

Figure 1. (A) Effect of pretreatment (30 minutes prior to occlusion of the middle cerebral artery; MCAO) with UPEI-100 (n=4/group) on infarct volume (mm³) calculated from TTC-stained 1 mm thick coronal sections throughout the extent of the infarct following I/R. Each bar represents the mean ± S.E.M and * indicates significance ($p \leq 0.05$) from the vehicle group (n=6). (B) Effect of UPEI-100 administered 30 minutes prior to permanent (6 hour) MCAO on infarct volume (mm³) calculated from TTC stained, 1 mm thick coronal slices throughout the extent of the infarct. Each bar represents the mean ± S.E.M and n=4/group.

Figure 2. Cardiovascular responses to UPEI-100 (0.1 mg/kg; n=3) or vehicle (25% EtOH; n=3) pretreatment (i.v.) 30 minutes prior to I/R. Graphs represent average changes in (A) mean arterial pressure (MAP; mmHg; (B) heart rate (HR; bpm) and (C) baroreflex sensitivity (BRS; bpm/mmHg) following phenylephrine injection (0.0025 μg/ml; i.v.). The up-arrow indicates time of UPEI-100 or vehicle injection (30 minutes pre MCAO). The first dashed line represents the time at which the MCA was occluded and the second line indicates when blood flow was returned (reperfusion). Each data point represents the mean ± S.E.M. and * indicates significance ($p \leq 0.05$) from the pre-MCAO values.

Figure 3. Effect of UPEI-100 (0.1 mg/kg) or vehicle (0.0125% EtOH) administered 15 minutes prior to the beginning of reperfusion (-15), immediately prior to suture removal and the start of reperfusion (0), or 30, 60, 90, 120 and 180 minutes
following reperfusion on infarct volume (mm³). Each bar represents the mean ± S.E.M. (n=4/group) and * indicates significance (p ≤ 0.05) from the vehicle control group at the same time point. There were no significant differences in the mean infarct volumes when vehicle was injected during MCAO or at any point during reperfusion (p ≥ 0.05), therefore, the vehicle data for all time points were pooled and is represented by a single bar.

**Figure 4.** Effect of direct cortical injections of UPEI-100 (n=4/group) or vehicle (0.0125% EtOH; n=6) 10 minutes pre-MCAO on infarct volume (mm³) calculated from TTC-stained 1 mm thick coronal sections throughout the extent of the infarct following I/R. Each bar represents the mean ± S.E.M. and * indicates significance (p ≤ 0.05) from the vehicle control group.

**Figure 5.** (A) Representative digital photomicrographs of TTC-stained 1 mm thick coronal sections following 30 minutes of MCAO illustrating the extent of the infarct size within the prefrontal cortex at progressive time points during 5.5 hours of reperfusion in vehicle (25% EtOH) and UPEI-100 (0.1 mg/kg) treated animals. (B) Graphic representation of the change in infarct volume (mm³) at progressive time points during the period of reperfusion following 30 mins of ischemia. Each data point (n=4 or 5) represents the mean ± S.E.M. and * indicates significance (p ≤ 0.05) between vehicle and UPEI-100 treated rats at the same time point.

**Figure 6.** Effect of pre-administration (30 minutes pre MCAO) of UPEI-100 (0.1 mg/kg) or
vehicle (25% EtOH) on (A) cytoplasmic superoxide dismutase (Cu/ZnSOD; SOD1) and (B) mitochondrial superoxide dismutase (MnSOD; SOD2) activity (% inhibition of chromogenic reduction); (C) total glutathione (GSH + GSSG) and (D) oxidized glutathione (GSSG) levels (µM/g protein); (E) hydrogen peroxide (H₂O₂) levels (µmol H₂O₂ per gram of protein); (F) total HNE-His protein adduct levels (µg/mL); (G) the total amount of DNA fragmentation (apoptosis; mean absorption at 405 nm of cytoplasmic nucleosomes) within the infarct area following ischemia/reperfusion (I/R) at progressive time points following the beginning of reperfusion. Each data point (n=5/group) represents the mean ± S.E.M. and * indicates significance ($p \leq 0.05$) between vehicle and UPEI-100 treated rats at the same time point.
A

Infarct Volume (mm³)

Veh  0.01  0.05  0.1  0.5

UPEI-100 (mg/kg)

B

Infarct Volume (mm³)

Veh  0.1  1.0

UPEI-100 (mg/kg)
Infarct Volume (mm$^3$) vs Injection time of UPEI-100 (mins)

- Veh
- -15
- 0
- 30
- 60
- 90
- 120
- 180

* indicates significant difference from Veh group.
Infarct Volume (mm$^3$) vs. UPEI-100 (µM)

- Veh
- 0.01
- 0.1
- 0.5
- 1

* indicates a significant difference compared to the vehicle control.
A

UPEI-100 (0.1 mg/ml)

<table>
<thead>
<tr>
<th>Time</th>
<th>Veh (25% EtOH)</th>
<th>UPEI-100 (0.1 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Infarct Volume (mm²)

- Veh (25% EtOH)
- UPEI-100 (0.1 mg/kg)

Reperfusion Time (hours)