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Ketone bodies protection against HIV-1 Tat-induced neurotoxicity

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Abstract

HIV-1 associated neurocognitive disorder (HAND) is a syndrome that ranges clinically from subtle neuropsychological impairments to profoundly disabling HIV-associated dementia. Not only is the pathogenesis of HAND unclear, but also effective treatments are unavailable. The HIV-1 transactivator of transcription protein (HIV-1 Tat) is strongly implicated in the pathogenesis of HAND, in part, because of its well-characterized ability to directly excite neurons and cause neurotoxicity. Consistent with previous findings from others, we demonstrate here that HIV-1 Tat induced neurotoxicity, increased intracellular calcium, and disrupted a variety of mitochondria functions, such as reducing mitochondrial membrane potential, increasing levels of reactive oxygen species, and decreasing bioenergetic efficiency. Of therapeutic importance, we show that treatment of cultured neurons with ketone bodies normalized HIV-1 Tat induced changes in levels of intracellular calcium, mitochondrial function, and neuronal cell death. Ketone bodies are normally produced in the body and serve as alternative energy substrates in tissues including brain and can cross the blood-brain barrier. Ketogenic strategies have been used clinically for treatment of neurological disorders and our current results suggest that similar strategies may also provide clinical benefits in the treatment of HAND.

Keywords

HIV-1 Tat; neurotoxicity; ketone bodies; mitochondrial membrane potential; oxidative stress; calcium homeostasis; ATP

Introduction

Over 40 million people worldwide are infected with human immunodeficiency virus-1 (HIV-1), the cause of acquired immune deficiency syndrome (AIDS). Of those infected with HIV-1, many develop HIV-1 associated neurocognitive disorder (HAND); a neurological condition with features ranging from subtle neuropsychological impairments to profoundly disabling HIV-1 associated dementia. Although combined antiretroviral therapies (CART)

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have increased longevity, the prevalence of HAND is increasing in HIV-1 infected individuals. Recent epidemiological data suggest that the prevalence of HAND in the U.S. is greater than 50% of HIV-1 infected individuals (Ellis et al. 2010; Heaton et al. 2010). Unfortunately, no effective treatments are available for the prevention or treatment of HAND.

A prominent pathological feature of HAND is neuronal injury, even though neurons themselves are not directly infected with HIV-1 virus (Nuovo et al. 1994; Mattson et al. 2005; Levine et al. 2009). Implicated in HIV-1 associated neuronal injury and the pathogenesis of HAND are soluble factors including HIV-1 viral products/proteins and pro-inflammatory mediators released from infected glia and monocytes (Ghafouri et al. 2006; King et al. 2006; Wallace 2006; Ances and Ellis 2007). The HIV-1 transactivator of transcription protein (HIV-1 Tat) is a nonstructural HIV-1 viral protein that is actively secreted by infected macrophages and microglia in the CNS, and can be transported across the blood-brain barrier from the systemic circulation (Kim et al. 2003; Banks et al. 2005). HIV-1 Tat directly excites neurons and is neurotoxic (Magnuson et al. 1995; Nath et al. 1996; Haughey et al. 1999; Nath et al. 2000; Buscemi et al. 2007). Although the precise molecular mechanisms underlying HIV-1 Tat-induced neurotoxicity are not fully understood, Tat deregulates intracellular calcium homeostasis (Kruman et al. 1998; Haughey et al. 1999; Nath et al. 2000; Bonavia et al. 2001; Haughey et al. 2001; Eugenin et al. 2003; Self et al. 2004b; Caporello et al. 2006; Wallace 2006; Zhu et al. 2009), and disrupts mitochondrial function (Kruman et al. 1998; Rappaport et al. 1999; Nath et al. 2000; Perry et al. 2005; Price et al. 2005; Zou et al. 2007; Norman et al. 2008; Malik et al. 2011). Both of which results in a decrease in cellular energy metabolism and an increase in the production of reactive oxidative species (Kruman et al. 1998; Aksenov et al. 2001; Perry et al. 2005; Aksenov et al. 2006; Wallace 2006; Agrawal et al. 2007; Malik et al. 2011; Merino et al. 2011); all are involved in the neurotoxicity associated with HIV-1 Tat.

Because ketone metabolism is neuroprotective and enhances bioenergetic status (Nakazawa et al. 1983; Masino et al. 2011), we tested the hypothesis that treatment of neurons with ketone bodies would protect against HIV-1 Tat-induced neurotoxicity. Here, we report that ketone bodies protected against HIV-1 Tat-induced neuronal cell death, reduced HIV-1 Tat-induced intracellular calcium overload and the production of reactive oxidative species, raised HIV-1 Tat-induced mitochondria membrane potential reduction, and normalized mitochondria bioenergetics. These results suggest that ketogenic strategies may be used as adjunct therapeutics in the treatment of HAND.

Methods and Materials

Primary hippocampal neuron cultures

Primary hippocampal neurons were cultured from embryonic day 18 Sprague Dawley rats (Harlan Laboratories Inc., WI) using a protocol approved by the University of North Dakota Animal Care and Use Committee adherent with the Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23). The pregnant dames were killed by asphyxiation with CO₂ and fetuses were removed, decapitated and meninges-free hippocampi were isolated, trypsinized, and plated onto poly-D-lysine-coated culture dishes. Neurons were grown in Neurobasal™ medium containing L-glutamine, antibiotic/antimycotic and B27 supplement, and maintained at 37°C and 5% CO₂ for 11–14 days *in vitro* at which time they were taken for experimentation. Typically, the purity of the neuronal cultures was greater than 95% as determined by morphology and staining for the neuronal markers NeuN and MAP-2, and the astrocyte marker GFAP. Neurons were treated with HIV-1 Tat_{1–72} (a gift from Dr. Avindra Nath) at a concentration of 100 nM (Haughey et al. 2001; Buscemi et al. 2007) for 0, 24, 48 and 96 hours. Neurons treated with mutant Tat

(Tat_{Δ31-61}, 100 nM) or phosphate-buffered saline (PBS) served as controls. Cultures subjected to ketone bodies (5 mM acetoacetate and 5 mM β-hydroxybutyrate from SigmaAldrich) were pretreated (Tieu et al. 2003; Thaler et al. 2010) for 24 hr prior to HIV-1 Tat exposure, co-treated with HIV-1 Tat for 96 hr, or post-treated for 48 hours after 48 hr of HIV-1 Tat exposure.

Neuronal cell viability assay

Neuronal cell viability was measured using a triple staining method as described (Buscemi et al. 2007). Briefly, neurons were stained with Hoechst 33342 (10 μg/ml), ethidium homodimer-1 (4 μM) and calcein (1 μg/ml). Hoechst 33342, which labels DNA, was used as a marker for identifying condensed nuclei characteristic of apoptotic cell death. Dead or dying cells as a result of loss of membrane integrity were unable to exclude ethidium homodimer dye. Cells were considered viable when cytoplasm was stained with green fluorescence after the cleavage of the non-fluorescent calcein acetoxymethyl ester to calcein. Fields were chosen at random, and at least five images from five separate fields from each of three separate culture dishes for every experimental condition were acquired with an Axiovert 200M fluorescence microscope (Zeiss) attached to a filter-based imaging system (III Imaging Systems). The number of dead or dying neurons (ethidium-labeled red nuclei and blue-condensed nuclei without green cytoplasmic staining) and total neuron numbers were counted manually, and percentage of neuronal viability was normalized to controls. Approximately 900 neurons were counted in each experimental condition.

Measurement of intracellular calcium

The level of free intracellular Ca²⁺ was determined using a Ca²⁺-specific fluorescent probe fura-2/AM (Invitrogen) as described previously (Haughey et al. 1999). Cells were incubated with 2 μM fura-2/AM for 40 min at 37°C. Cells were excited at 340 and 380 nm, and emission was recorded at 510 nm using a filter-based calcium imaging system (Zeiss). Images were acquired every 2 s that allowed for the quantification of baseline and peak increases in free intracellular calcium. Stallion software (Ver. SB 4.1.0, Stallion Software Systems) was used to normalize excitation intensity ratios of 340/380nm to represent free intracellular calcium concentration.

Mitochondrial membrane potential measurement

Mitochondrial membrane potential was measured using fluorescent dye (JC-1, Invitrogen) in neurons grown in 96-well plates. Following treatment, cultured neurons were incubated with 10 μM JC-1 for 20 min at 37°C, washed three times with Locke's solution, prior to measuring fluorescence. Fluorescence was measured using a SpectraMax Plus 384 plate-reader (Molecular Devices) with excitation/emission wavelengths set at 485/530nm for the JC-1 monomer and 535/570nm for the J-aggregates. JC-1 is a lipophilic cationic dye that selectively enters the mitochondria and reversibly changes color. JC-1 aggregates at regions of high membrane potential and remains as a monomer in regions of low membrane potential. Fluorescence intensity of J-aggregates and JC-1 monomers was quantified and normalized to control ratios. The ratio of JC-1 aggregates to JC-1 monomers in control cultures were assigned a value of 1. Experiments were repeated using separate neuronal cultures.

Measurement of reactive oxidative species (ROS)

The levels of reactive oxygen species was measured in 96-well plates using the fluorescent dye carboxy-H₂DCFDA (Sigma). When this dye is oxidized by reactive oxygen species it is deacetylated and converted to the fluorescence compound 5-(and 6-) carboxy-2',7'-dichlorofluorescein (carboxy-DCF) that stains cell cytoplasm bright green. Following

treatment, neurons were incubated with 100 μ M H₂DCFDA for 45 min at 37°C, washed three times with PBS, and fluorescence emission was quantified using a SpectraMax Plus 384 plate-reader (Molecular Devices) at excitation/emission wavelengths of 485/530nm. H₂DCFDA fluorescence intensity was normalized to control values, in which control cultures were assigned a value of 1. Each experiment was conducted in triplicate and experiments were repeated using separate cultured neurons.

ATP measurement

Cellular levels of ATP were measured using a high performance liquid chromatography method as described previously (Hammer et al. 1988; Bough et al. 2006). To eliminate breakdown of nucleotides, medium was removed and the plates containing neurons were snap frozen on liquid nitrogen. The cells were scraped off the dishes and transferred into ice-cold trichloroacetic acid (2%, 700 μ l/100 mm dish, Supelco) for extraction and protein precipitation. After homogenization and centrifugation (12,000 $\times$ g, 10 min, 4°C), the pellets were dissolved overnight in 700 μ l of 1 N NaOH and protein concentrations were measured with a DC protein assay (BioRad). Supernatants were immediately transferred into equal amounts of dichloromethane/trioctylamine (3:1, by Vol.) and samples were mixed then centrifuged (12,000 $\times$ g, 5 min 4°C). The upper layers containing nucleotides were transferred into a tube containing an equal amount of dichloromethane/trioctylamine and centrifuged again at 12,000 $\times$ g, 5 min 4°C. The upper layer was transferred to a fresh tube and 100 μ l of each sample was used to quantify the ATP using an HPLC (Beckman Coulter, Fullerton, CA). The ATP was isolated on a Waters Sunfire ODS column (5 μ M, 250 $\times$ 4.6 mm, Milford, MA) using a mobile phase of 0.1 M KH₂PO₄ at a flow rate set at 1.0 ml per minute. Pump control and peak integration was achieved using the 32 Karat™ software (Ver. 7.0, build 1048, Beckman Coulter, Inc., Fullerton, CA). ATP levels were quantified spectrophotometrically at an absorbance of 254 nm based on known retention times and standard curves. Data were reported as nmoles of ATP per mg protein.

Statistics

All data were expressed as means $\pm$ S.D. Statistical significance for multiple comparisons was determined by one-way ANOVA and a Tukey post hoc test (GraphPad Software, San Diego, CA). Statistical significance was set at $p < 0.05$.

Results

HIV-1 Tat directly excites neurons and is neurotoxic (Magnuson et al. 1995; Nath et al. 1996; Kruman et al. 1998; Haughey et al. 1999; Bonavia et al. 2001; Aksenov et al. 2006; Buscemi et al. 2007; Eugenin et al. 2007). Here, we determined the extent to which pretreatment with ketone bodies protected against HIV-1 Tat-induced neuronal cell death. We found that a 48 h treatment with HIV-1 Tat₁₋₇₂ (100 nM) decreased the neuronal survival to $83.4 \pm 4.8\%$ of control levels ($p < 0.05$) (Figure 1A). Following a 96 h HIV-1 Tat treatment, neuronal viability was decreased to $64.7 \pm 2.8\%$ of control levels ($p < 0.001$) (Figure 1A). No statistically significant increases in neuronal cell death were observed with either mutant Tat_{Δ31-61} (100 nM) or PBS; data consistent with previous reports that this deletion mutant of Tat is not overtly toxic to neurons (Buscemi et al. 2007).

A 24 h pretreatment with the ketone bodies, β -hydroxybutyrate and acetoacetate at concentrations of 5 mM each, did not alter neuronal viability when measured 96 h after the treatment was initiated (Figure 1A). However, pretreatment with ketone bodies did reverse the HIV-1 Tat-induced decreases in neuronal survival at 96 h ($p < 0.001$) (Figure 1). Lower concentrations of ketone bodies (0.5 and 1 mM) failed to protect against HIV-1 Tat induced neuronal cell death (data not shown). When co-treated with HIV-1 Tat, ketone bodies (β -

hydroxybutyrate and acetoacetate at concentrations of 5 mM each) also reversed the HIV-1 Tat-induced decrease in neuronal survival at 96 h (Figure 1B). When post-treated for 48 hr after 48 hr exposure of HIV-1 Tat, ketone bodies (β -hydroxybutyrate and acetoacetate at concentrations of 5 mM each) did not afford statistically significant protection against HIV-1 Tat-induced decreases in neuronal survival at 96 h (Figure 1B). When acetoacetate (ACA, 5 mM) and β -hydroxybutyrate (BHB, 5 mM) were used separately, we found that only pre-treatment of β -hydroxybutyrate, but not acetoacetate, exerted protective effects against HIV-1 Tat-induced decreases in neuronal survival (Figure 1B). However, only partial protection against HIV-1 Tat induced neuronal cell death was observed in β -hydroxybutyrate pre-treatment as compared with pretreatment with the ketone bodies (β -hydroxybutyrate and acetoacetate at concentrations of 5 mM each) ($p < 0.05$, Figure 1B).

HIV-1 Tat evokes calcium release from IP₃-sensitive intracellular calcium stores, and causes a leak of calcium through what appears to be activation of NMDA, and potentially voltage-gated receptors (Milani et al. 1996; Nath et al. 1996; Menegon et al. 1997; Haughey et al. 1999; Haughey et al. 2001; Haughey and Mattson 2002). HIV-1 Tat also potentiates the effect of other depolarizing agents that increase the rate and magnitude of rise in intracellular calcium (Nath et al. 2000). Here, we tested the extent by which HIV-1 Tat acted synergistically with KCl to increase levels of intracellular calcium, and the ability of ketone bodies to reverse this phenomenon. Neurons exhibited increased resting levels of intracellular calcium by 47.9% after 24 h, and 48.2% after 48 h of Tat exposure (both $p < 0.001$) (Figure 2A). Treatment of neurons with HIV-1 Tat for 24 or 48 h potentiated the calcium response to KCl (50 mM) by 87.4% and 125%, respectively (both $p < 0.001$) (Figure 2B). Pretreatment of neurons with the 5 mM of β -hydroxybutyrate and acetoacetate attenuated the HIV-1 Tat-induced increase in resting levels of intracellular calcium by 15% after 24 h and 12% after 48 h of Tat treatments (both $p < 0.05$) (Figure 2A), and reduced KCl-evoked increases in levels of intracellular calcium by 28% after 24 h and 32% after 48 h of Tat treatments ($p < 0.01$ at 24h, $p < 0.001$ at 48 h) (Figure 2B).

The disruption in mitochondrial function associated with HIV-1 Tat induced neurotoxicity includes a decrease in the mitochondrial membrane potential (Kruman et al. 1998; Langford et al. 2004; Perry et al. 2005; Norman et al. 2008; Malik et al. 2011) and an increase in oxidative stress (Perry et al. 2005; Aksenov et al. 2006; Wallace et al. 2006; Agrawal et al. 2007; Malik et al. 2011; Merino et al. 2011). Ketone bodies protect against impaired mitochondria function and abnormal levels of oxidative stress (Maher and Davis 1996; Sullivan et al. 2004; Kim do et al. 2007; Maalouf et al. 2007; Maalouf et al. 2009; Cheng et al. 2010; Samartsev and Kozhina 2010). Accordingly, we measured the extent to which pretreatment with ketone bodies attenuated HIV-1 Tat-induced mitochondrial dysfunction. Treatment of neurons with HIV-1 Tat for 24 h and 48 h decreased mitochondrial membrane potential by 44% when compared to controls. The normalized JC-1 ratio was decreased from 1.0 ± 0.1 to 0.56 ± 0.1 ($p < 0.01$) (Figure 3). Treatment of neurons with HIV-1 Tat for 48 h further reduced the mitochondrial membrane potential by 62% compared to controls (0.38 ± 0.07) ($p < 0.001$) (Figure 3). Pretreatment of neurons with ketone bodies reversed the HIV-1 Tat induced decrease in mitochondrial membrane potential back to control values ($p < 0.05$) (Figure 3).

We next determined the extent by which pretreatment with ketone bodies attenuated the HIV-1 Tat-induced elevation of reactive oxygen species. A 24 h treatment of neurons with HIV-1 Tat increased levels of reactive oxygen species by 31% compared with control cultures; the normalized H₂DCFDA fluorescent intensity was increased to 1.3 ± 0.2 from control levels of 1.0 ± 0.1 ($p < 0.05$) (Figure 4). A 48 h treatment with HIV-1 Tat resulted in a 38% greater increase in reactive oxygen species (1.4 ± 0.4) compared to controls ($p <$

0.01) (Figure 4). Pretreatment with ketone bodies prevented the HIV-1 Tat-induced increase in reactive oxygen species ($p < 0.05$) and returned these values to control levels (Figure 4).

Deficits in cellular bioenergetics increase the resting levels of intracellular calcium by a decrease in the activity of ATP-dependent calcium pumps, a decrease in mitochondrial membrane potential through a reduction in electron transport chain function, and/or by an increase in the production and release of reactive oxygen species (Nicholls and Budd 2000; Nicholls et al. 2007). Since ketone bodies could serve as an alternative energy source, we measured the extent to which pretreatment with ketone bodies protected against HIV-1 Tat-induced decrease in neuronal bioenergetics. A 24 h treatment with HIV-1 Tat reduced ATP levels by 22% from control values of 16.3 ± 2.2 nmol ATP per mg protein to 12.8 ± 3.5 nmol ATP per mg protein ($p < 0.05$) (Figure 5). Ketone bodies pretreatment reversed the HIV-1 Tat-induced decrease in levels of ATP. The ATP level of HIV-1 Tat-treated cultures with pretreatment of ketone bodies was 15.5 ± 0.9 nmol per mg protein, which were slightly higher than that found in the HIV-1 Tat treated cultures but not different from control cultures (Figure 5).

Discussion

The focus of these studies was to determine the extent to which ketone bodies pretreatment protected against HIV-1 protein Tat-induced neurotoxicity. Our major findings show that pretreatment with ketone bodies attenuated neuronal cell death, reduced KCl-evoked increases in intracellular calcium, and preserved mitochondrial functions and ATP levels that were altered by HIV-1 Tat treatments. These findings suggest that mitochondrial disruption, redox reactions abnormality and neuronal bioenergetics impairment are all involved in HIV-1 Tat-induced neuronal cell death and that ketogenic strategies might be considered as a potential therapeutic intervention against HAND. CART decreases death rate in HIV infected individuals, but also increases the incidence of HAND (Bouwman et al. 1998; McArthur 2004; Ghafouri et al. 2006; Kraft-Terry et al. 2009; Xu and Ikezu 2009; Lindl et al. 2010). This situation has renewed efforts not only to determine mechanisms underlying this neurological disorder, but also to identify effective therapeutic strategies.

HIV-1 Tat is a nonstructural transcriptional regulator essential for the replication of HIV-1. The first exon of HIV-1 Tat encodes for the first 72 amino acids and the second exon encodes for another 14 to 32 amino acids. Nanomolar concentrations of HIV-1 Tat have been reported in sera of HIV-1 infected patients, but these levels are almost certainly underestimated given how avidly HIV-1 Tat binds to proteins and cells (Westendorp et al. 1995; Xiao et al. 2000). HIV-1 Tat can be transported across the blood-brain barrier from the systemic circulation (Kim et al. 2003; Banks et al. 2005), can be secreted by infected macrophages and microglia, and has been detected in brain of patients with HAND (Westendorp et al. 1995; Ellis et al. 2000; Nath 2002). HIV-1 Tat protein causes neuronal excitation and toxicity and is implicated in the pathogenesis of HAND (Nath et al. 1996; Cheng et al. 1998; Kruman et al. 1998; Haughey et al. 1999; Gavril et al. 2000; Buscemi et al. 2007). We have shown that the neurotoxic component of Tat resides within the basic region of exon 1 (Nath et al. 1996). Since then, most studies have used Tat₁₋₇₂ at the concentration of about 100 nM for neurotoxicity studies (Buscemi et al. 2007). There may be differences in the transactivating potential of different forms of Tat, but there is no additional toxicity with Tat₁₋₈₆ or Tat₁₋₁₀₁.

Although mechanisms underlying the direct neurotoxic effect of Tat are not fully understood, an important role of altered calcium homeostasis is implicated (Nath et al. 1996; New et al. 1997; Kruman et al. 1998; Shi et al. 1998; Bonavia et al. 2001; Haughey et al. 2001; Chauhan et al. 2003; Langford et al. 2004; Singh et al. 2005). Tat-induced calcium

dysfunction can also result from abnormal calcium influx (Cheng et al. 1998; Haughey et al. 2001; Song et al. 2003; Self et al. 2004a; Chandra et al. 2005; Li et al. 2008) and/or enhanced release from intracellular calcium stores (Haughey et al. 1999; Mayne et al. 2000; Haughey et al. 2001; Fotheringham et al. 2004; Self et al. 2004a). Elevated levels of intracellular calcium promotes neuronal cell death by disrupting mitochondrial functions (Rizzuto et al. 1993; Pivovarova et al. 2004; Dong et al. 2006), impairing bioenergetics (Kruman et al. 1998; Perry et al. 2005; Aksenov et al. 2006; Wallace et al. 2006; Norman et al. 2007; Norman et al. 2008) and activating cellular death effectors (Kruman et al. 1998; Singh et al. 2005). Moreover, the neurotoxic effects of Tat are exaggerated by other factors such as glutamate, other HIV-1 proteins, neuronal depolarization, and drugs of abuse (Haughey et al. 2001; Aksenov et al. 2006).

Consistent with these findings, we demonstrated here that HIV-1 Tat increased intra-neuronal calcium, decreased mitochondrial membrane potential, increased levels of reactive oxygen species, and decreased cellular levels of ATP. These events likely contributed to Tat-induced neurotoxicity since all occurred prior to neuronal cell death. Although the exact molecular mechanisms are still unknown, some have been proposed including mitochondrial dysfunction, intra-neuronal calcium overload (Nicholls and Budd 2000), decreased levels of intracellular ATP, and/or changes in mitochondrial membrane potential (Ferri et al. 2000; Maragos et al. 2002; Langford et al. 2004; Perry et al. 2005; Rumbaugh et al. 2006; Norman et al. 2007; Rumbaugh et al. 2007; Norman et al. 2008).

Ketone bodies are endogenous products of liver fatty acid β -oxidation that can cross the blood brain barrier (BBB) with the assistance of monocarboxylic acid transporters (MCT1) and enter neurons through high-affinity monocarboxylic acid transporters (MCT2) (Morris 2005). Under physiological conditions, serum concentrations of ketone bodies are too low to replace glucose as a primary brain energy source. However, levels of ketone bodies can rise significantly during fasting and upon ingestion of a ketogenic diet (Morris 2005). Ketone bodies, especially acetoacetate and β -hydroxybutyrate enter mitochondria where they are oxidized more efficiently to acetyl-CoA than is glucose (Lund et al. 2009). Ketogenic diets and caloric restriction exhibit anti-seizure features and are neuroprotective against a number of neurodegenerative disorders (Freeman et al. 1998; Mattson et al. 2002; Massieu et al. 2003; Cheng et al. 2007), most likely by increasing serum levels of acetoacetate and β -hydroxybutyrate (Laffel 1999). In this regard, a variety of mechanisms are proposed to explain the therapeutic mechanism of ketone supplementation including increasing the levels of ATP and adenosine (Nakazawa et al. 1983; Masino et al. 2011) and reducing oxidative damage (Kashiwaya et al. 2000; Suzuki et al. 2001).

To our knowledge, this is the first demonstration that pretreatment and co-treatment with the ketone bodies can protect against HIV-1 Tat-induced neurotoxicity. Our findings indicate that the neuroprotective effects of ketone bodies were achieved mainly by the presence of β -hydroxybutyrate because acetoacetate exerted little protective effect by itself. Because β -hydroxybutyrate only partially protected against HIV-1 Tat induced neurotoxicity as compared with combined treatment of acetoacetate and β -hydroxybutyrate, an additive or possibly synergistic effect might be present between β -hydroxybutyrate and acetoacetate. Our finding contrasts with the findings of others that acetoacetate by itself is neuroprotective against NMDA-induced neurotoxicity (Noh et al. 2006; Thaler et al. 2010). However, this might simply mean that HIV-1 Tat has neurotoxic actions in addition to NMDA receptor activation which has been reported on by many others. It might also indicate there are differences in actions between acetoacetate lithium salt which was used by others and acetoacetate sodium salt which was used in our studies because neuroprotective effects may have been caused by the lithium (Valdes and Weeks 2009). Mechanistically, we have demonstrated that ketone bodies attenuate early pathological events associated with HIV-1

Tat-induced neurotoxicity including an increase in intra-neuronal calcium, a decrease in mitochondrial membrane potential, an increase in the level of reactive oxygen species, and a decrease in cellular levels of ATP. These results are consistent with others showing that mitochondria dysfunction may contribute to HAND and that ketone bodies can help to restore mitochondrial function and exert neuroprotection by preserving ATP levels and decreasing oxidative stress (Perry et al. 2005; Kim do et al. 2007; Haces et al. 2008; Kim do and Rho 2008).

Although the molecular mechanisms and sequential events whereby ketone bodies exert their neuroprotective effects are not fully understood, it is likely that ketone bodies first protect mitochondrial function. As an alternative energy fuel to glucose, ketone bodies can by-pass glycolysis and enter directly into mitochondria, where they are oxidized to acetyl-CoA. Thus, ketone bodies are more efficient in producing ATP than is glucose and can stimulate mitochondrial biogenesis via the up-regulation of genes encoding energy metabolism and mitochondrial enzymes (Kashiwaya et al. 1994; Sato et al. 1995; Veech et al. 2001). In addition, metabolism of ketone bodies produces less reactive oxygen species than does glucose (Mobbs et al. 2007). Our observations that ketone bodies attenuate the HIV-1 Tat-induced drop in ATP levels, decreases in mitochondrial membrane potential, increases in levels of reactive oxygen species, and elevation in intracellular calcium seem to support this premise. Such observations are consistent with other reports that ketone bodies can prevent ATP decline following cellular oxidative challenges (Massieu et al. 2003; Haces et al. 2008) and inhibition of mitochondrial respiration in neurons (Kim do et al. 2010). Our recent findings that increased levels of adenosine, a known neuroprotective agent, is associated with ketogenic diet and/or ketone bodies reducing seizures (Masino et al. 2011), raised another possibility that ketone bodies could exert their neuroprotective effects by raising levels of adenosine, and investigating such a mechanism is warranted in future studies.

Our results demonstrate that ketone bodies protect against neurotoxicity. However we demonstrated that ketone bodies exerted little protection against HIV-1 Tat induced dendritic/synaptic degeneration (data not shown). Such findings indicate that dendritic/synaptic alterations and neuronal cell death may be regulated by different mechanisms, and that ketone body treatment is more effective in protecting against neuronal cell death. While they are not without some drawbacks, ketogenic strategies are widely used and are generally considered safe. Accordingly, such strategies might be adopted as an adjunct therapeutic strategy in the treatment of HAND in the future.

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Abbreviation List

HIV-1	human immunodeficiency virus-1
AIDS	acquired immune deficiency syndrome
HAND	HIV-1 associated neurocognitive disorder
HIV-1 Tat	HIV-1 transactivator of transcription protein
KB	ketone bodies
CART	combined antiretroviral therapies
BBB	blood brain barrier

MCT monocarboxylic acid transporters

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Figure 1.

Ketone bodies (KB; β -hydroxybutyrate and acetoacetate, 5 mM each) protected against HIV-1 Tat-induced neurotoxicity. (A) HIV-1 Tat₁₋₇₂ (Tat; 100 nM, closed circles) produced significant ($p < 0.05$) decreases in neuronal cell viability after 48 h treatment and caused a maximum of about 60% neuronal cell death after 96 h treatment when compared with neurons treated with mutant Tat (Tat_{Δ31-61}, 100 nM, open triangles) or PBS (open circles) as controls. Ketone bodies treatment alone for 96 h did not affect neuronal survival (open squares), but a 24 h ketone bodies pretreatment attenuated significantly ($p < 0.001$) HIV-1 Tat-induced neuronal cell death at an interval of 96 h treatment (closed squares). (B) When co-treated with HIV-1 Tat, ketone bodies (β -hydroxybutyrate and acetoacetate at concentrations of 5 mM each) also reversed the HIV-1 Tat-induced decrease in neuronal survival at 96 h. When post-treated for 48 hr after 48 hr exposure of HIV-1 Tat, ketone bodies (β -hydroxybutyrate and acetoacetate at concentrations of 5 mM each) protected, to

some extent, against HIV-1 Tat-induced decrease in neuronal survival at 96 h. When acetoacetate (ACA, 5 mM) and β -hydroxybutyrate (BHB, 5 mM) were used separately, we found that only pre-treatment of β -hydroxybutyrate, but not acetoacetate, exerted protective effects against HIV-1 Tat-induced decrease in neuronal survival. However, β -hydroxybutyrate pretreatment only partially protected against HIV-1 Tat induced neuronal cell death when compared with pre-treatment of both β -hydroxybutyrate and acetoacetate. (* p <0.05 vs. Tat 96h, *** p <0.001 vs. Tat 96h, ### p <0.01 vs. Control, ^ p <0.05 vs. Pre-KB 24h + Tat 96h)

Figure 2.

Pretreatment with ketone bodies attenuated the HIV-1 Tat-induced increases in free intracellular calcium and decreased HIV-1 Tat-induced potentiation of KCl-induced increase in intracellular calcium. (A) In the absence of KCl, HIV-1 Tat₁₋₇₂ treatment (100 nM) for 24 h or 48 h increased significantly levels of free intracellular calcium when compared with control neurons treated with PBS (***, $p < 0.001$), whereas ketone bodies (KB, 5 mM) pretreatment for 24 h alleviated significantly (#, $p < 0.05$) Tat-induced increases in levels of intracellular calcium. (B) In the presence of KCl (50 mM), Tat₁₋₇₂ (100 nM) treatment for 24 h or 48 h enhanced significantly KCl-induced increases in intracellular calcium levels when compared with control neurons treated with PBS (***, $p < 0.001$), whereas ketone

bodies (KB, 5 mM) pretreatment for 24 h mitigated significantly Tat-induced increases in levels of intracellular calcium (##, $p < 0.01$; ### $p < 0.001$).

Figure 3.

Pretreatment with ketone bodies rectified the HIV-1 Tat-induced decrease in mitochondrial membrane potential. HIV-1 Tat₁₋₇₂ (100 nM) treatment for 24 h and 48 h decreased significantly mitochondrial membrane potential as indicated by normalized JC-1 fluorescent values of control cultures treated with PBS (**, $p < 0.01$; ***, $p < 0.001$), whereas ketone bodies (KB, 5 mM) pretreatment rectified significantly Tat-induced decreases in mitochondrial membrane potential (#, $p < 0.05$).

Figure 4.

Pretreatment with ketone bodies attenuated the HIV-1 Tat-induced increase in reactive oxygen species. HIV-1 Tat₁₋₇₂ (100 nM) treatment for 24 h and 48 h increased significantly levels of reactive oxygen species as indicated by normalized H₂DCFDA fluorescence of control cultures treated with PBS (*, $p < 0.05$; **, $p < 0.01$), whereas ketone bodies (KB, 5 mM) pretreatment attenuated significantly Tat-induced increases in levels of reactive oxygen species (#, $p < 0.05$).

Figure 5. Pretreatment with ketone bodies rectified the HIV-1 Tat-induced decrease in intracellular ATP. HIV-1 Tat₁₋₇₂ (100 nM) treatment for 24 h decreased significantly (*, $p < 0.05$) levels of ATP when compared with control cultures treated with PBS, and these Tat-induced decreases in levels of ATP were rectified by ketone bodies (KB, 5 mM) pretreatment.