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Stereo-selective neuroprotection against stroke with vitamin A derivatives

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Abstract

In this study we evaluated the neuroprotective potential of vitamin A (all-trans retinol), and its geometric isomers, all-trans retinoic acid and 9-cis retinoic acid, in a focal model of ischemia. A 60 minute middle cerebral artery occlusion in C57 mice resulted in over 50% hemispheric infarction. Vitamin A (retinol) and its derivatives were administered as two i.p. injections immediately prior to and following ischemia. A reduction in infarct volume was observed with all-trans retinol, in a dose dependent manner: maximum protection was observed with a 10 mg/kg dose. A similar protective profile was observed with all-trans retinol, but not the stereoisomer 9-cis retinoic acid. Administration of the derivatives 1 hour following ischemia did not produce significant protection. Taken together these data suggest a possible use of vitamin A derivatives as an acute neuroprotective strategy for stroke.

Keywords

Retinoid; ischemia; Retinol; C57; stroke

Introduction

A considerable amount of effort has been devoted to the discovery of pharmaco-therapeutics to reduce brain injury following cerebral ischemia. While many novel and potent therapies have been identified in pre-clinical animal models, few have been successful in larger human trials [20]. One potential problem with this strategy may be the quest for selective potent antagonists of a single protein receptor or channel/enzyme. Hence attention has been focused on identifying currently available potential therapeutics which may have multiple therapeutic targets.

One such example is retinol, or vitamin A which has been shown to have multiple potential neuroprotective properties. While a toxic dose of vitamin A has the well know property of intracranial hypertension, in therapeutic doses, retinol has antioxidant properties and has been shown to block voltage gated calcium channels [11,13,25]. Individual blockers of these

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cellular mechanisms have been attributed to neuroprotection individually [7,8,12]. Trials involving cocktails of compounds are not usually investigated because of the complexity of determining the therapeutic effect of each component. Hence retinol, with its potential multiple biological effects in a single molecule may have neuroprotective potential.

It has been shown that patients admitted to hospital following cerebral ischemia have reduced plasma levels of vitamin A [1,3,16]. In addition, in a previous study 9-cis retinoic acid was shown to be protective in a rat model of focal ischemia when administered by an intra-cerebral ventricular route [6] and in an in vitro model of ischemia [9]. Taken together there is a clear rationale for determining the effects of retinol and its metabolites all-trans-retinoic acid and 9-cis retinoic acid as potential neuroprotective agents for stroke. In this study we investigate the effect of retinol, and its metabolites on infarct volume in a murine focal ischemia model and determine that retinol is neuroprotective, but that the neuroprotection of its geometric isomers is stereo-selective depending on the *cis-trans* configuration of the side chains double bonds.

Results

The vitamin A (all-trans retinol) was administered intra-peritoneally (i.p.) to mice to determine its acute neuroprotective potential. The retinol was administered as two doses of 0.5, 1.5 or 5 mg/kg immediately before and immediately after 60 min MCAO (total dose 1, 3 and 10 mg/kg, i.p. respectively). A 60 min MCAO produced a robust infarction (58% of hemisphere), as determined by a decrease in defined TTC staining in the ipsilateral hemisphere. Using transcranial Doppler we observed an average 80% reduction in blood flow during MCAO. Using this model, all-trans retinol produced a dose-dependent protection against 60 min MCAO (Fig 1).

To determine whether the protective effects of all-trans retinol were via retinol or one of its metabolites we investigated the effect of all-trans retinoic acid and its stereoisomer 9-cis retinoic acid. The retinoic acid derivatives were administered as two doses of 0.5, 1.5 or 5 mg/kg immediately before and immediately after 60 min MCAO (total dose 1, 3 and 10 mg/kg, i.p. respectively). All-trans retinoic acid (1.0 mg/kg) produced a dose dependent protection against 60 min MCAO modeled ischemia (Fig 2). In contrast, no protection was observed with the stereoisomer 9-cis retinoic acid at any doses investigated (1.0, 3.0 and 10.0 mg/kg, i.p. total doses) (Fig 2). To directly compare the effect of all-trans retinol vs. all-trans retinoic acid, a two way ANOVA with post-hoc Bonferroni's test was performed on infarct volume following stroke. There was no significant difference between the protective potency of all-trans-retinol vs. all-trans retinoic acid. This suggests that the protective effect of retinoic acid is mediated through the all-trans isomer when delivered via intraperitoneal administration.

We further investigated whether retinol or all-trans retinoic acid may have utility as a post ischemic neuroprotective agent. The maximal effective dose of 10 mg/kg was administered as a single bolus i.p. 1 hour following 60 min MCAO. Brain infarction following ischemia was reduced when all-trans retinol or all-trans retinoic acid was administered post ischemia, however the protection was modest and not significant. In contrast, no evidence for protection was observed when animals were administered 9-cis retinoic acid 10 mg/kg i.p. one hour post MCAO (data not shown).

Discussion

Thus in our study we observed a neuroprotective potential of all-trans retinol and all-trans retinoic acid, but not 9-cis retinoic acid in a murine model of focal ischemic, when the drug

is administered via an intra-peritoneal route at the time of stroke. Given the stereoselectivity of the protective effect, a distinct binding site mechanism of action may be more relevant to its therapeutic effects.

Retinol and its derivatives exert their biological actions via specific nuclear receptors (RARs and RXRs) which regulate gene transcription [11]. Retinoic acid receptors can also interact with other nuclear receptors which have neuroprotective properties, for example 1, 25 dihydroxyvitamin D(3) is neuroprotective against stroke [24] and RXR receptors form heterodimers with the vitamin D₃ receptor (RXR-VDR). In addition, RXR can form a dimer with thyroid hormone receptors, and thyroid hormone derivatives are protective against infarction [5]. Hence the neuroprotective effects of retinol and retinoic acid may be mediated, at least in part, by its formation of heterodimers with other nuclear receptors.

In addition to its effect on nuclear receptors, retinol exerts acute effects on other potentially protective biological sites including calcium channels [18,25], gap junction channels [26] and has anti-oxidant properties [13]. Calcium channel antagonists [7,8] and anti-oxidants protect the brain from ischemia in animal models of stroke [12,21]. Retinoic acid also induced the expression of midkine which is protective against brain ischemia [6]. Neither calcium channel antagonists nor anti-oxidant classes of drugs have proven to be successful in stroke clinical trials. The reasons behind the apparent failure of these drugs in clinical trials are multiple [19,20]. However, by combining multiple potential modes of action within a single compound may yield a more efficacious therapeutic compound. Hence, retinol may be worthy of further investigation as an anti-stroke therapeutic.

Our data contrasts with the study of Harvey et al 2004 [6] who show in an in vitro ischemia model and a focal ischemia model in rat, that 9-cis but not all-trans retinoic acid was significantly protective. The studies while offering different conclusions are not really comparable. Harvey et al did not study retinol and used intra-cerebral injection [6], 24 hours prior to experimental stroke. As Harvey et al make the point that retinoic acid protection may occur via induction of neurotropic factors and their study is designed to induce midkine, Harvey et al's icv administration, 24 hours before stroke vs. our intraperitoneal administration at the time of stroke (designed to effect acute modulators of stroke – calcium channels blockade and antioxidant effects) likely study different biologic effects of similar compounds.

There has been much interest in “natural” supplements which may reduce the incidence of stroke. Vitamin A is found in many foods including liver, dairy products, eggs and fish. Vitamin A plasma levels are reduced in patients following cerebral ischemia [1,3,16] suggesting that boosting the levels of vitamin A may be beneficial to stroke patients. Although the role of the vitamin A, whether it is already reduced prior to the stroke, or reduced as a result of the stroke is not clear. In order to address this issue some studies have investigated the effect of prophylactic vitamin A therapy, in the form of beta-carotene (a precursor of retinol). Such long term prophylactic studies show mixed results at reducing stroke incidence. In a recent review of controlled clinical studies using vitamin A only one study in 4 showed a protective effect of vitamin A (in the form of beta-carotene) [4]. Interestingly, in the high risk study subjects there was a reduced incidence of cerebral infarction in the group receiving beta carotene (20 mg/day) [10]. However, these studies have not investigated the acute effects of a large dose of retinol or retinoic acid following a stroke. Antioxidant vitamins have also been shown to reduce skeletal muscle damage following ischemia [17]. Our study suggests that in the acute setting of cerebral ischemia vitamin A derivatives may be a useful therapy to help reduce the damage following cerebral ischemia, a caveat however is the restricted time window for administration.

Experimental procedure

All animal procedures were performed at Legacy Research (Portland, OR) a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and in accordance with protocols approved by the Legacy Institutional Animal Care and Use Committee, as well as the principles outlined in the National Institute of Health *Guide for the Care and Use of Laboratory animals*. We used a murine focal ischemia model to determine the potential protective effects of retinol and its derivatives. In this model a 60 minute period of middle cerebral artery occlusion results in significant infarction of the ipsilateral hemisphere, as determined by staining with the vital dye triphenyltetrazolium chloride (TTC). Male adult C57 mice (average mass 23 g) were anesthetized using 5% isoflurane, and then maintained at 2% isoflurane. Depth of anesthesia was determined by periodic (every 10 minutes) assessment of respiration and pinch withdrawal reflex. Brain temperature was measured using a temoralis muscle-implanted thermocouple (Omega, Stamford, CT) and controlled within the range of $37\pm0.5^{\circ}\text{C}$. The middle cerebral artery (MCA) was occluded by threading silicone-coated 6-0 monofilament nylon surgical suture through the external carotid to the internal carotid, and finally blocking its bifurcation into the MCA and anterior cerebral artery [23]. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow (CBF) in the area of the right middle cerebral artery. CBF was monitored through a disposable microtip fiber optic probe (diameter 0.5 mm) connected through a Master Probe to a laser Doppler computerized main unit (PF5001, Perimed, Sweden) and analyzed using PSW Perisoft 2.5 (Kawano et al. 2006). Animals that did not show a CBF reduction of at least 70% were excluded from the experimental groups, as well as animals that died after ischemia induction.

A dose response profile was first established for all-trans retinol (Fluka®Buchs SG, Switzerland) (1.0-10.0 mg/kg i.p.) all-trans retinoic acid (Sigma® St Louis, MO, US) (1.0-10.0 mg/kg i.p.) or 9-cis retinoic acid (Sigma® St Louis, MO, US) (1.0-10.0 mg/kg i.p.), dissolved in DMSO and administered intraperitoneally, immediately before and after 60 minutes MCAO. As a control some animals were administered 10% DMSO vehicle. Half of each drug dose was administered prior to MCAO and the other half following the period of MCAO. To assess post stroke administration potency some animals received the maximal effective dose of 10 mg/ kg i.p. dose of all-trans retinol, all-trans retinoic acid or 9-cis retinoic acid one hour following the termination of MCAO. Body temperature was determined during recovery from ischemia, but no differences were observed between control and retinoid treated groups. Infarct volume was assessed 24 hours following MCAO using a triphenyltetrazolium hydrochloride (TTC) method [2]. Animals were sacrificed by isoflurane overdose, brains were rapidly removed, sectioned coronally at 2 mm intervals and immersed in TTC (2 %) at 37°C for 20 minutes, followed by formaldehyde (4%) for 15 minutes. The hemispheric infarct area in each section was calculated by subtracting the area of normal TTC stained brain in the ipsilateral cortex from the contralateral area using NIH image [14,15,22]. Statistical analysis was performed via a one way or a two way ANOVA with post hoc Bonferroni's test as noted in the specific figure legends and results section (Graphpad Prism version 4.0 for Windows).

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Abbreviations

MCAO	middle cerebral artery occlusion
TTC	triphenyltetrazolim hydrochloride
ANOVA	analysis of variance
sem	standard error of the mean
DMSO	dimethyl sulfoxide
i.p	intra peritoneal

Figure 1.

Dose-dependent neuroprotection in stroke by all-trans retinol. a) Structure of all-trans retinol. b) Infarct volume of mice subject to 60 min MCAO and treated with DMSO vehicle (n=10) 1.0 (n=8) 3.0 (n=6) or 10.0 (n=6) mg/kg all-trans retinol. Infarct volume was determined by TTC staining. Data shown are mean ± sem, * denotes significant difference of P<0.05 and ** denotes P<0.01 vs. vehicle group (one-way ANOVA with post hoc Dunnet's test). c) Photograph of representative series of TTC stained brain sections treated with either vehicle control or all-trans retinol (1.0-10.0 mg/kg).

Figure 2.

Dose dependent neuroprotection in stroke by all-trans retinoic acid, but not 9-cis retinoic acid. a) Structure of all-trans retinoic acid. b) Infarct volume of mice subject to 60 min MCAO and treated with DMSO vehicle (n= 10), 1.0 mg/kg (n=10) 3.0 mg/kg (n=9) or 10.0 mg/kg (n=11) all-trans retinoic acid. Infarct volume was determined by TTC staining. Data shown are mean $\pm$ sem, * denotes significant difference of $P < 0.05$ and ** denotes $P < 0.01$ vs. vehicle group (one-way ANOVA with post hoc Dunnet's test). c) Structure of 9-cis retinoic acid. d) Infarct volume of mice subject to 60 min MCAO and treated with DMSO vehicle (n= 10), 1.0 mg/kg (n=7) 3.0 mg/kg (n=5) or 10.0 mg/kg (n=12) 9-cis retinoic acid. Infarct volume was determined by TTC staining. Data shown are mean $\pm$ sem, no groups were significant from vehicle control (one-way ANOVA with post hoc Dunnet's test). e) Photograph of representative series of TTC stained brain sections treated with vehicle, all-trans retinoic acid (1.0-10 mg/kg) or 9-cis retinoic acid (1.0-10.0 mg/kg).