OXIDISED LOW DENSITY LIPOPROTEIN DECREASES HIT-T15 PANCREATIC BETA CELLS VIABILITY VIA DECREASE IN ANTIOXIDANT ACTIVITIES

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ABSTRACT

Increased serum levels of oxidised low density lipoprotein (oxLDL) as found in patients with Type 2 diabetes, can induce severe oxidative stress in exposed cells which then can lead to cell death. Our study aimed to determine how the decreased cells antioxidant status of pancreatic beta cells induced by oxLDL affects their viability. We used various concentrations of CuCl₂ oxidised LDL to determine its cytotoxic effect as well as the influence on the antioxidant enzymes activity in HIT-T15 pancreatic beta cells. A significant cellular formation of reactive oxygen species (ROS) was detected within 3 hours incubation of HIT-T15 pancreatic beta cells with 1.5 mg/mL of oxLDL. The formation of ROS was accompanied by a simultaneously loss of cellular glutathione. However, a significant reduction in cell viability was only measured after 10 hours incubation with oxLDL. The enzymatic activities of catalase and peroxidase remained unchanged for 24 hours. These results suggest that loss of glutathione induced by oxLDL is not sufficient to cause HIT-T15 cell death as they can be still protected from cytotoxic effects of oxLDL by the other antioxidant defenses such as catalase and peroxidase.

Key words: Oxidised low density lipoprotein, reactive oxygen species, glutathione, catalase, peroxidase

INTRODUCTION

The progressive dysfunction and destruction of pancreatic beta-cells are hallmarks of the onset and progression of type 2 diabetes mellitus (T2DM) (Plaisance et al., 2016). Chronic excess of cholesterol and metabolic fuels (such as glucose and non-esterified palmitate) together with genetic factors are among the common causes that elicit beta-cell damage over time (Favre et al., 2011). High levels of oxLDL are strongly correlated with obesity-associated metabolic syndrome (Holvoet et al., 2004) and/or the development of T2DM (Nakhjavani et al., 2010). Several studies have shown that low plasma levels of high density lipoprotein (HDL) together with increased levels of oxLDL are strongly linked to T2DM (Cubedo et al., 2015; Rohrer et al., 2004). Oxidative stress caused by oxLDL was 23% higher in obese and 56% higher in T2DM patients (Marin et al., 2015). Additionally, specific antibodies against oxLDL were found in patients with T2DM while oxLDL receptors were found in both human and rodent islet beta-pancreatic cells (Bellomo et al., 1995; Vavuli et al., 2016).

A previous study showed that exposure of pancreatic beta cell lines to oxLDL leads to reduced gene expression with subsequent reduced in insulin protein production and therefore insulin secretion (Ceriello, 2000). Kaneto et al. (2010) found that oxLDL activates mitogen-activated protein kinase (MAPK) 8 (also known as c-Jun amino terminal kinase, JNK) which is one of the hallmarks of oxidative stress. Activation of JNK signaling by oxLDL leads to decrease in the PDX-1 DNA binding activity to the insulin promoter which causes reduction of insulin gene transcription. This event leads to impaired insulin expression and consequently pancreatic beta cell dysfunction. OxLDL also induces the inducible cyclic AMP repressor (ICER) that silences the expression of genes containing cAMP response elements (CRE) and ultimately causes defect in cell insulin genes expression (Favre et al., 2011). These cumulative evidences further strengthen the association of
Mammals have several antioxidant defense systems to detoxify ROS which include (a) low-molecular-mass antioxidants such as glutathione, uric acid, and vitamins C and E; (b) antioxidant enzymes; and (c) sequestration and repairing systems. Oxidative stress induced by oxLDL might be affecting the antioxidant status in the pancreatic beta cells which among the body tissues has the lowest levels of intrinsic antioxidant defenses (Pi et al., 2010). Adachi et al. (2004) showed that the expression of the antioxidant enzymes such as catalase (CAT), glutathione (GSH) reductase, GSH peroxidase, and superoxide dismutase (SOD) is decreased in various tissues of long-term diabetics.

Exposure of pancreatic beta cells to oxLDL is detrimental to the cells. Herein, we provide evidence that oxLDL, exerts deleterious effects on HIT-T15 cells by stimulating the production of ROS which is depleting glutathione levels and reducing the activity of antioxidant enzymes in pancreatic beta cells and thus affecting the cell survival. Delineating the effect of oxidative stress conferred by the oxLDL on the islet cells will give further insight in understanding the progression of diabetes mellitus.

MATERIALS AND METHODS

All reagents and chemicals were AR grade or better obtained from the Sigma Chemical Company (St Louis, USA) or Fisher Scientific (New Jersey, USA) unless otherwise stated. All solutions were prepared using ultra-pure water generated by PURELAB Option Q water system from ELGA (UK).

Preparation of oxidized low density lipoprotein (oxLDL)

Human LDL was purified by a single 22 hours density gradient ultracentrifugation in a Sorvall™ WX ultracentrifuge from EDTA-plasma as previously described (Gieseg et al., 2010). EDTA plasma was prepared from whole blood taken from healthy volunteers (nonsmoker) following an overnight fasting. The ethical approved for the blood collection was obtained from UNIMAS Ethic Committee (Bil 2/2013). The LDL concentration (total mass) was measured using the cholesterol reagent kit assay supplied by AMS Diagnostic (CT, USA) and protein concentration was measured using the bicinchoninic acid protein determination kit from Pierce (Thermo Scientific, MA, USA). For oxidation, the LDL was transferred to dialysis tubing and 0.5 mM CuCl₂ was added to it. The dialysis tube was put in 1 litre of phosphate buffer saline (PBS) containing 0.5 μM CuCl₂ and incubated at 37°C with gentle shaking for 24 hours. While still in the dialysis tubing, the oxLDL was dialysed against three changes of chelex-100 in PBS. The oxLDL was filter-sterilised using a 0.22 μm membrane filter (Sartorius) and stored at 4°C.

Cell culture

The hamster pancreatic islet cell line HIT-T15 (ATCC: CRL-1777) was maintained in a humidified 5% CO₂ incubator at 37°C. The cells were kept in Ham F12K medium (ATCC, USA) supplemented with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 87.5%; dialyzed horse serum, 10%; fetal bovine serum, 2.5%; pen-strep, 0.5%. For experiments the cells were seeded at a density of 1 x 10⁶ cells per well of 12 wells plates. After an attachment period of 12 hours the cells were washed and then either incubated with various concentrations of oxLDL for 24 hours or with the IC50 concentration of oxLDL for various time points.

Cell viability

Cell viability was determined by the MTT reduction analysis (Mosmann, 1983). After removal of the incubation medium, the cells were washed twice with PBS then growth medium containing 0.5 mg/mL MTT (Thermo Scientific, USA) was added and the cells were incubated at 37°C for 2 hours. The purple insoluble MTT-formazan crystals were dissolved with 10% (w/v) SDS in 0.01 M HCl and the absorbance was read at 570 nm using spectrophotometer (Cecil 7400 Double Beam UV/Vis Scanning Spectrophotometer, USA).

Cellular glutathione (GSH) level

Cell supernatant was used for measuring the GSH level, catalase and peroxidase activities. Cells were harvested using a rubber policeman and lysed by sonication in a cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) for catalase, PBS for peroxidase and lysis buffer for GSH. Cell lysates were then centrifuged at 10,000 x g for 15 minutes at 4°C and the supernatant was collected for the enzymatic assays.

Cellular glutathione was assayed using glutathione detection assay kits (Molecular Research) that utilizes a monochlorobimane (MCB). MCB bound to the reduced glutathione in the reaction that is catalyzed by glutathione S-transferase (GST) and form a strongly fluorescent adducts. Fluorescent adduct was detectable at excitation and emission 380 nm and 461 nm respectively.

Catalase activity

Catalase (CAT) activity was measured using an assay kit from Cayman (USA) according to the
manufacturer’s instruction. The method is based on the enzymatic breakdown of H$_2$O$_2$ in the presence of methanol. The produced formaldehyde was measured calorimetrically with a spectrophotometer (Cecil 7400 Double Beam UV/Vis Scanning Spectrophotometer, USA) at 540 nm with 4-aminomethylhydrazine-5-mercapto-1,2,4-triazole (Purpald) as the chromogen.

**Peroxidase enzyme activity**

Peroxidase activity was assayed using Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Oregon, USA) which uses the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect peroxidase activity and H$_2$O$_2$. In the presence of peroxidase, the Amplex red reagent reacts with H$_2$O$_2$ in 1:1 stoichiometry to produce the red fluorescent oxidation product, resorufin, which is then detected using microplate reader (Infinite M200 PRO Tecan, USA) at excitation maximum of 571 nm and emission maximum of 585 nm.

**Superoxide production**

The generation of superoxide was detected by dihydroethidine (DHE) staining (Burnaugh et al., 2006). DHE is a highly specific dye for superoxide anion (O$_2^-$) with low affinity for hydrogen peroxide. The cells were grown in 6 wells plate at 1.0 × 10$^6$ cells/mL and incubated with 1.5 mg/mL oxLDL. After the experiments, cells were incubated with DHE in the dark for 20 min. The cells were viewed under the inverted fluorescence microscope (Olympus, USA) using 20 x objective with fluorescent filter set for propidium iodide (PI).

**Statistical analysis**

Statistical analyses were performed by using SPSS version 20.0 where comparison among treatments were performed using one-way analysis of variance (ANOVA). All data represented are mean±SD of triplicate treatments. Where appropriate significance is indicated as *** p < 0.05.

**RESULTS**

**OxLDL reduced HIT-T15 cell viability**

Incubation of HIT-T15 cells for 24 hours with 1.0 mg/mL oxLDL caused a 22% increase in cell viability (Figure 1), while 50% of HIT-T15 cells viability was lost when exposed to 1.5 mg/mL oxLDL and less than 5% cell viability remained with 2.0 mg/mL oxLDL. Since 1.5 mg/mL oxLDL caused approximately 50% cells viability loss, this was the concentration chosen in experiments examining the effect of oxLDL on HIT-T15 cells. The appearance of the cells corresponded with the cell viability as 1.0 mg/mL oxLDL caused the cells to look foamy and large (Figure 2). Incubation of the cells with 1.5 mg/mL of oxLDL caused the cells to shrink and significant number of released apoptotic bodies were observed in the presence of 2.0 mg/mL oxLDL.

![Fig. 1. Loss of HIT-T15 cells viability after 24 hours incubation with increasing concentrations of oxLDL. HIT-T15 cells at 1.0 × 10$^6$ cells/mL were incubated with increasing concentrations of oxLDL for 24 hours. The cell viability was measured by MTT assay. Significance is indicated as compared to the control. Results are displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.](image-url)
A time course of oxLDL-mediated damage was conducted by incubating HIT-T15 cells with 1.5 mg/mL oxLDL for 24 hours (Figure 3). Cell death in HIT-T15 cells was initiated within the first 6 hours of incubation with oxLDL, however, the observed changes were not significant. About 35% of cell viability were lost after 10 hours and from there onwards a rapid decline in cell viability was observed. This would suggest that oxLDL doesn’t have a direct effect on the cells but that downstream events initiate one or several apoptotic pathways. The loss of cell viability matched with the observation of the cells under the microscope, where the cells appeared normal morphologically up to 6 hours incubation with 1.5 mg/mL oxLDL (Figure 4). Under the microscope, significant apoptotic bodies were observed after 10 hours incubation of HIT-15 cells with 1.5 mg/mL oxLDL.

**OxLDL decreased the glutathione content in HIT-T15 cells**

Glutathione is a major non enzymatic regulator of intracellular redox homeostasis in cells. As observed for the cell viability studies (Figure 1), 1 mg/mL of oxLDL concentration did not affect the HIT-T15 cells viability, but it was potent enough to disrupt the intracellular glutathione level (Figure 5). Incubation with 1 mg/mL oxLDL caused approximately 40% loss in the glutathione level of HIT-T15 cells. At 2 mg/mL oxLDL, the intracellular glutathione was only 10% of control and glutathione was totally lost at concentration of 3 mg/mL oxLDL.

The intracellular glutathione loss in HIT-T15 cells over time was examined by incubating HIT-T15 cells with 1.5 mg/mL oxLDL for 24 hours. In contrast with the cell viability studies, the glutathione loss was very rapid with 53% reduction after 3 hours (Figure 6). After 24 hours, the glutathione level was reduced by 60%.

**OxLDL decreased major antioxidant enzymes: catalase and peroxidase**

Time course studies for the effects of 1.5 mg/mL oxLDL on catalase and peroxidase activities were done to determine their sensitivity toward oxLDL. Both catalase and peroxidase activities did not show a significant drop in activities when compared to controls. Time course study for the effects of 1.5 mg/mL of oxLDL on catalase and
Fig. 3. Time course of oxLDL-induced cell viability loss in HIT-T15 cells. HIT-T15 cells at $1.0 \times 10^6$ cells/mL were incubated with 1.5 mg/mL oxLDL concentration. At various times (0, 3, 6, 10 and 24 hours), the cell viability was measured by MTT reduction assay. Significance is indicated as compared to the time zero point. Results are displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.

Fig. 4. Time course changes in HIT-T15 cells morphology during incubation with oxLDL. HIT-T15 cells at $1.0 \times 10^6$ cells/mL were incubated with 1.5 mg/mL oxLDL for 24 hours period. The cells were viewed under the microscope (20X magnification) at (a) 0 hours, (b) 3 hours (c) 6 hours (d) 10 hours and (e) 24 hours in tissue culture wells using an inverted fluorescence microscope (X70i, Olympus).
Fig. 5. Loss of glutathione activity in HIT-T15 cells after 24 hours incubation with increasing concentrations of oxLDL. HIT-T15 cells at 1.0 × 10⁶ cells/mL were incubated with increasing concentrations of oxLDL for 24 hours. Data were expressed as a percentage of zero hour levels (control). Significance is indicated from 0 mg/mL oxLDL (control) with p<0.05. Results are displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.

Fig. 6. Time course of oxLDL-induced glutathione loss in HIT-T15 cells. HIT-T15 cells at 1.0 × 10⁶ cells/mL were incubated with 1.5 mg/mL oxLDL. At various times, the glutathione levels of HIT-T15 cells were analysed by GSH assay kits (Molecular Research). Significance is indicated as compared to the time zero point. Results are displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.

Peroxidase activities suggested that these two enzymes were quite robust and able to withstand the toxicity of oxDL (Figure 7 and 8). This is in contrast with glutathione which was quite sensitive to the toxic effect of oxLDL. Catalase and peroxidase were probably very potent antioxidants in protecting the HIT-T15 cells compared to glutathione.

OxLDL induced formation of intracellular superoxide anion reactive oxygen species

It was suspected that oxLDL decreased the antioxidant status of HIT-T15 cells by generation of reactive oxidant species especially superoxide anion. A significant increase in DHE fluorescence was found after 3 hours treatment of the cells with
1.5 mg/mL oxLDL. This confirmed that oxLDL induced oxidative stress over the first 3 hours (Figure 9). This also suggests that oxidative stress occurred a few hours before the cells started losing their viability i.e. after 10 hours incubation with 1.5 mg/mL oxLDL (Figure 3).

DISCUSSION

There are many evidences confirming the adverse effects of proatherogenic oxLDL on beta pancreatic cells (Ceriello, 2000; Favre et al., 2011). At low concentration, oxLDL is nontoxic and initiate the

Fig. 7. Effect of 1.5 mg/mL of oxLDL on catalase activity in HIT-T15 cells. HIT-T15 cells at $1.0 \times 10^6$ cells/mL were incubated with 1.5 mg/mL oxLDL concentration. At various times, the catalase activities of HIT-T15 cells were measured by Cayman catalase kits. Significance is indicated with $p<0.05$ by independent t-test. Results are displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.

Fig. 8. Effect of 1.5 mg/mL of oxLDL on peroxidase activity in HIT-T15 cells. HIT-T15 cells at $1.0 \times 10^6$ cells/mL were incubated with 1.5 mg/mL oxLDL. At various times, the peroxidase activities of HIT-T15 cells were analysed by Amplex Red Hydrogen Assays. Significance is indicated with $p<0.05$. Results are displayed as mean ± SD of triplicates from a single experiment, representative of three separate experiments.
proliferation of cells. OxLDL can have dual effects on cells where it can signal both cell proliferation and death of the cells depending on oxLDL concentration and its exposure time (Galle et al., 2000; Xu et al., 2010). In this study, we showed that oxLDL decreases pancreatic beta cell viability by affecting the status of the glutathione levels. OxLDL induced oxidative stress in HIT-T15 through a production of superoxide anions which were detected with DHE staining. Superoxide anion generation was detected as early as 3 hours exposure with 1.5 mg/mL oxLDL (Figure 9). The oxidative stress caused by the oxidants generated leads to 53% loss of glutathione within 3 hours of exposure to 1.5 mg/mL oxLDL (Figure 5) suggesting that glutathione is very sensitive to the oxidants generated. Glutathione was totally loss when HIT-T15 cells were exposed to higher concentration of 3 mg/mL oxLDL. This was similar to that seen in human monocytes derived macrophages (Gieseg et al., 2010; Wang et al., 2006), U937 cells (Katouah et al., 2015) and human aortic vascular smooth cells (HASMC) (Sukhanov et al., 2006). In addition, the time course of the cell viability and glutathione loss (Figure 3 and 6) showed that the loss of glutathione occurred immediately and at all time points were at a faster rate than the loss of the cell viability. This could indicate that the gradual collapse of the glutathione level induced by oxLDL preceded HIT-T15 cells death. This could also suggest that glutathione depletion was required for oxLDL-induced HIT-T15 cells toxicity. These observations were similar to studies with human macrophages demonstrating that depletion of reduced glutathione enhanced oxLDL cytotoxicity in human macrophages (Darley-Usmar & White, 1997; Gotoh et al., 1993; Wang et al., 2006). It is likely that the depletion of reduced glutathione by oxLDL alters the glutathione thiol redox state (GSH/GSSG ratio) (Wang et al., 2006). Since GSH/GSSG ratio is one of the principal determinants of the cellular redox environment, any alteration in the redox environment can lead to cellular dysfunction and cell death (Schafer & Buettner, 2001).

In this study, we found that a significant loss of HIT-T15 cell viability occurs only after 10 hours exposure to 1.5 mg/mL oxLDL (Figure 1). Loss of glutathione does not cause the total loss of cell viability as to a certain time, the cells are still protected from the oxLDL-induced damage by the presence of the antioxidant enzymes catalase and peroxidase.

In this study, we measured the effect of oxLDL on catalase and peroxidase that catalyse the reactions of H₂O₂. Besides superoxide, H₂O₂ is a major oxidative component of oxLDL-induced ROS (Sukhanov et al., 2006) and therefore could be involved in reduced viability of HIT-T15 cells. In catalase reaction, H₂O₂ is converted to nontoxic products; water and oxygen. Peroxidase reacts by mechanisms similar to catalase where for many of these enzymes the optimal substrate is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides.
This study shows that the effect of oxLDL on peroxidase activity of HIT-T15 cells seems to be similar to its effect on catalase activity. Unlike glutathione, catalase and peroxidase enzymes were more robust towards the damaging effects of 1.5 mg/mL oxLDL since their activities dropped only after 24 hours incubation with oxLDL. The very significant drop of the cell viability at 24 hours could suggest that these two enzymes are counteracting the effect of increased free radical formation. Catalase is a radical scavenging agent found to contribute to the observed protection against vascular smooth muscle cells induced by oxLDL (Hsieh et al., 2001). Previous study showed that catalase was a very potent endogenous oxidant (Sukhanov et al., 2006) where pretreatment of HASMC with catalase efficiently suppressed oxLDL-induced H₂O₂ formation to below basal levels. Without pretreatment with catalase, oxLDL suppresses catalase’s protein and activity of HASMC.

Enzymatic antioxidant defenses such as superoxide dismutase (SOD), catalase, thioredoxin, and glutathione peroxidase (GPx) are shown to have antioxidant ability to convert ROS into less noxious compounds (Talalay, 2000; Arner & Holmgren, 2000). Other enzymatic antioxidant defenses against ROS is provided by enzymes such as Glutathione S transferase (GST), aldo-keto reductase and aldehyde dehydrogenase (Kuhn & Borcert, 2002). These enzymes detoxify and eliminate reactive metabolites using efflux pump such as glutathione S conjugate transporter (Akerboom et al., 1991). Apart from generation of superoxide anion, oxLDL also causes formation of highly reactive oxidised lipid and protein species that overwhelm the cell antioxidant capacity and induce redox imbalances leading to changes in various signaling pathways and gene expression and subsequent cell death via caspase-independent necrosis (Baird et al., 2005).

GSH (tri peptide γ-glutamlycysteinylglycine) is a major nonenzymatic regulator of intracellular redox homeostasis in cell. GSH exists in two forms which are reduced (GSH) or oxidised (GSSG) form. Under normal cellular redox condition, it mainly exists as reduced form and is distributed in nucleus, endoplasmic reticulum and mitochondria. GSH play a role in cell defense in which it involved in glutathionylation by binding with proteins and also can acts as coenzyme of numerous enzymes (Pompella et al., 2003). Glutathione directly scavenge free radicals or act as a substrate for GPx and GST during the detoxification of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds.

CONCLUSION

This study shows that in loss cell viability induced by oxLDL, a decrease in antioxidant defenses has the indispensable role. Most significantly, it also shows that the reduced levels of antioxidants enzyme activities of pancreatic beta cells definitely cause them to be more susceptible to elevated level of ROS induced by oxLDL. Excessive generation of ROS without adequate antioxidant defense leads to oxidative stress and cell function’s damage which underline numerous diseases, development abnormalities and aging (Forman & Torres, 2001; Martin & Barrett, 2002; Thannickal & Fanburg, 2000).

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