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ScienceDirect
Experimental Neurology 203 (2007) 196–204
Experimental Neurology
www.elsevier.com/locate/yexnr

Acetaldehyde promotes rapamycin-dependent activation of $p70^{S6K}$ and glucose uptake despite inhibition of Akt and mTOR in dopaminergic SH-SY5Y human neuroblastoma cells

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Received 27 March 2006; revised 27 July 2006; accepted 1 August 2006
Available online 7 September 2006

Abstract

Alcohol intake is one of the important lifestyle factors for the risk of insulin resistance and type 2 diabetes. Acetaldehyde, the major ethanol metabolite which is far more reactive than ethanol, has been postulated to participate in alcohol-induced tissue injury although its direct impact on insulin signaling is unclear. This study was designed to examine the effect of acetaldehyde on glucose uptake and insulin signaling in human dopaminergic SH-SY5Y cells. Akt, mammalian target of rapamycin (mTOR), ribosomal-S6 kinase ($p70^{S6K}$), the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and insulin receptor substrate (IRS)-2 were evaluated by Western blot analysis. Glucose uptake and apoptosis were measured using [^{3}H]-2-deoxyglucose uptake and caspase-3 assay, respectively. Short-term exposure (12 h) of acetaldehyde (150 μM) facilitated glucose uptake in a rapamycin-dependent manner without affecting apoptosis, IRS-2 expression and insulin-stimulated glucose uptake in SH-SY5Y cells. Acetaldehyde suppressed basal and insulin-stimulated Akt phosphorylation without affecting total Akt expression. Acetaldehyde inhibited mTOR phosphorylation without affecting total mTOR and insulin-elicited response on mTOR phosphorylation. Rapamycin, which inhibits mTOR leading to inactivation of $p70^{S6K}$, did not affect acetaldehyde-induced inhibition on phosphorylation of Akt and mTOR. Interestingly, acetaldehyde enhanced $p70^{S6K}$ activation and depressed 4E-BP1 phosphorylation, the effect of which was blunted and exaggerated, respectively, by rapamycin. Collectively, these data suggested that acetaldehyde did not adversely affect glucose uptake despite inhibition of insulin signaling cascade at the levels of Akt and mTOR, possibly due to presence of certain mechanism(s) responsible for enhanced $p70^{S6K}$ phosphorylation.

Keywords: Acetaldehyde; Glucose uptake; IRS-2; Akt; mTOR; $p70^{S6K}$; 4E-BP1

Introduction

The population of diabetes mellitus is expected to rise from 171 million in 2000 to 366 million by 2030 (Wild et al., 2004). The prevalence of diabetes and the whole cluster of metabolic syndromes characterized by insulin resistance has been closely linked to various lifestyle factors such as obesity, cigarette smoking, satiety, physical inactivity and alcohol intake (Fan et al., 2006a; Daskalopoulou et al., 2004; Marks, 2003; Poirier et al., 2006). Epidemiological evidence has revealed a U-shaped association between alcohol consumption and the risk of type 2 diabetes, indicating alteration of insulin sensitivity following alcohol intake (Koppes et al., 2005, 2006). While light to moderate alcohol intake reduces the risk of insulin resistance and diabetes, heavy drinking (≥48 g/day) leads to compromised insulin sensitivity and increased incidence of metabolic syndrome especially diabetes (Fan et al., 2006b; Koppes et al., 2005). Although several hypotheses have been speculated for alcohol-induced insulin resistance including accumulation of norepinephrine and epinephrine, fatty acid ethyl esters, impaired insulin binding and hepatic insulin sensitizing substance (Ting and Lautt, 2006), none of these scenarios has been fully validated by clinical and experimental evidence as direct triggers for alcoholism-induced insulin resistance. Acetaldehyde, the very first oxidized metabolic product of ethanol, is one of the candidates for alcohol-

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doi:10.1016/j.expneurol.2006.08.002

induced tissue and cell injury along with formation of protein-aldehyde adducts, accumulation of fatty acid ethyl esters and modification of lipoprotein and apolipoprotein particles. It is far more reactive than ethanol and contributes to alcohol-induced tissue injury and protein damage (Zhang et al., 2004; Quertemont et al., 2005; Hunt, 1996; Deitrich, 2004). Although acetaldehyde has been demonstrated to inhibit insulin-stimulated glucose oxidation *in vitro* (Lomeo et al., 1988), the concentration of acetaldehyde (~55 mM) required to inhibit glucose oxidation was too high to be realistic for alcoholics. Blood acetaldehyde levels usually achieve 30–125 μM (with 500 μM being the highest level reported) in individuals with defective mitochondrial aldehyde dehydrogenase ($ALDH_2$) following alcohol intoxication compared with normal individuals (5 μM) (Watanabe et al., 1985; Nishimura et al., 2002; Chen et al., 1999). Up-to-date, acetaldehyde is not favorably considered as a contributing factor to the pathogenesis of alcohol-induced insulin insensitivity or resistance (Ting and Lautt, 2006). Given the predominant role of acetaldehyde in alcohol-induced tissue injury, the aim of the present study was to examine the effect of pathophysiological level of acetaldehyde (150 μM) on glucose uptake and insulin signaling in dopaminergic SH-SY5Y human neuroblastoma cells. The rationale of selecting SH-SY5Y cells in this study was two-fold. First, the impact of the ethanol metabolite acetaldehyde on insulin sensitivity has been rarely elucidated in neuronal cells, given the well-known neuronal cytotoxicity of ethanol and acetaldehyde including acetaldehyde-mediated accumulation of reactive oxygen species and impairment of microtubule systems in the brain (Pratt et al., 1990). Secondly, SH-SY5Y human neuroblastoma cells have been relatively well characterized for insulin signaling cascade. It was demonstrated that SH-SY5Y cells lack insulin receptor substrate (IRS)-1 but use IRS-2 as the main substrate for insulin signaling (Kurihara et al., 2000; Kim et al., 1998). Following insulin stimulation, IRS-2 is tyrosine phosphorylated and quickly associates with Grb2 and p85. Phosphatidylinositol-3 (PI-3) kinase is then recruited to IRS-2 initiating activation of protein kinase B/Akt and participation of multiple downstream signaling molecules including mammalian target of rapamycin (mTOR), ribosomal-S6 kinase ($p70^{S6K}$), the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) in cell growth and glucose transport (Kurihara et al., 2000; Kim et al., 1998; Chang et al., 2004).

Materials and methods

SH-SY5Y cell culture and drug treatment:

SH-SY5Y human neuroblastoma cells purchased from American Type Culture Collection (Manassas, VA) were cultured in a complete medium containing minimum essential media (MEM), Hams F-12 media and Hanks Balanced Salt Solution (HBSS, Gibco-BRL) with a 2:1:1 ratio (Shavali et al., 2003). The media contained 10% fetal bovine serum along with penicillin (50 U/ml) and streptomycin (50 μg/ml). The cells were cultured in flasks and were kept in a humidified incubator containing 5% CO_2 in air at 37°C. The media were changed every 2–3 days. For drug treatment, SH-SY5Y cells were starved (with only 0.1% fetal bovine serum instead of 10%) for 18 h before drug incubation including acetaldehyde (150 μM), insulin (100 nM), the mammalian target of rapamycin inhibitor rapamycin (200 nM) or a combination of these agents. Following overnight (12 h) drug incubation, caspase-3 assay, glucose uptake and gel electrophoresis were performed in SH-SY5Y neuroblastoma cells. To minimize acetaldehyde evaporation, culture flasks were tightly sealed with parafilm as a common practice in our research laboratory as well as others (Aberle and Ren, 2003; Roman et al., 2000).

Western blot analysis

SH-SY5Y cells were lysed by sonication on ice in 0.5 ml RIPA lysis buffer containing 150 mM NaCl, 0.25 deoxycholic acid, 1% NP-40, 1 mM EDTA, 50 mM Tris–HCl, 2 mM sodium orthovanadate and 1% protease inhibitor cocktail before centrifugation (12,000×*g* for 10 min) to remove precipitated material. Protein concentration was determined in the supernatant containing the soluble proteins using Bradford assay. The soluble proteins (50 μg/lane) were separated on 10% [Akt, pAkt, $p70^{S6K}$, phospho-$p70^{S6K}$ ($pp70^{S6K}$), phosphor-4E-BP1] or 7% [mTOR, phospho-mTOR (pmTOR)] SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and were transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBS-T, and then incubated with anti-Akt (1:1000), anti-pAkt (Thr308) (1:1000), anti-mTOR (1:1000), anti-pmTOR (Ser2448, 1:1000), anti-$p70^{S6K}$ (1:1000), anti-$pp70^{S6K}$ (Thr-389, 1:1000), anti-p4E-BP1 (Thr70, 1:1000) and anti-IRS-2 (1:1000) antibodies. All antibodies were obtained from Cell Signaling Technology (Beverly, MA) or Upstate (Lake Placid, NY). The antigens were detected by the luminescence method (ECL Western blotting detection kit, Amersham) with peroxidase-linked anti-rabbit IgG (1:5000 dilution). After immunoblotting, the film was scanned and the intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer (Model: GS-800) (Fang et al., 2005).

Glucose uptake measurement

The cells were washed 3 times with Krebs-Ringer-*N*-[2-hydro-ethyl]-piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) (KRH, 136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10 mM HEPES, pH 7.4) buffer and incubated with 2 ml KRH buffer at 37°C for 30 min. Some cells were exposed to insulin (100 nM, 10 min). Glucose uptake was initiated with addition of 0.1 ml KRH buffer and 2-deoxy-D-[^{3}H] glucose (0.2 μCi/ml with a specific activity of 10 Ci/mmol) and 5 mM glucose. Glucose uptake was terminated 30 min later by washing the cells 3 times with cold PBS. Our earlier observations indicated that 2-deoxy-D-[^{3}H] glucose uptake is still linear over the 30 min duration (Davidoff et al., 2004; Dong et al., 2006). The cells were lysed overnight with 0.5 ml 0.5 M NaOH and 0.1% SDS (w/v). The radioactivity retained by cell lysates was determined by a scintillation counter (1 cpm = 0.888×10^{-12} Ci, Beckmann LC