Acetaldehyde promotes rapamycin-dependent activation of p70S6K and glucose uptake despite inhibition of Akt and mTOR in dopaminergic SH-SY5Y human neuroblastoma cells

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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 (p70S6K), 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 [3H]-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 p70S6K, did not affect acetaldehyde-induced inhibition on phosphorylation of Akt and mTOR. Interestingly, acetaldehyde enhanced p70S6K 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 p70S6K phosphorylation.

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Keywords: Acetaldehyde; Glucose uptake; IRS-2; Akt; mTOR; p70S6K; 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-
induced tissue and cell injury along with formation of protein-acetaldehyde 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 alcohols. Blood acetaldehyde levels usually achieve 30–125 μM (with 500 μM being the highest level reported) in individuals with defective mitochondrial aldehyde dehydrogenase (ALDH2) 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 (Praat 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 kinase (PI3-K) 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 (p70S6K), 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% CO2 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 (12,000g 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, p70S6K, phospho-p70S6K (pp70S6K), 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-p70S6K (1:1000), anti-pp70S6K (Thr389, 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 CaCl2, 1.25 mM MgSO4, 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-[3H] 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-[3H] 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.
Caspase-3 assay

The caspase-3 activity was determined according to the published method (Li et al., 2004). Briefly, 1 ml PBS was added to a flask containing SH-SY5Y cells and the monolayer was scraped and collected in a microfuge tube. Cells were pelleted by centrifugation at 10,000 × g at 4°C for 10 min. The supernatant was discarded, and cells were lysed in 100 μl of ice-cold cell lysis buffer [50 mM HEPES, pH 7.4, 0.1% [3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1% nonidet P-40 (NP40)]. The assay was carried out in a 96-well plate with each well containing 30 μl of cell lysate, 70 μl of assay buffer (50 mM HEPES, 0.1% CHAPS, 100 mM NaCl, 10 mM DTT and 1 mM EDTA) and 20 μl of caspase-3 colorimetric substrate Ac-DEVD-pNA (Sigma). The 96-well plate was incubated at 37°C for 1 h, during which time the caspase in the sample was allowed to cleave the chromophore pNA from the substrate molecule. Absorbency was detected at 405 nm with caspase-3 activity being proportional to color reaction. Protein content was determined using the Bradford method (Bradford, 1976). The caspase-3 activity was expressed as picomoles of pNA released per μg of protein per minute.

Data analysis

Data were mean ± SEM. Statistical significance (p < 0.05) was determined by analysis of variance (ANOVA) with a Dunnett’s post hoc test.

Results

Effect of acetaldehyde on apoptosis and basal- as well as insulin-stimulated glucose uptake

Short-term (12 h) incubation of acetaldehyde (ACA, 150 μM), insulin (100 nM) and rapamycin (Rapa, 200 nM) along or in combination did not induce overt apoptosis in SH-SY5Y cells (Fig. 1A). Results in Fig. 1B indicated that short-term incubation of acetaldehyde elicited a subtle, however, significant elevation in 2-deoxy-D-[3H] glucose uptake, which was blocked by rapamycin, which inhibits mTOR leading to inactivation of p70S6K (Guertin and Sabatini, 2005). Similarly, insulin (100 nM) elicited a significant elevation in glucose uptake, the effect of which was ablated by rapamycin. Combination of acetaldehyde and insulin did not elicit any additive effect compared with each agent alone. Rapamycin itself significantly reduced glucose uptake.
Effect of acetaldehyde on IRS-2 expression

As shown in Fig. 2, expression of IRS-2 was not significantly affected by short-term incubation of acetaldehyde (ACA, 150 μM), insulin (100 nM) or both. Neither did rapamycin (Rapa, 200 nM) affect the pattern of response acetaldehyde- or insulin-elicited on IRS-2 expression.

Effect of acetaldehyde on activation of Akt, mTOR, p70S6K and 4E-BP1

Western blot analysis depicted in Fig. 3 revealed that short-term incubation of acetaldehyde (ACA, 150 μM), insulin (100 nM) and rapamycin (Rapa, 200 nM) either alone or in combination exhibited little effect on total Akt expression. Insulin significantly enhanced Akt phosphorylation (pAkt) by 2.4-fold, which was not affected by rapamycin. Acetaldehyde suppressed both basal and insulin-stimulated Akt phosphorylation, the effect of which was not altered by rapamycin. Rapamycin itself did not have any significant effect on Akt phosphorylation. Our data further revealed that acetaldehyde significantly reduced mTOR phosphorylation without affecting total mTOR expression. Although insulin itself did not affect total mTOR and mTOR phosphorylation, it nullified acetaldehyde-induced inhibition of mTOR phosphorylation. Although rapamycin significantly upregulated total mTOR expression, it did not affect the pattern of response acetaldehyde or insulin elicited on total mTOR. In addition, rapamycin did not affect acetaldehyde-elicited inhibition on mTOR phosphorylation but significantly unmasked an insulin-induced inhibition on mTOR phosphorylation (Fig. 4). We also evaluated expression and activation of the downstream signal of Akt and mTOR, p70S6K. Results shown in Fig. 5 depicted that both acetaldehyde (ACA, 150 μM) and insulin (100 nM) stimulated phosphorylation of p70S6K without affecting the expression of total p70S6K. There was no additive effect on p70S6K phosphorylation from acetaldehyde and insulin. However, co-incubation of acetaldehyde and insulin significantly upregulated total expression of p70S6K. As expected, rapamycin (200 nM) abolished the stimulatory effect of acetaldehyde and insulin on p70S6K phosphorylation. As expected, rapamycin itself attenuated activation of p70S6K. We also evaluated the effect of acetaldehyde on phosphorylation of 4E-BP1, a signaling molecule downstream of mTOR (Aoki et al., 2001). Data in Fig. 6 show that acetaldehyde (ACA, 150 μM) inhibited 4E-BP1 phosphorylation. Although insulin (100 nM) exerted little effect...
on basal 4E-BP1 phosphorylation, it nullified acetaldehyde-induced inhibition of 4E-BP1 phosphorylation. Rapamycin (Rapa, 200 nM) itself inhibited basal 4E-BP1 phosphorylation and induced an additional depression of acetaldehyde-induced inhibition of 4E-BP1 phosphorylation.

Discussion

Our current study revealed that short-term incubation of acetaldehyde interferes with basal and insulin-stimulated Akt phosphorylation, basal phosphorylation of mTOR and 4E-BP1 but enhanced basal (not insulin-stimulated) p70S6K phosphorylation in the absence of overt abnormality in IRS-2, glucose uptake and cell survival in SH-SY5Y human neuroblastoma cells. These results suggested possible interruption of insulin signal at levels of Akt, mTOR and 4EBP1, which may be countered by some compensating mechanisms leading to enhanced p70S6K phosphorylation and glucose uptake.

Acetaldehyde is the very first metabolic product of ethanol from alcohol dehydrogenase and is considered one of the toxins contributing to the pathogenesis of alcoholic complications due to its high reactivity (Zhang et al., 2004; Deitrich, 2004; Hunt, 1996; Quertemont et al., 2005). Elevated blood acetaldehyde levels and antibodies to acetaldehyde-protein adducts have been found in alcoholics (Nuutinen et al., 1983; Harcombe et al., 1995). However, the advance of the acetaldehyde toxicity theory has been stalled over the past years due to practical constraints of acetaldehyde manipulation such as low boiling point, high volatile property and high chemical reactivity. Acetaldehyde is usually metabolized rapidly (~5 times faster than ethanol) to keep a low blood acetaldehyde level following ethanol intoxication. Levels ranging from 30 to 125 μM (with 500 μM being the highest level reported) following ethanol intoxication have been documented in Asians and African American populations lacking the low Km ALDH2 (Chen et al., 1999; Nishimura et al., 2002; Watanabe et al., 1985). Our present study employed 150 μM of acetaldehyde, a pathophysiological level which may be achieved following ethanol intoxication, to examine its short-term effect on IRS-2 (IRS-1 is lacking in SH-SY5Y cells; Kim et al., 1998) and insulin signaling. Our finding is somewhat consistent with the previous notion that acetaldehyde may not be an essential player in alcohol-induced insulin resistance (Ting and Lautt, 2006). Nonetheless, our data revealed that acetaldehyde may interrupt...
Akt, mTOR and 4E-BP1 signaling, three critical components in post-insulin receptor signaling cascade, in the absence of overt cell death. Moreover, our data suggested that acetaldehyde may elicit subtle but significant increase in glucose uptake while displaying little effect on insulin-stimulated glucose uptake in SH-SY5Y human neuroblastoma cells. This seems to be consistent with the observation of an enhanced p70S6K phosphorylation in response to acetaldehyde exposure, similar to that of insulin.

Perhaps the most puzzling findings from our study are the disparities between p70S6K and Akt/mTOR/4E-BP1 response following acetaldehyde incubation, given that p70S6K is a known target of Akt/mTOR (Chang et al., 2004; Aoki et al., 2001). Although no precise mechanism can be offered at this point, it may be speculated that certain Akt/mTOR-independent mechanism(s) may participate in acetaldehyde-initiated subcellular signal transduction leading to activation of p70S6K. Acetaldehyde may work through the mitogen-activated protein (MAP) kinase pathway independent of its action on PI-3 kinase/Akt/mTOR pathway. Our earlier study has demonstrated activation of extracellular signal-regulated kinase (ERK1/2), c-Jun NH2-terminal kinase (JNK) and p38 MAP kinase family by 100–200 μM acetaldehyde (Li et al., 2004, 2006). Activation of MAP kinase may lead to downstream activation of protein kinase C, known to be activated by acetaldehyde (Wyatt et al., 2000). Vice versa, different protein kinase C isoforms have been shown to stimulate ERK1/2 and p70S6K phosphorylation and cell proliferation (Ghosh et al., 2004). Therefore, protein kinase C and MAP kinase activation may contribute to the Akt/mTOR-independent activation of p70S6K in response to acetaldehyde exposure. Similar to p70S6K, the eukaryotic initiation factor 4E-BP1 is a downstream target of mTOR. Phosphorylation of 4E-BP1 due to Akt/mTOR phosphorylation may lead to downstream activation of protein kinase C, known to be activated by acetaldehyde (Wyatt et al., 2000). Vice versa, different protein kinase C isoforms have been shown to stimulate ERK1/2 and p70S6K phosphorylation and cell proliferation (Ghosh et al., 2004). Therefore, protein kinase C and MAP kinase activation may contribute to the Akt/mTOR-independent activation of p70S6K in response to acetaldehyde exposure. Similar to p70S6K, the eukaryotic initiation factor 4E-BP1 is a downstream target of mTOR. Phosphorylation of 4E-BP1 due to Akt/mTOR phosphorylation leads to inactivation of the negative regulator of translation (Aoki et al., 2001). Our data of reduced 4E-BP1 phosphorylation in response to acetaldehyde is consistent with acetaldehyde-induced reduction in mTOR phosphorylation and is also supported by rapamycin-induced decrease in 4E-BP1 phosphorylation. In conjunction with the p70S6K data (which contradict findings of Akt/mTOR), our study seems to suggest existence of both Akt/mTOR-dependent and -independent mechanisms in acetaldehyde-induced response on post-receptor insulin signaling. Our result shown in Fig. 4 indicated that neither insulin nor rapamycin alone affects mTOR phosphorylation, although a combination of the two significantly decreases mTOR phosphorylation. Careful review of the data revealed that both insulin and rapamycin elicited a
In our study, combination of insulin and acetaldehyde exerts no additive effect on glucose uptake beyond either stimulus alone (Fig. 1B), indicating a shared mechanism between the two molecules. However, acetaldehyde represses Akt phosphorylation whereas insulin stimulates it (Fig. 3). This apparent conundrum may indicate that involvement of distinct signaling pathway(s) in acetaldehyde- and insulin-stimulated glucose uptake. As mentioned earlier, acetaldehyde may work through MAP kinase pathway independent of the PI-3 kinase/Akt/mTOR pathway (Li et al., 2004, 2006). MAP kinase has been demonstrated to regulate glucose uptake (Heo and Han, 2006). Furthermore, in addition to PI-3 kinase/Akt activation, activation of AMP-activated protein kinase (AMPK) may also contribute to glucose uptake. AMPK is known to potentiate insulin-induced phosphorylation of glycogen synthase kinase 3β (GSK3β), p70S6K and IRS (Longnus et al., 2005) although no information is available interaction between acetaldehyde and AMPK. Discrepant mechanism in MAP kinase and AMPK may contribute to acetaldehyde- and insulin-stimulate glucose uptake independent of their effects on Akt phosphorylation. Last but not the least, acetaldehyde-induced effect on insulin signaling appears to be somewhat inconsistent with that elicited by its parent compound ethanol. Lang and colleagues revealed certain tissue-specific variations in ethanol-induced effect on post-receptor insulin signaling cascade. While alcohol impairs insulin or insulin-like growth factor I (IGF-1)-induced phosphorylation of p70S6K in both hearts and skeletal muscles, it inhibits IGF-1-induced phosphorylation of 4E-BP1 in hearts but not skeletal muscles (Lang et al., 2003; Kumar et al., 2002). This is in contrary to our present experimental findings of enhanced p70S6K in response to acetaldehyde exposure in SH-SY5Y cells. In addition, acute alcohol intoxication-induced effects on p70S6K and 4E-BP1 were independent of alcohol metabolism (Lang et al., 2004), supporting the notion that acetaldehyde may not play a major role in alcohol-induced loss of insulin sensitivity (Ting and Lautt, 2006).

**Experimental limitations**

In our study, we used sealed parafilm to minimize evaporation of the volatile acetaldehyde. Although previous studies from our laboratory as well as others indicated that sealed container is effective against acetaldehyde escape (Aberle and Ren, 2003; Roman et al., 2000), it is unlikely that we can maintain consistent levels of acetaldehyde through the 12 h of incubation. Paradoxically, if acetaldehyde is eliminated approximately 12 h after alcohol intoxication (Zuba et al., 2002). Therefore, our data may not necessarily reflect the effect of a true “150 μM” of acetaldehyde through the entire duration of incubation. Moreover, pharmacological dose of insulin (100 nM) was used. This is essentially based on our previous experience of cell culture study to provide sufficient nutrients to cells (Ren et al., 2003) although a non-physiological factor was introduced. Last but not the least, we only focused on the major insulin signaling components while leaving others especially non-Akt dependent pathways untouched.

In summary, our study provided evidence that acetaldehyde interferes with post-receptor insulin signaling (at levels of Akt, mTOR and 4E-BP1 but not p70S6K) in SH-SY5Y cells in the absence of apoptosis and glucose uptake defect. It should be pointed out that a conclusive statement cannot be made on whether acetaldehyde plays a role in the pathogenesis of alcoholism-induced alteration of insulin signaling and insulin sensitivity without further study using transgenic model (such as altered acetaldehyde metabolism) with elevated blood or tissue acetaldehyde levels. Although it may be difficult to rationalize the mechanism responsible for discrepant responses acetaldehyde induced on glucose uptake and post-receptor insulin signaling, it is pertinent to elucidate the precise role of acetaldehyde in alcoholism-associated insulin resistance so that optimal clinical management can be arranged.
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References


