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Overexpression of mitochondrial antioxidant manganese superoxide dismutase (MnSOD) provides protection against AZT- or 3TC-induced endothelial dysfunction

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ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) are considered the backbone of current combination therapies for HIV. These therapies have significantly decreased mortality and morbidity in HIV-infected patients, but some are associated with cardiovascular complications, including endothelial dysfunction, an early marker for atherosclerosis. Our prior studies demonstrated that co-treatment of cells with an antioxidant therapy reversed NRTI-induced endothelial injury. Thus, as a proof of concept that mitochondrially-targeted antioxidants may be useful in preventing NRTI toxicity, in the current study, mice overexpressing a mitochondrial antioxidant, manganese superoxide dismutase (MnSOD), were compared with wild-type (WT) mice. Mice were treated chronically with either zidovudine (AZT), lamivudine (3TC), or tenofovir (TDF) to determine whether overexpression of MnSOD protected them from endothelial dysfunction. Endothelial function was assessed using vessel reactivity experiments on thoracic aortas as well as measures of endothelium derived factors nitric oxide (NO), endothelin-1 (ET-1), and prostacyclin. Oxidative stress was evaluated as levels of plasma 8-isoprostane. Alterations in vessel reactivity, NO, and ET-1 in WT mice treated with AZT or 3TC were noted. Overexpression of MnSOD offered protection from decreases in vessel reactivity and increases in ET-1. These findings indicate that mitochondrial oxidative stress induced by AZT or 3TC plays a major role in mediating NRTI-induced endothelial dysfunction, and suggest that the use of targeted antioxidants administered in conjunction with NRTIs may attenuate these effects.

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) are a major component of the current combination therapy for HIV. These combination therapies have significantly decreased mortality (Hammer et al., 1997) and reduced viral load in HIV-infected patients (Hirsch et al., 1999). Long term treatment with these drugs, however, has also been associated with cardiovascular complications including endothelial dysfunction, an early marker of atherosclerosis (Jiang et al., 2006; Lewis, 2004; Sutliff et al., 2002). Endothelial dysfunction is characterized by an imbalance of endothelium-derived contracting (EDCF) and relaxing factors (EDRF). This imbalance may be due to decreased EDRF, increased EDCF, or a combination of the two (Furchgott and Vanhoutte, 1989; Furchgott and Zawadzki, 1980). Two of the primary EDRF are nitric oxide (NO) and prostacyclin, and a major EDCF is endothelin-1 (ET-1). Studies have demonstrated that oxidative stress can alter the EDRF/EDCF balance *via* effects on any or all of these factors (Feletou and Vanhoutte, 2006).

In vitro analyses in our lab have demonstrated that NRTI-treated endothelial cells produce decreased NO and increased ET-1 levels, indicative of an EDRF/EDCF imbalance and endothelial dysfunction. Additionally, these cells generated increased reactive oxygen species (ROS), reduced ATP production, and decreased electron transport chain activity, suggesting that mitochondrial oxidative stress is a key player in this dysfunction (Xue et al., 2013). Oxidative stress occurs when the production of ROS exceeds the ability of antioxidants to scavenge and neutralize them. Superoxide radical, generated primarily in mitochondria as a consequence of normal metabolic processes, is the primary oxygen radical produced in the cell, and its production may be increased in pathological conditions (Turrens and Boveris, 1980). The cell has a number of protective mechanisms to prevent

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toxicity from superoxide (Madamanchi et al., 2005), with the superoxide dismutases (SOD) acting as the primary scavenging enzymes. These enzymes catalyze the conversion of superoxide to molecular oxygen and hydrogen peroxide (Halliwell and Gutteridge, 1984), and exist in three major forms, each localized to specific intracellular and extracellular compartments: extracellular Cu/ZnSOD, mitochondrial MnSOD, and cytosolic Cu/ZnSOD (Crapo et al., 1992; Weisiger and Fridovich, 1973). Studies have indicated that with aging, insufficient MnSOD alters endothelium-dependent vessel reactivity, a well-established measure of endothelial function (Brown et al., 2007).

Animal studies have demonstrated that a number of NRTIs induce endothelial dysfunction, and that this dysfunction is associated with increased ROS (Jiang et al., 2007, 2006; Sutliff et al., 2002). The current study utilized mice overexpressing MnSOD to determine the protective effects of this mitochondrial enzyme on endothelial function *in vivo* after chronic administration with zidovudine (AZT), lamivudine (3TC), or tenofovir disoproxil fumarate (TDF). Following treatment, vessel reactivity experiments utilizing thoracic aortas were employed to evaluate endothelial function, and plasma was collected to evaluate endothelium derived factors and oxidative stress. The use of NRTIs from differing subclasses, i.e., a thymidine (AZT) and a cytidine (3TC) analog, compared to a nucleotide reverse transcriptase inhibitor (TDF), enabled us to assess any differential toxic effects of the drugs on the endothelium, as well as any differential protective effects of the overexpressed antioxidant enzyme between groups. We hypothesized that overexpression of MnSOD would protect against oxidative stress, and would, by extension protect against endothelial dysfunction.

Our findings indicate that two of the drugs tested induced endothelial dysfunction, and that MnSOD overexpression offered variable degrees of protection for these treatments. It is not clear whether these differences are due to additional toxic mechanisms or simply a greater degree of mitochondrial-induced oxidative stress for one drug compared to another. Further studies are needed to investigate the possibility of additional mechanisms of toxicity. However, the findings reported here suggest a possible benefit of mitochondrially-targeted antioxidants as an adjunct therapy for preventing the endothelial dysfunction induced by NRTI treatments.

2. Materials and methods

2.1. Drugs

AZT, 3TC, and TDF were purchased from Morris & Dickson Co. (Shreveport, LA).

2.2. Animals

Transgenic mice overexpressing human MnSOD were a generous gift from Yunfeng Zhao at LSU Health Sciences Center. These mice were developed at the University of Kentucky (Yen et al., 1996). Animals were bred on a C57BL/6 background and were maintained in an AALAC-accredited animal facility. Animal care and use was in accordance with NIH guidelines and all procedures were approved in advance by the LSUHSC Institutional Animal Care and Use Committee. Both male and female mice were utilized for experiments, with mice of each sex stratified as evenly as possible across treatment groups. Dosing of animals was initiated at 6–10 wk of age.

2.3. NRTI administration

Mice were treated with AZT (100 mg/kg/day), TDF (50 mg/kg/day), or 3TC (50 mg/kg/day) in their drinking water for 6–8 wk. These doses were selected by extrapolation from human dosages, using normalization to body surface area (Reagan-Shaw et al., 2008). The method is based on FDA guidelines for estimating doses for clinical trials following animal experimentation (FDA, 2005). To maximize the use of animals and to ensure that our analyses were representative of a human population, male and female mice were randomized as evenly as possible across all groups. Following treatment, mice were anesthetized with pentobarbital (50 mg/kg, i.p.). Anesthesia was confirmed by lack of toe pinch response, and animals were sacrificed by pneumothorax. Blood was collected from the inferior vena cava, and thoracic aortas were excised.

2.4. Measurement of plasma drug levels

Blood for measuring drug levels in mice was collected in EDTA. Plasma drug levels were determined by HPLC using a modification of a previously described method (Jiang et al., 2006; Notari et al., 2006).

2.5. Measurement of plasma biomarkers

Blood for measuring NO metabolites was collected in heparin. After centrifugation, plasma was mixed 1:5 (v/v) with a stabilizing buffer containing 800 mM potassium ferricyanide, 100 mM N-ethylmaleimide, and 10% Nonidet-P40, to preserve nitrite. Samples (were injected into a purge vessel containing a solution with potassium iodide (66.8 mM), iodine (28.5 mM) and acetic acid (78% v/v) to convert nitrite to NO for analysis using a Sievers 280i Nitric Oxide Analyzer (GE, Boulder, CO).

Blood for measuring all other biomarkers was collected in EDTA. Plasma ET-1 and 6- keto prostaglandin F1α were measured using ELISA kits from Enzo Life Sciences (Farmingdale, NY) and Cayman Chemical (Ann Arbor, MI), respectively. Alanine aminotransferase (ALT) and Blood Urea Nitrogen (BUN) were measured using kits from Eagle Diagnostic (DeSoto, TX). Plasma creatinine was measured using a BioVision fluorometric assay kit (Milpitas, CA).

The measurement of 8-isoprostane (8-isoPGF$_{2\alpha}$) required saponification and extraction of F_2-isoprostanes from the plasma (Jiang et al., 2009). 8-isoPGF$_{2\alpha}$ was then measured in the extracts using an ELISA kit from Cayman.

2.6. Vessel reactivity experiments

Thoracic aortas were kept at 37 °C in Krebs Henseleit solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.0 mM KH_2PO_4, 27 mM $NaHCO_3$, and 11.1 mM D-glucose), pH 7.4, and the solution was aerated with 95% O_2 and 5% CO_2 for the duration of the experiments. Aortas were cut into rings and were connected to a force displacement transducer interfaced to a Power Lab data acquisition system (ADInstruments). Baseline tension was set at 1 g and vessels were equilibrated for 1 h before each treatment. Rings were pre-contracted with phenylephrine to achieve 70–80% maximal relaxation, followed by relaxation using increasing doses of acetylcholine (to measure endothelium-dependent relaxation) or sodium nitroprusside (to measure endothelium-independent relaxation).

2.7. Statistics

Data are means ± SD. Statistical analyses were performed using GraphPad Prism software. Treatment and strain were compared by 2-way ANOVA, followed by Fisher LSD posthoc tests. Levels of individual drugs were analyzed between mouse strains using unpaired *t*-test. $P < 0.05$ was considered significant.