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Improved antioxidative defence protects insulin-producing cells against homocysteine toxicity

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ABSTRACT

Homocysteine (HC) is considered to play an important role in the development of metabolic syndrome complications. Insulin-producing cells are prone to HC toxicity and this has been linked to oxidative stress. However, the exact mechanisms remain unknown. Therefore it was the aim of this study to determine the nature of reactive oxygen species responsible for HC toxicity.

Chronic exposure of RINm5F and INS1E insulin-producing cells to HC decreased cell viability and glucose-induced insulin secretion in a concentration-dependent manner and led to a significant induction of hydrogen peroxide generation in the cytosolic, but not the mitochondrial compartment of the cell. Cytosolic overexpression of catalase, a hydrogen peroxide detoxifying enzyme, provided a significant protection against viability loss and hydrogen peroxide generation, while mitochondrial overexpression of catalase did not protect against HC toxicity. Overexpression of CuZnSOD, a cytosolic superoxide dismutating enzyme, also protected against HC toxicity. However, the best protection was achieved in the case of a combined overexpression of CuZnSOD and catalase. Incubation of cells in combination with alloxan resulted in a significant increase of HC toxicity and an increase of hydrogen peroxide generation. Overexpression of CuZnSOD or catalase protected against the toxicity of HC plus alloxan, with a superior protection achieved again by combined overexpression. The results indicate that HC induces oxidative stress in insulin-producing cells by stimulation of superoxide radical and hydrogen peroxide generation in the cytoplasm. The low antioxidative defence status makes the insulin-producing cells very vulnerable to HC toxicity.

1. Introduction

Many factors can contribute to dysfunction of pancreatic beta cells in diabetes, ranging from increased secretory demand to toxic effects of elevated circulating glucose and lipid concentrations as well as exposure to environmental chemicals [1–4]. Although hyperhomocysteinaemia occurs typically during the development of cardiovascular diseases [5–14], recent reports suggest that chronically elevated concentrations of homocysteine (HC) can be frequently observed in obesity-diabetes [15] and T1DM [16] may also play a contributory role. Thus acute and chronic exposure of insulin-secreting cells to HC results in defective insulin secretion and beta cell demise [16–19]. We have previously shown that HC generates reactive oxygen species in a redox cycling reaction with alloxan [19] explaining the decline in viability of insulin-secreting cells, leading to reduced glucokinase phosphorylating ability, diminished insulin secretory responsiveness and cell death [19].

One of the most widely studied beta-cell toxins is alloxan, which is toxic through redox cycling [20]. Alloxan and its redox cycling products target SH groups, forming disulfide bonds thereby causing beta-cell dysfunction and death [21,22]. Sulphur-containing HC serves also as a redox cycling partner for alloxan thereby potentiating alloxan toxicity [19].

Various mechanisms have been proposed to account for the events leading to beta-cell dysfunction and death. Arguably, the

Abbreviations: Cat, catalase; CuZnSOD, copper/zinc superoxide dismutase; CytoCat, cytosolic catalase; FBS, fetal bovine serum; Gpx, glutathione peroxidase; H_2O_2, hydrogen peroxide; HBSS, Hanks balanced saline solution; HC, homocysteine; MitoCat, mitochondrial catalase; MnSOD, manganese superoxide dismutase; MTT, 3–[4,5- dimethylthiazol-2yl]- 2,5 – diphenyl tetrazolium bromide; ROS, reactive oxygen species; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus.

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most favoured view is that chronic oxidative stress can damage beta-cells [23–26]. Like primary beta-cells, permanent insulin-secreting cell lines are very sensitive to oxidative stress due to their low antioxidative defence status [23,26]. While both cytosolic CuZnSOD and mitochondrial MnSOD superoxide dismutases are relatively well expressed, the expression of the H_2O_2 detoxifying enzymes catalase and glutathione peroxide is extremely low, leading to an imbalance between the generation and detoxification of H_2O_2, especially in the face of stress conditions like cytokine toxicity (T1DM) [25,27] or lipotoxicity (T2DM) [24,28,29].

The aim of the present study was to define the role of oxidative stress in the susceptibility of insulin-producing cells towards HC toxicity and to determine the nature of the reactive oxygen species formed in the presence of HC. For this purpose, we used insulin-producing cell clones overexpressing different antioxidant enzymes and a specific method of intracellular H_2O_2 measurement. We show that the low antioxidative defence status of insulin-producing cells makes them particularly vulnerable to the toxic action of HC and related molecules.

2. Materials and methods

2.1. Materials

Penicillin, streptomycin, fetal calf serum (FCS), Hanks balanced saline solution (HBSS), RPMI-1640 tissue culture medium (supplemented with 0.3 g/l L-glutamine), and trypsin/EDTA were from Gibco Life Technologies, (Paisley, Strathclyde, UK). Geneticin (G418), zeocin, blasticidin, lipofectamine were from Invitrogen. HyPer vectors were from Evrogen (Moscow, Russia). All other chemicals were from BDH or Sigma-Aldrich chemicals.

2.2. Stable overexpression of antioxidant enzymes in insulin-producing RINm5F cells

Insulin-producing RINm5F cells were cultured in RPMI medium supplemented with 10 mM glucose, 10% (v/v) fetal calf serum (FCS), penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. Overexpressing RINm5F cells (ATCC) were generated through stable transfection using lipofectamine as described earlier [27,30–32]. Briefly, the pCMV/myc/mito-plasmid was used to overexpress mitochondrially-targeted human catalase cDNA and the pcDNA3 vector was used to overexpress human catalase or CuZnSOD in the cytosolic cell compartment. Positive clones were selected by resistance to antibiotics (G418 and/or zeocin) and the expression of various antioxidative enzymes was verified by Western blotting (data not shown).

2.3. Determination of cell viability

Cell viability of control and transfected RINm5F cells as well as INS1E cells was determined after incubation with HC or cysteine in the absence or presence of alloxan (50 μM), using a microplate-based 3–[4,5- dimethylthiazol-2yl]- 2,5 – diphenyl tetrazolium bromide (MTT) assay [33]. The range of concentrations of chemical compounds used in this study was selected based on the preliminary studies of the concentration (0–10,000 μM)- and time (6, 12, 24, 48, 72 h)- dependency experiments (data not shown).

2.4. Analysis of H_2O_2 levels in different subcellular compartments

pHyPer-Mito and pHyPer-Cyto eukaryotic expression vectors (Evrogen) were used for mitochondrial and cytoplasmic expression, respectively, of the fluorescent H_2O_2 sensor protein HyPer as described in detail earlier [25]. Briefly, positive clones were selected by resistance to G418 (250 μg/ml) (Invitrogen) (RIN-HyPerCyto and RIN-HyPerMito) or to G418 and blasticin (Invitrogen) (CytoCat-HyPerCyto) and verified by fluorescence measurements. The HyPer protein has two excitation peaks (427 nm and 475 nm) and one emission peak (520 nm). Upon exposure to H_2O_2 the ratio of fluorescence F475/F420 rises proportionally to the H_2O_2 concentration. RINm5F cells expressing HyPer proteins or RINm5F cells expressing CytoCat and pHyPerCyto were seeded onto 96-well black plates. Plates were analyzed at 475/427 nm excitation and 520 nm emission using the Victor^2 1420 Multilabel Counter fluorescence reader (Perkin Elmer). The data were expressed as mean values of the F475/F427 ratio.

2.5. Insulin content and secretion measurements

Insulin-producing INS1E cells were cultured in fully supplemented RPMI medium as described earlier [34]. Cells were seeded onto 6-well plates 24 h before addition of chemical compounds. Cells were incubated with HC or Cys (5000 μM) for 72 h, or with alloxan and HC or Cys (250 μM) for 1 h, followed by a 1 h incubation period with glucose as given in the legend to Fig. 5. Thereafter supernatants were collected for measurements by radioimmunoassay with rat insulin as standard as described earlier [34]. Insulin content was determined in cell pellets. Insulin values were normalized to the DNA content of the incubated cells, as measured by the PicoGreen assay (Promega).

2.6. Statistical analyses

Results are presented as mean ± standard error of the mean (S.E.M). Statistical analyses were done using one way ANOVA followed by Dunnett's test or Tukey-Kramer test for multiple comparisons. EC_{50} values were calculated from non linear regression analyses using the one phase exponential decay algorithm of Prism 4 analysis program (GraphPad, San Diego, CA).

3. Results

3.1. Effects of homocysteine or cysteine on viability of insulin-producing RINm5F cells overexpressing cytoplasmic catalase (CytoCat) or mitochondrial catalase (MitoCat)

HC induced a significant concentration-dependent loss of viability in control insulin-producing RINm5F cells (Fig. 1a) with an EC_{50} value of 4287 μM. Similar toxicity (EC_{50} value of 4295 μM) was observed in RINm5F cells overexpressing catalase in the mitochondria (MitoCat) (Fig. 1a). Interestingly, however, RINm5F cells overexpressing catalase in the cytoplasm (CytoCat) were protected against HC cytotoxicity (Fig. 1a), as evident from a significantly higher EC_{50} value of 9127 μM. In contrast cysteine toxicity was minimal in RINm5F cells (Fig. 1a). Even at the highest concentration of cysteine tested (10,000 μM) the reduction in viability was less than 30% (Fig. 1a). Overexpression of catalase in either the mitochondrial or in the cytoplasmic compartment did not reduce the toxicity of cysteine (Fig. 1a).

3.2. Effects of homocysteine or cysteine on viability of insulin-producing RINm5F cells overexpressing CuZnSOD alone or in combination with cytoplasmic catalase (CuZnSOD plus CytoCat)

RINm5F cells overexpressing the cytosolic isoform of superoxide dismutase, CuZnSOD, were significantly protected against the loss of viability induced by HC (Fig. 1b). The reduction of viability was around 40% in the presence of the highest concentration of HC (10,000 μM) (Fig. 1b). In cells overexpressing both CuZnSOD and