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Hyperglycemia-induced inflammation caused down-regulation of 8-oxoG-DNA glycosylase levels in murine macrophages is mediated by oxidative-nitrosative stress-dependent pathways

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ABSTRACT

High glucose-induced increase in production of reactive oxygen/nitrogen species (ROS/RNS) is recognized as a major cause of the clinical complications associated with diabetes. ROS/RNS apart from being redox agents, cause an unwanted severe physiological load to cells, also act as cellular messengers, and play a key role in activation of circulating macrophages. However, the molecular mechanisms of activation of macrophages by hyperglycemic conditions are currently unclear. In the present study, we report that high glucose (HG) causes a dramatic increase in the production of inflammatory cytokines and chemokines, at least in part through enhanced mRNA transcription. The increase in levels of inflammatory cytokines/chemokines corresponds to increased levels of ROS/RNS, which is accompanied by increased activities of Akt, ERK1/2, tuberin, down regulation of 8-oxoG-DNA glycosylase (OGG1), and increase in 8-hydroxydeoxyguanosine (8-OHdG) accumulation in DNA. Elevated levels of ROS/RNS are triggering alteration in antioxidants level, biomolecules damage, cell cycle dysregulation, and apoptosis in macrophage cells. Pretreatment of antioxidants caused decrease in the levels of ROS/RNS leads to an increase in the levels of antioxidants, decrease in biomolecules damage, alterations in Akt, ERK1/2, tuberin, upregulation of OGG1, and decrease in 8-OHdG accumulations in DNA. Further, antioxidants treatments inhibit the effects of HG on the transcriptional activity of cytokines and chemokines. Our results demonstrate that intracellular signaling pathways mediated by ROS/RNS are linked to each other by elevated glucose in macrophages activation leading to inflammation. These findings provide a mechanistic explanation of how ROS/RNS cooperate to conduct inflammatory intracellular signals in macrophages related complications in hyperglycemic conditions.

1. Introduction

Diabetes is increasingly afflicting the human population all around the world in general and with more severity the developed countries in particular. However, the Indian population had garnered the dubious distinction of having maximum number of diabetics in the world (Kaveeshwar and Cornwall, 2014). Hyperglycemic conditions have been correlated with at least two-fold or higher risk of cardiovascular diseases including atherosclerosis, which accounts for the majority of deaths in individuals with diabetes (Steinberg and Schertzer, 2014; Seshasai et al., 2011). Atherosclerosis is initiated by onset of inflammatory processes in the endothelial cells, leading to accumulation of lipids and immune cells within the intimal space of arteries (Woollard, 2013). Interestingly, the orchestration of adaptive and innate immune cell interactions is critical for controlling metabolic inflammation in atherosclerosis (Lumeng, 2013; Lumeng et al., 2009). Different immune cell types have been linked to the progression of diabetes and related cardiovascular complications. Macrophages are a key source of inflammatory mediators that directly contribute to causes of diseases like atherosclerosis, insulin resistance, immune homeostasis, and tolerance that prevent tissue or organ damage (Swirski and Nahrendorf, 2013; Bhargava and Lee, 2012).

A number of hypotheses have been propagated in order to explain the adverse effects of hyperglycemia and oxidative stress is one of them (Russell and Cooper, 2015; Kumar et al., 2016). High glucose in the blood stream could accelerate the generation of many free radicals and peroxide species by autooxidation of glucose, causing oxidative stress to the all cell types of an individual in general including circulating monocytes (Kashiwagi et al., 1996; Tesfamariam and Cohen, 1992). Many studies have demonstrated that the levels of end products of free radicals attack

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are reliable and relatively straightforward indicators of oxidative stress due to hyperglycemia (Kumar et al., 2016; Simone et al., 2008). The redox changes induced by hyperglycemia, DNA damage, protein carbonylation (PC), and lipid peroxidation (LPO) have been shown to alter cellular functions via activation of key signal transduction pathways (Kumar et al., 2014). It is likely that hyperglycemia-induced intra- and extracellular changes lead to alterations of signal transduction pathways, affecting gene expression and protein function leading to cellular dysfunction. Recent publications have revealed that mitochondria and nuclei are two major targets of oxidative/nitrosative stress, which contain a variety of DNA repair enzymes (Evans et al., 2004). Apoptosis occurs when the endogenous antioxidant network and DNA repair systems are overwhelmed with oxidative overload and cannot recover from the damages inflicted to the cells (Maiese et al., 2007). 8-OHdG, a modified base, is formed in DNA due to attacks on guanosines by hydroxyl radicals (•HO) produced as intermediary byproduct of aerobic metabolism (Sheng et al., 2012). Smart et al. (2006) have demonstrated that the 8-OHdG incorporated in DNA is removed primarily via the DNA base excision repair pathway. DNA repair enzyme that recognizes and excises out 8-OHdG is OGG1 (Rosenquist et al., 1997). Apart from structural changes in biomolecules, a number of cell signal transduction pathways are activated in hyperglycemic conditions. Activation of phosphatidylinositol3-kinase (PI3-K) and phosphorylation of Akt by certain agonists lead to inactivation of tuberin (Dan et al., 2002). The PI3-K/Akt and ERK1/2MAPK pathway is activated in diabetes, which is redox dependent in different cell types (Kumar et al., 2016).

Recent studies show that tissue-resident macrophages are key instigators in the secretion of cytokines that impair insulin action in metabolically active organs (Olefsky and Glass, 2010) and promote foam-cell formation during atherosclerosis (Kaveeshwar and Cornwall, 2014). Further, Steinberg and Schertzer (2014) demonstrated a coincidence of nutrient overload with increased infiltration of metabolic tissues and the vascular endothelium by macrophages which promote formation of a proinflammatory environment, including increased levels of cytokines like tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β) and enzymes like inducible nitric oxide synthase (iNOS). Although it has been known for years that cardiovascular tissues can release a large amount ROS/RNS, the role of oxidative-nitrosative stress-dependent pathways in atherogenesis has received increasing attention only in recent years. Moreover, very few studies have examined the signaling pathways and molecular mechanisms leading to these events. In this study we demonstrate that HG causes an increased in the levels of free radicals in macrophage cells, which dependents upon the HG concentration as well as on the duration. High glucose causes a dramatic increase in the expression of inflammatory cytokines and chemokines, corresponds to increased levels of ROS/RNS, which is accompanied by increased activities of Akt, ERK1/2, tuberin, down regulation of OGG1, and increase in 8-OHdG accumulation in DNA. Also, we demonstrate that pretreatment of antioxidants like *N*-acetyl cysteine (NAC) and hydralazine hydrochloride (Hyd.HCl) counteract the injurious effects inflicted by HG challenge in these cells.

2. Materials and methods

2.1. Reagents and cell culture

D-glucose, mannitol, NAC, Hyd.HCl, and PI3-K/Akt inhibitors (LY294002 [LY], Wortmannin [WT] were purchased from Sigma-Aldrich. ERK1/2 inhibitors (PD98059 [U1], U0126 [U2]) and different antibodies like phospho- and total ERK1/2, JNK, P38, Akt, tuberin, ATM, ATR, p53, H2A.X, β-actin and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (USA). OGG1 and 8-OHdG from Abcam (UK), cytochrome C, Catalase (CAT), and superoxide dismutase (SOD) were obtained from Imgenex, India. Murine macrophage (RAW264.7) cells and human monocyte (THP-1) cells, obtained from National Centre for Cell Science, India, were maintained in Dulbecco's modified eagle medium (Invitrogen, USA) containing 10% heat-inactivated fetal bovine serum (Invitrogen, USA), and 5.5 mM basal glucose (BG). Cells were exposed to different concentrations of additional D-glucose (5, 10, 20, 25, and 30 mM) for 24 h and 25 mM glucose for indicated times. Moreover, cells were grown in 1% serum containing media overnight before subjecting to one of the inhibitors before subjecting them to 25 mM of glucose for indicated durations. The THP-1 cells were maintained at 37 °C in 5% CO_2 in RPMI-1640 medium (Invitrogen, USA) containing 10% heat-inactivated fetal bovine serum and differentiated into the adherent macrophages after treatment of phorbol myristate acetate (20 nmol/l) for 24 h.

2.2. Oxidative stress assay

ROS level was determined by Nitrotetrazolium blue (NBT) reduction assay (Cury-Boaventura and Curi, 2005). Briefly, treated cells were harvested and incubated with NBT solution (10 mM) for 1 h. After centrifugation at 12,000 × *g*, reduced intracellular NBT was solubilized by adding 50% (v/v) acetic acid. Cell debris was finally pelleted and the absorbance of NBT in the supernatant was determined. Free •HO assay was carried out according to Manikandan et al. (2010). Briefly, 300 μl (80 μg protein) of cell lysate was mixed with 700 μl phosphate buffered saline (PBS, pH 7.8), 0.1 M EDTA, 2 mM sodium salicylate, 40 μl 10 N HCl and 0.25 g NaCl. Further, equal volume of ice cold diethyl ether was added and then incubated for 30 min at 25 °C. Absorbance was recorded at 510 nm. Free •HO generation was expressed as absorbance at 510 nm/30 min. Level of nitric oxide (NO) was determined by measuring the nitrite production (Yoo et al., 2005). Briefly, 100 μl of culture media was mixed with 100 μl Griess reagent and absorbance was recorded at 520 nm. Intracellular ROS and RNS production was assayed by using the Dihydroethidium (DHE), and Dihydrorodamine 123 (DHR) probes, respectively. Treated cells were harvested and incubated with 5 μM solution of either DHE or DHR for 30 min and fluorescence was measured with a fluorescence-activated cell sorting (FACS) analyzer (Becton Dickinson) (Sagar et al., 2014). For cytological analysis, cells were seeded onto glass coverslips, stained with DHE/DHR, and fixed with 3.7% paraformaldehyde. The glass cover slip was mounted and observed under a microscope (Olympus BX61).

2.3. Assay of antioxidants

Antioxidants activities in cell lysates were estimated by appropriate biochemical techniques (Kumar et al., 2014; Weydert and Cullen, 2010). For CAT activity assay, protein was dissolved in 50 mM of PBS, and then H_2O_2 was added to make final concentration of 30 mM just before recording the absorbance rate kinetics. For in-gel enzyme activity assay, treated cells were harvested and 80 μg of total protein was resolved on 8% native polyacrylamide gel and then incubated with 0.003% (v/v) H_2O_2 solution for 10 min in dark. After rinsing with distilled H_2O, gel was incubated with 2% ferric chloride and 2% potassium ferricyanide stain. When achromatic bands began to appear, stain was decanted and then rinsed the gel extensively with distilled H_2O and photographed. GSH content was measured as described by Kapoor and Kakkar (2012). Briefly, treated cells were lyzed in 0.2% Triton X-100 in PBS, then spun at 8000 × *g* for 10 min. Five hundred μl of reaction mixture was mixed