Intermittent high glucose exacerbates the aberrant production of adiponectin and resistin through mitochondrial superoxide overproduction in adipocytes

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Abstract

Hypoadiponectinemia and hyperresistinemia may be important in mediating signals from adipocytes to insulin-sensitive tissue and vasculature. However, the mechanism that mediates the aberrant production of adipokines remains poorly understood. In this study, we have investigated the effect of intermittent high glucose on the expression of adiponectin and resistin, and the production of 8-hydroxydeoxyguanosine (8-OHdG) and nitrotyrosine in the adipocytes, either in the presence or in the absence of Mn(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) or thenoyltrifluoroacetone (TTFA). 3T3-L1 adipocytes were incubated for 72 h in media containing different glucose concentrations: 5 mmol/l, 20 mmol/l, 5 mmol/l alternating with 20 mmol/l glucose, with or without MnTBAP and TTFA. We measured the expression of resistin and adiponectin. The production of nitrotyrosine and 8-OHdG as oxidative stress parameter was measured. Both constant and intermittent high glucose significantly suppressed the expression and secretion of adiponectin, and increased expression and secretion of resistin in mature adipocytes compared to normal glucose conditions. However, these effects were significantly greater under intermittent high glucose conditions compared to constant high glucose. The levels of nitrotyrosine and 8-OHdG were significantly elevated under both intermittent and constant high glucose conditions, the effect being greater under intermittent high glucose. In addition, the antioxidants MnTBAP or TTFA reversed the aberrant production of adiponectin and resistin, as well as overproduction of nitrotyrosine and 8-OHdG in adipocytes induced by constant or intermittent high glucose. Intermittent high glucose exacerbates the aberrant production of adiponectin and resistin through reactive oxygen species overproduction at the mitochondrial transport chain level in adipocytes, indicating that glycemic variability has important pathological effects on the secretion of adipokines.

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Introduction

Adipose tissue is a major endocrine organ, which releases a wide range of protein signals and factors termed adipokines. A number of adipokines, including leptin, adiponectin, resistin, plasminogen activator inhibitor-1, are linked to inflammation and the inflammatory response. The adipokines appear to be involved in a wide range of physiological processes, including hemostasis, lipid metabolism, blood pressure regulation, insulin sensitivity, and angiogenesis (Fruhbeck *et al.* 2001, Trayhurn & Wood 2004). The aberrant production of inflammatory adipokines is increasingly considered to be etiologically important in the development of diseases linked to obesity, particularly type 2 diabetes, cardiovascular diseases, and the metabolic syndrome (Hotamisligil 2003, Yudkin 2003). Thus, type 2 diabetes and atherosclerosis, as well as the other components of the metabolic syndrome, have been causally linked to inflammation.

Aberrant production of adipose-derived adipokines, especially hypoadiponectinemia and elevated resistin

levels, is now believed to play a causative role in the development of obesity-associated insulin resistance and cardiovascular disorders (Trayhurn & Wood 2004, Lam & Xu 2005). Various proinflammatory cytokines, such as tumor necrosis factor- α and interleukin-6, and reactive oxygen species (ROS) have been shown to increase expression of adipokines in adipocytes (Lin *et al.* 2005). Oxidative stress, endoplasmic reticulum stress, and nuclear factor kappa B (NF- κ B) activation pathways also operate in adipocytes (Van Gaal *et al.* 2006). Oxidative stress and inflammation in adipose tissue can be exacerbated by hyperglycemia (Lin *et al.* 2005).

The increased production of superoxide by the mitochondrial electron transport chain plays a causative role in hyperglycemic damage (Nishikawa *et al.* 2000). A possible involvement of this pathway in the hyperglycemia-induced aberrant production of inflammatory adipokines from adipocytes has not yet been reported. Therefore, the purpose of this study was to verify a possible connection between mitochondrial ROS production and aberrant production of adiponectin and resistin induced by both intermittent and

constant high glucose. To this aim, 3T3-L1 adipocytes culture medium was enriched with the superoxide dismutase (SOD) mimetic Mn(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP; Quagliaro *et al.* 2003), a cell-permeable superoxide dismutase mimetic or thenoyltrifluoroacetone (TTFA), an inhibitor of mitochondrial complex II (Nishikawa *et al.* 2000). Nitrotyrosine and 8-hydroxy-deoxyguanosine (8-OHdG) have been measured as an index of oxidative damage.

Materials and methods

Cell culture

3T3-L1 cells (Wuhan University Type Culture Collection) were cultured in high glucose DMEM supplemented with 10% FBS (Sigma Chemicals) and antibiotics. The differentiation of 3T3-L1 preadipocytes to mature adipocytes was performed using insulin, dexamethasone, and 3-isobutyl-1-methylxanthine as described previously (Lin *et al.* 2005). On the 10th day post-induction, a vast majority of cells ($\geq 90\%$) had accumulated into lipid droplets (data not shown). Then they were exposed to the experimental conditions for 72 h. The cells were randomly divided into three groups: 1) continuous normal glucose medium (5 mmol/l), alternating 5 mmol/l glucose fresh media every 6 h; 2) continuous high glucose medium (25 mmol/l), alternating 25 mmol/l glucose fresh media every 6 h; 3) intermittent high glucose medium (5/25 mmol/l), alternating normal (5 mmol/l) and high glucose (25 mmol/l) media every 6 h; and 10 μ mol/l TTFA and 100 μ mol/l MnTBAP, were also added individually to the three media previously described.

Determination of 8-OHdG

8-OHdG amount was determined in 3T3-L1 adipocytes DNA digests using Bioxytech 8-OHdG-EIA Kit, a competitive ELISA. Adipocytes DNA was isolated using DNAzol Reagent, according to the manufacturer's instructions, and quantified using a spectrophotometer. Samples containing 400 μ g of DNA were resuspended in 50 μ l reaction mixture, containing 100 mmol/l sodium acetate (pH 5.0) and 5 mmol/l MgCl_2 , and digested with 1 μ l DNaseI, for 10 min at room temperature. DNA-digested samples were added to the microtiter plate precoated with 8-OHdG, and the assay was performed according to the manufacturer's instructions.

Determination of nitrotyrosine

Nitrotyrosine content was evaluated by ELISA. Briefly, an identical amount of protein from cell lysates (50 μ g) was applied to a Maxisorp ELISA plate (NUNC Brand

Products) together with nitrated BSA standard and allowed to bind overnight at 4 °C. After blocking, wells were incubated at 37 °C for 1 h with a mouse monoclonal antibody anti-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY, USA; 5 μ g/ml) and then for 45 min at 37 °C with a peroxidase conjugated goat anti-mouse IgG secondary antibody diluted 1:1000. After washing, peroxidase reaction product was generated using 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate.

Adiponectin and resistin quantification

The conditioned culture medium from 3T3-L1 adipocytes was collected at different time points as indicated, and the concentrations of adiponectin and resistin in the conditioned medium were measured using the mouse adiponectin and resistin assay kits from Linco Research Inc. (St Charles, MO, USA). The assay was performed using the sandwich ELISA according to the manufacturers' protocols.

Analysis of adiponectin and resistin gene expression

Total RNA was isolated from cultured 3T3-L1 cells using the TRIZOL method (Invitrogen) and reverse transcribed using the reverse transcription system. In brief, the reverse transcription mixture contained 1 mg total RNA, 0.5 mg oligod (T) primer, 4 ml $5\times$ RT buffer, 0.5 mmol/l deoxynucleotides, 50 U of RNase inhibitor, and 200 U reverse transcriptase (Promega) in a total volume of 20 μ l. The reaction mixture underwent PCR carried out at 42 °C for 1 h followed by heat inactivation at 95 °C for 5 min. The number of cycles and reaction temperatures used in the PCR assay were optimized to provide a linear relationship between the amount of input template and the amount of PCR product. The amount of PCR products formed in each cycle was evaluated on the basis of SYBR Green I fluorescence. All amplification reactions were performed using the Mx3000 Multiplex Quantitative PCR System. Results were analyzed with Stratagene Mx3000 software. The primers used for amplification, together with their specific optimum cycling conditions, were as follows: mouse adiponectin (a 219 bp product): sense primer 5'-AAGGACAAGGCCGTTCTCT-3'; antisense primer 5'-TATGGGTAGTTGCAGTCAGTTGG-3'; annealing temperature 61 °C; 35 cycles. Mouse resistin (a 175 bp product): sense primer 5'-GTACCCACGGGATGAAG-AACC-3'; antisense primer 5'-CAAGGTTGGATGG-GGCCGAAGGG-3'; annealing temperature 60 °C; 33 cycles. β -actin (a 489 bp product) sense primer 5'-CCAGGGTGTGATGGTGAATG-3'; antisense primer 5'-CGCAGGATTCCCTCTCAGCTG-3'; annealing temperature 59 °C; 33 cycles.

Statistical analysis

All experimental conditions were replicated fivefold. Results are expressed as mean $\pm$ s.d. The inter-group comparisons were made by Student's *t*-test or one-way ANOVA. Analyses were performed by the software package, SPSS 13.0 (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered significant.

Results

Intermittent high glucose markedly decreases adiponectin production in adipocytes

To examine the effects of high glucose on the expression of adiponectin, fully differentiated 3T3-L1 adipocytes were cultured in the constant or intermittent high glucose condition for different time periods. Quantitative real-time PCR demonstrated that constant and intermittent high glucose significantly decreased the steady-state mRNA abundance of the adiponectin gene in a time-dependent manner. A modest reduction was observed at 12 h, and further deteriorated afterwards (Fig. 1A).

Quantitative ELISA analysis showed that the adiponectin concentrations in the conditioned media were suppressed by 16 and 43% at 12 h after constant and intermittent high glucose treatment (Fig. 1C), and reached the maximum suppression at 48 h. The expression of adiponectin showed a great decrease in constant high glucose condition in comparison with normal glucose condition, and this decrease was even more marked in intermittent glucose condition. This result suggests that constant and intermittent high glucose treatment not only decreases the mRNA level of the adiponectin gene, but also inhibits its protein synthesis and secretion from adipocytes, but in the latter condition the decrease was more marked.

MnTBAP and TTEA can prevent the hyperglycemia-induced reduction in the adiponectin concentrations. The content of adiponectin in cell culture supernatants of constant and oscillating high glucose conditions after 48 h became comparable to the content in normal glucose conditions (Fig. 2A).

Figure 1 Time-dependent effects of intermittent and constant high glucose on the gene expression and protein production of adiponectin and resistin in 3T3-L1 adipocytes. N, constant normal glucose (5 mmol/l); H, constant high glucose (25 mmol/l); H/N, 5 mmol/l alternating with 25 mmol/l glucose. Fully differentiated 3T3-L1 adipocytes were cultured in intermittent or constant high glucose condition for 12, 24, 48, and 72 h. Total RNA purified from these samples was subjected to quantitative PCR analysis to determine the mRNA levels of adiponectin (A) and resistin (B). The protein concentrations of adiponectin (C) and resistin (D) in the conditioned medium were quantified as described in Materials and methods. For comparison, the levels of the adiponectin and resistin mRNA from 12-h time point in 5 mmol/l glucose condition were arbitrarily set at 1. * $P < 0.01$ vs 5 mmol/l glucose, # $P < 0.01$ versus constant high glucose ($n = 5-8$).

Figure 2 The effects of MnTBAP and TIFA on protein production of adiponectin (A) and resistin (B) in 3T3-L1 adipocytes cultured in intermittent or constant high glucose condition for 48 h. N, constant normal glucose (5 mmol/l); H, constant high glucose (25 mmol/l); H/N, 5 mmol/l alternating with 25 mmol/l glucose. * $P < 0.01$ vs 5 mmol/l glucose, # $P < 0.05$ versus constant high glucose ($n = 5-8$).

Intermittent high glucose markedly increases resistin expression in adipocytes

In contrast to adiponectin, both the steady-state mRNA abundance of the resistin gene and its protein concentration in the conditioned medium were markedly elevated in both constant and intermittent high glucose in a time-dependent manner, but in the latter condition the increase was more marked (Fig. 1B and D). The significant increase in mRNA abundance of resistin was observed at 12 h after constant and intermittent high glucose treatment, further deteriorated afterwards and reached the maximum expression at 48 h. Resistin protein concentration was elevated at 12 h and reached the maximum expression at 48 h. This result suggests that constant and intermittent high glucose treatment not only increases the mRNA level of the adiponectin gene,

but also inhibits its protein synthesis and secretion from adipocytes.

MnTBAP and TIFA produced an equally marked reduction in protein concentration of the resistin gene obtained in cells, cultured in constant high glucose and intermittent high glucose in comparison with the same conditions without the addition of the inhibitor (Fig. 2B).

Nitrotyrosine and 8-OHdG measurement

After 48 h, when no oxidative stress inhibitory substance was added, the concentration of nitrotyrosine in cell protein extract revealed an increase in the constant

Figure 3 The effects of MnTBAP and TIFA on production of nitrotyrosine (A) and 8-OHdG (B) in 3T3-L1 adipocytes cultured in intermittent or constant high glucose condition for 48 h. N, constant normal glucose (5 mmol/l); H, constant high glucose (25 mmol/l); H/N, 5 mmol/l alternating with 25 mmol/l glucose. * $P < 0.01$ vs 5 mmol/l glucose, # $P < 0.05$ versus constant high glucose ($n = 5-8$).

high glucose condition and more in intermittent high glucose condition in comparison with the normal glucose condition. The adding of the inhibitory substances, MnTBAP and TTFA, equally inhibited the increase of nitrotyrosine in the constant and intermittent high glucose condition as regards the same conditions where no inhibitor was added (Fig. 3A).

Similarly, after 48-h culture, the amount of 8-OHdG increased both in the constant and in the intermittent high glucose condition, but in the latter the content was more than doubled (Fig. 3). The presence of the inhibitors (MnTBAP and TTFA) equally blocked the 8-OHdG production in both the constant and intermittent high glucose conditions (Fig. 3B).

Discussion

In the present studies, we have demonstrated that both the constant and intermittent high glucose markedly suppressed adiponectin mRNA expression and its protein secretion, and increased resistin production in mature adipocytes.

3T3-L1 adipocytes are a widely used cell culture model of white adipocytes (Rosen & Spiegelman 2000). In mice, resistin is highly and specifically expressed in white adipose tissue (Steppan *et al.* 2001a). Overall, human resistin is only 53% identical with its murine counterpart, but identity is highest in the C-terminal signature sequence region (Steppan *et al.* 2001b). Surprisingly, unlike murine resistin, the expression level of resistin mRNA in human adipose tissue is extremely low but detectable by real-time PCR and is about 1/250 of that in the mouse. Remarkably, resistin mRNA is abundant in mononuclear blood cells (Savage *et al.* 2001). Resistin is highly expressed in pre-adipocytes isolated from plastic surgery patients, and resistin expression decreased during adipogenesis, contrary to the results in mice (Janke *et al.* 2002). Plasma resistin concentration is reported to be in the range of 3–13 ng/ml in healthy subjects with levels approaching 40 ng/ml in obese individuals (Pfutzner *et al.* 2003). McTernan *et al.* (2002) detected 4.2-fold higher resistin expression and protein than in peripheral fat depots in thigh and breast in human. Resistin mediates insulin resistance, but this role may be limited to rodents. In humans, data on the role of this adipocytokine in insulin sensitivity and obesity are controversial. Some authors indicated that increased serum resistin levels are associated with increased obesity, visceral fat (Pagano *et al.* 2005), and type 2 diabetes (Burnett *et al.* 2006), while other groups failed to observe such correlations (Zou *et al.* 2005). Verma *et al.* (2003) demonstrated that resistin activates vascular endothelium, causing release of endothelin-1.

Additionally, resistin treatment increased endothelial cell expression of VCAM-1 and MCP-1. Thus, resistin exerts proinflammatory changes in vascular endothelium in experimental settings. As a result, resistin has become an inflammatory marker of atherosclerotic disease (Reilly *et al.* 2005). Hence, resistin may play a prominent role in the pathogenesis of diabetes-related vascular disease. Adiponectin is a protein hormone secreted almost exclusively by adipocytes from mice and human, which exhibits antiatherogenic and insulin-sensitizing properties (Kadowaki & Yamauchi 2005). Lower adiponectin concentrations were found in patients with obesity and diabetes (Cavusoglu *et al.* 2006, Fantuzzi & Mazzone 2007), and the treatment of apolipoprotein E-deficient mice with an adiponectin-expressing adenovirus has proven to reduce atherosclerotic plaque formation (Fantuzzi & Mazzone 2007). Hypoadiponectinemia are an independent predictor of all-cause mortality, cardiac mortality, and myocardial infarction in patients presenting with chest pain (Kadowaki & Yamauchi 2005).

In this study, we found that intermittent high glucose induces a greater aberrant production of adiponectin and resistin than constant high glucose. These findings suggest that intermittent high glucose could be more deleterious to the dysregulation of adipokines from adipocytes than constant high glucose, supporting a pathophysiologic link between intermittent high glucose and increased cardiovascular risk (Ceriello *et al.* 2008). Intermittent high glucose may be more dangerous for the cells than constant high glucose. Mesangial cells cultured in periodic high glucose concentration increase matrix production more than the cells cultured in high stable glucose (Takeuchi *et al.* 1995). Similarly, fluctuations of glucose display a more dangerous effect than stable high glucose on both tubulointerstitial cells and human renal cortical fibroblasts, in terms of collagen synthesis and cell growth (Jones *et al.* 1999). New diabetes therapies focused on reducing postprandial hyperglycemia have become available and may benefit glycemic control and cardiovascular disease risk factor levels (Bastyr *et al.* 2000). It is now recognized that both hyperglycemia at 2 h during an oral glucose challenge and glucose fluctuation (Ceriello 2004) are strong predictors of cardiovascular disease and microangiopathic complications (Singleton *et al.* 2003), and it has been suggested that these 'hyperglycemic spikes' may play a direct and significant role in the pathogenesis of diabetic vascular complications (Ceriello 1998).

The present study also addresses the signaling pathways responsible for dysregulation of adipokines induced by high glucose treatment in adipocytes. We found that constant high glucose produced an increase in oxidative stress generation. Moreover, the pathway involved in the damaging effect of intermittent high

glucose on adipocytes is, at least in part, the same one working in stable high glucose concentrations. It appears noteworthy that it is enhanced in intermittent high glucose conditions. We confirm that intermittent glucose enhances oxidative stress generation and worsens the effects of high glucose on dysfunction of adipocytes. Dysregulation of adiponectin and resistin expression is accompanied by an increase of both nitrotyrosine and 8-OHdG, and is reversed by the SOD mimetic MnTBAP. This study showed that inhibiting the mitochondrial electron transport complex II preserves adipocytes from dysregulation of adipokines expression induced by high glucose treatment, both constant and intermittent. The effect of TTFA, a specific antioxidant active at mitochondrial level, in normalizing dysregulation of adipokines expression, as well as nitrotyrosine and 8-OHdG, was equivalent to those of MnTBAP, suggesting that an overproduction of free radicals at mitochondrial level is the mediator of the dysfunction of adipocytes. Actually, MnTBAP and SOD were active against ROS originating in different districts inside the cell. The fact that a specific mitochondrial oxidative stress inhibitor achieves the same results suggests that the major source of ROS inside the cell, due to high glucose exposition, is the mitochondrial electron transport chain. To be specific, TTFA selectively inhibits mitochondrial complex II activity; inhibition of entrance of electron to ubiquinone from complex II blocks off ROS generation, preventing all downstream processes.

At present, the molecular mechanisms specifically triggered on cultured adipocytes by periodically changing glucose concentrations are not known. A possible explanation may be that during chronic exposure to high glucose, some metabolic variations induced by this constant situation might change or feedback regulatory cell controls, partially counteracting the glucose toxic effect. Intermittent exposure to high glucose might reduce such adaptation, causing more pronounced toxicity.

In conclusion, our study shows that the exposure of adipocytes to intermittent high glucose produces the dysregulation of several adipokines, and supports the hypothesis that glucose fluctuation may be involved in the aberrant secretion of adipokines. Moreover, our data suggest that this phenomenon is related to overproduction of mitochondrial superoxide in adipocytes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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