Enhancement of homocysteine toxicity to insulin-secreting BRIN-BD11 cells in combination with alloxan

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Abstract

Previous studies have shown that homocysteine (HC) has a detrimental impact on insulin secretion and pancreatic beta cell function. The aim of the present study was to determine the role of reactive oxygen species (ROS) in the *in vitro* toxic effects of HC on insulin secretion and function of BRIN-BD11 insulin-secreting cells. In this study, insulin secretion from BRIN-BD11 cells was determined radioimmunologically, cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and glucokinase activity by a glucose phosphorylation assay following culture with HC plus alloxan (Alx). Treatment with HC resulted in concentration-dependent inhibition of insulin secretion induced by glucose and other insulinotropic agents. HC in combination with Alx resulted in a more pronounced decline in insulin secretion, including that induced by 20 mM alanine, by 43% ($P < 0.001$)

and 30 mM KCl by 60% ($P < 0.001$), compared with control culture. The glucokinase phosphorylating capacity in cells cultured with HC plus Alx was significantly lower, compared with control cells. The cells also displayed a significant 84% ($P < 0.001$) decline in cell viability. Prolonged, 72-h culture of insulin-secreting cells with HC followed by 18-h culture without HC did not result in full restoration of beta cell responses to insulinotropic agents. *In vitro* oxygen consumption was enhanced by a combination of Alx with HC. The study arrived at the conclusion that HC generates ROS in a redox-cycling reaction with Alx that explains the decline in viability of insulin-secreting cells, leading to reduced glucokinase phosphorylating ability, diminished insulin secretory responsiveness and cell death.

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Introduction

Homocysteine (HC) is a sulphhydryl amino acid with a well-established important role in various pathological situations. Raised plasma HC is now generally accepted as an important biological marker for arterial and venous vascular disease (Eikelboom *et al.* 1999). Elevated plasma HC levels have been reported in patients suffering from diseases associated with the metabolic syndrome (Sanchez-Margalet *et al.* 2002). Increased HC levels have also been detected in obese diabetic patients (Sanchez-Margalet *et al.* 2002). HC exerts detrimental effects on a number of cell lineages including endothelial cells (Blundell *et al.* 1996) and neuronal cells (Kim & Pae 1996), at least partially mediated through production of reactive oxygen species (ROS; Skurk & Walsh 2004), which induce DNA damage and apoptosis (Suhara *et al.* 2004). Studies using insulin-secreting BRIN-BD11 pancreatic beta cells demonstrated that both acute and prolonged exposure to HC had detrimental effects on beta cell glucose metabolism, insulin secretory responsiveness and cell viability (Patterson *et al.* 2006a,b, 2007a,b,c). The exact mechanisms through which HC induces irreversible damage remain unclear.

Various mechanisms have been proposed to account for the events leading to beta cell dysfunction, demise and destruction. The most favoured view is that oxidative stress can damage vulnerable beta cells (Lenzen 2008a). One of the most widely studied beta cell toxins is alloxan (Alx; Lenzen 2008b). Alx-induced diabetes has been used commonly to induce insulin-dependent diabetes where the cytotoxic action of Alx is preceded rapidly by the formation of ROS (Lenzen 2008b).

The present study examines the mechanisms underlying the detrimental effects of HC on insulin secretion induced by glucose or other insulin secretagogues. The study also examines the effect of HC on glucokinase activity and signalling function as well as cell viability in the absence or presence of the beta cell toxin Alx.

Materials and Methods

Penicillin, streptomycin, foetal bovine serum, 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. All other chemicals were from BDH and Sigma–Aldrich Chemicals.

Culture of BRIN-BD11 insulin-secreting cells

Clonal BRIN-BD11 beta cells were utilised in these studies, the features of which have been described elsewhere (McClenaghan *et al.* 1996). For experimentation, BRIN-BD11 cells were routinely maintained in RPMI-1640 tissue culture medium supplemented with 11.1 mM glucose, 10% FCS, 100 µmol/ml penicillin, and 0.1 mg/ml streptomycin in a 37 °C incubator with 5% CO₂ and 95% air (McClenaghan *et al.* 1996). To harvest BRIN-BD11, the cells were gently washed in 10 ml HBSS prior to detachment from culture flasks by 5 min incubation (37 °C) with 3 ml of pre-warmed 0.025% (w/v) trypsin containing 1 mM EDTA.

Determination of insulin release

BRIN-BD11 cells were resuspended in tissue culture medium, and then seeded in each well of 24-well plates at 1.5×10^5 cells/well. The following day, the tissue culture medium was removed and 1 ml of Krebs Ringer Bicarbonate (KRB) buffer, supplemented with 0.1% (w/v) BSA and 1.1 mM glucose, was added to each well. Cell monolayers were preincubated for 40 min at 37 °C, and then the buffer was removed and fresh KRB test buffer (1 ml) supplemented with glucose, and test agents were added to each well as noted in the Figures. After acute 20 min incubation at 37 °C, 900 µl aliquots of test buffer were collected from each well and stored at -20 °C. Insulin was measured by dextran-charcoal RIA (Flatt & Bailey 1981), using guinea-pig anti-porcine insulin serum and rat insulin standard.

Determination of cell viability

Cell viability was determined 72 h after incubation in the presence or absence of HC, using a microplate-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (Mosmann 1983).

Measurement of glucose phosphorylating enzyme

Cells were homogenised by sonication in PBS (pH 7.4) and glucokinase enzyme activity was measured in soluble fractions using an enzyme-coupled photometric assay (Lenzen *et al.* 1987). Glucokinase activity was determined by subtracting the hexokinase activity measured at 1 mmol/l glucose from the activity measured at 100 mmol/l glucose. The protein concentration was quantified by a Bio-Rad protein assay.

Oxygen uptake

Oxygen uptake by Alx in the presence of cysteine (CE) and HC was measured by using a wtw oxi 340/set dissolved oxygen meter (WTW, Weilheim, Germany). The buffer used was 50 mM potassium phosphate at pH 7.4, and the temperature was maintained at 25 °C. Recordings of oxygen

uptake were started immediately after addition of Alx to the buffered thiol solutions (Elsner *et al.* 2008).

Statistical analysis

Results are presented as mean $\pm$ S.E.M. for a given number of observations. Statistical analyses were done using one-way ANOVA followed by the Tukey's test for multiple comparisons using GraphPad (PRISM Software, San Diego, CA, USA). Differences were considered significant if $P < 0.05$.

Results

Concentration-dependent acute inhibitory effects of HC on glucose-induced insulin secretion

Raising the glucose concentration from basal 1.1 to 5.6 and 16.7 mM caused an increase in insulin release, which was significant with 16.7 mM glucose ($P < 0.001$; Fig. 1). Treatment with HC resulted in a concentration-dependent inhibition of insulin secretion at both glucose concentrations.

Effects of chronic exposure (72 h) to HC on the secretory responses of BRIN-BD11 cells to various insulin secretagogues and the effect of a withdrawal period (18 h)

The effects of a range of test agents on insulin secretion in the presence of glucose (16.7 mM) were studied in cells cultured for 72 h in the absence or presence of HC (250 µM; Fig. 2).

Figure 1 Concentration-dependent inhibitory effect of acute homocysteine (HC) exposure on glucose-induced insulin secretion. Following 40 min incubation of BRIN-BD11 insulin-secreting cells at 1.1 mM glucose, the effects of 0–1000 µM HC were assessed at 1.1, 5.6 or 16.7 mM glucose during 20 min incubations. Values are mean $\pm$ S.E.M. ($n = 6$) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ compared with control. $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with 1.1 mM glucose.

Figure 2 Effects of chronic exposure to homocysteine (HC) on the insulin secretory responses to various secretagogues and the effect of a withdrawal period (18 h). BRIN-BD11 insulin-secreting cells were cultured for 72 h in the absence (A) or presence (B) of 250 μ M HC or with an 18-h recovery period (C) control culture conditions. Following 40 min preincubation in 1.1 mM glucose, cells were exposed to an acute 20 min incubation with 16.7 mM glucose together with 20 mM alanine (Ala), arginine (Arg) or keto-isocaproic acid (KIC), 200 μ M tolbutamide (Tol), 30 mM potassium chloride (KCl) or 7.68 mM calcium (Ca^{2+}) with or without of 250 μ M HC. Values are mean $\pm$ S.E.M. ($n=6$). ** $P<0.01$, *** $P<0.001$ compared with control. $\Delta\Delta P<0.01$, $\Delta\Delta\Delta P<0.001$ compared with effect without HC. + $P<0.05$, ++ $P<0.01$ compared with corresponding test agent in control culture.

Though still significant, the insulin secretory responses to all agents were significantly reduced in the presence of HC (Fig. 2). Withdrawal (18 h) of HC did not result in recovery of the attenuated responses to any of the insulin secretagogues tested (Fig. 2).

Effects of HC culture in combination with Alx on glucose-induced insulin secretion

The secretory responses to increasing glucose concentrations (5.6 and 16.7 mM as compared with 1.1 mM) were not significantly reduced by exposure to 250 μ M HC or 50 μ M Alx during 72 h culture (Fig. 3).

When, however, the cells were cultured with a combination of HC (250 μ M) plus Alx (50 μ M), there was a nearly 50% reduction ($P<0.05$) of insulin secretion at both 5.6 and 16.7 mM (Fig. 3).

Effects of HC in combination with Alx on hexokinase and glucokinase enzyme activities

Following 72 h culture of intact BRIN-BD11 cells with a combination of HC (250 μ M) with Alx (50 μ M), enzyme

activity was determined in cytosolic fractions. There was a more than 50% reduction ($P<0.001$) of glucokinase (measured at 100 mM glucose) activity after culture with HC (250 μ M) with Alx (50 μ M) compared with control culture cells. Exposure to HC (250 μ M) alone showed a much smaller inhibitory effect on glucokinase activity (Fig. 4). HC (250 μ M) in the presence and absence of Alx (50 μ M) increased hexokinase activity (Fig. 4).

Effects of HC in combination with Alx and the modulatory role of methionine on BRIN-BD11 cell viability

A significant inhibitory effect of Alx (50 μ M) on cell viability was also observed in the presence of HC (250 μ M; Fig. 5). HC (250 μ M) alone was not toxic (Fig. 5). Methionine (Met; 10 mM), another sulphur-containing amino acid, did not reduce cell viability, either alone or in the presence of Alx (50 μ M; Fig. 5).

Effects of chronic exposure (72 h) to HC plus Alx on secretory responses to various secretagogues

Cells cultured in the absence or presence of HC (250 μ M) or Alx (50 μ M) or a combination of both were used to assess the insulin-releasing effects of 16.7 mM glucose with (A) alanine (Ala; 20 mM), arginine (Arg; 20 mM), keto-isocaproic acid (KIC; 20 mM), (B) Tol (0.2 mM), 30 mM KCl (30 mM) or 7.68 mM calcium (Ca^{2+} ; 7.68 mM; Fig. 6). Each agent significantly ($P<0.05$ – $P<0.001$) stimulated insulin secretion when compared with control cells, incubated in the presence of 16.7 mM glucose alone. In contrast, cells cultured in the presence of HC or Alx exhibited reduced insulin secretory responsiveness to all agents tested (Fig. 6). However, culture

Figure 3 Effects of chronic exposure to homocysteine (HC) in combination with alloxan (Alx) on glucose-induced insulin secretion. BRIN-BD11 insulin-secreting cells were cultured for 72 h in the absence or presence of 250 μ M HC, 50 μ M Alx or with HC plus Alx. Cells were then acutely exposed to 1.1, 5.6 or 16.7 mM glucose. Values are S.E.M. ($n=6$). *** $P<0.001$ compared with control culture $\Delta\Delta\Delta P<0.001$ compared with 1.1 mM glucose. ** $P<0.01$ compared with Alx culture.

Figure 4 Effects of chronic exposure to homocysteine (HC) in combination with alloxan (Alx) on hexokinase and glucokinase enzyme activities. BRIN-BD11 insulin-secreting cells were cultured for 72 h in various culture conditions in the absence or presence of 250 μ M HC with or without 50 μ M Alx. Cellular protein was then extracted and the glucokinase assay was performed with addition of 1 mM glucose (hexokinase) or 100 mM glucose (glucokinase). The rate of reaction was measured at 340 nm. Glucokinase activity was determined by subtracting the hexokinase activity measured at 1 mM glucose from the activity measured at 100 mM glucose. Values are mean $\pm$ S.E.M. ($n=6$). * $P<0.05$, *** $P<0.001$ compared with control culture. $\Delta P<0.05$, $\Delta\Delta P<0.01$ compared with glucokinase. $\Psi\Psi P<0.01$ compared with corresponding culture in the absence of 50 μ M Alx.

with a combination of HC plus Alx evoked particularly severe inhibitory effects on insulin secretion induced by all insulin secretagogues by around 50–80% when compared with cells cultured under normal conditions (Fig. 6).

Oxygen uptake by Alx in the presence of HC or CE

To obtain direct evidence for redox cycling of Alx with HC or CE, the initial oxygen uptake was measured. Oxygen was consumed in a solution of Alx with HC or CE. The initial rate of oxygen uptake by Alx (50 μ M) with HC (1 mM) was 24.5 ± 2.1 μ mol/l per min, whereas for CE (1 mM) the rate of oxygen consumption was 14.4 ± 2.2 μ mol/l per min ($n=4$, $P<0.01$).

Discussion

Consistent with previous data, HC reduced insulin output at basal, intermediate and high glucose levels in a concentration-dependent manner with substantial reductions in insulin output at higher HC concentrations (100–1000 μ M). Oxidative stress is widely accepted as playing a key mediatory role in the development and progression of diabetes and its complications, due to increased production of free radicals and impaired antioxidant defences (Maritim *et al.* 2003). The diabetogenic agent, Alx, is selectively toxic to insulin-secreting cells causing beta cell toxicity (Lenzen 2008b) through generation of ROS in a cyclic reaction with its reduction product dialuric acid (Elsner *et al.* 2006). As such, Alx toxicity may largely be accounted for by intracellular

generation of oxygen radicals through redox cycling (Elsner *et al.* 2006). The present data demonstrate marked effects of HC in the presence of Alx, consistent with the view that HC can interact with Alx, reducing it to dialuric acid and generating superoxide radicals and hydrogen peroxide, thereby altering BRIN-BD11 insulin-secreting functional integrity. The chemical redox reactions between Alx and thiols such as HC, CE and GSH leading to the generation of the cytotoxic radicals follow the flow diagram of chemical reactions depicted in the review by Lenzen (2008b).

The effect of 72 h culture with HC in combination with Alx resulted in the most detrimental effects on insulin secretion in comparison with cells cultured with HC or Alx alone. Alx alone is not damaging to the beta cells at submillimolar concentrations (Lenzen 2008b), as used in this present study. Alx needs a thiol-containing partner to redox cycle with in order to generate ROS (Lenzen 2008b). HC redox-cycles vigorously with Alx, as demonstrated by this study. The 72-h culture of HC in the presence of Alx resulted in a significant decrease in cell viability, due to the generation of ROS. As a result of the general demise of the beta cell, the ability of glucokinase to phosphorylate glucose was diminished. Thus the loss of glucokinase enzyme activity within this beta cell-damaging process (Lenzen 2008b) results in reduced glucose oxidation and ATP generation (Gunnarsson & Hellerström 1973), and this also blunts the ATP signal required to initiate the stimulus–secretion coupling pathway for insulin secretion (Lenzen 1992). Since the detrimental effect of HC is not specific for glucose-induced insulin secretion but reduces insulin secretion induced by a number of other agents studied, this shows that the inhibitory effect of

Figure 5 Effects of chronic exposure to homocysteine in combination with alloxan (Alx) and the modulatory role of methionine (MET) on cell viability. BRIN-BD11 insulin-secreting cells were cultured for 72 h with 50 μ M Alx or 10 mM MET or 10 mM MET plus 50 μ M Alx with or without HC. *** $P<0.001$ compared with control culture. Values are S.E.M. ($n=6$) $\Delta\Delta\Delta P<0.001$ compared with absence of HC. +++ $P<0.001$ compared with Alx culture.

Figure 6 Effects of chronic exposure to homocysteine (HC) plus alloxan (Alx) on acute insulin secretory responses to various secretagogues. BRIN-BD11 insulin-secreting cells were cultured for 72 h in the absence or presence of 250 μ M HC, 50 μ M Alx or with HC plus Alx. Cells were then acutely exposed to (A) 20 mM alanine (Ala), arginine (Arg) or keto-isocaproic acid (KIC), (B) 200 μ M Tol, 30 mM potassium chloride (KCl) or 7.68 mM calcium (Ca^{2+}). Values are S.E.M. ($n=6$) * $P<0.05$, *** $P<0.001$ compared with control culture (16.7 mM glucose). $\Delta P<0.05$, $\Delta\Delta P<0.01$, $\Delta\Delta\Delta P<0.001$ compared with control (no secretagogues). + $P<0.05$ compared with HC culture.

HC is not solely the result of a glucokinase inhibition but rather that of a general toxic action of extracellularly generated hydrogen peroxide through Alx redox cycling, which causes unspecific damage to insulin-secreting cells. Hydrogen peroxide is membrane permeable (Lenzen 2008a) and after entering the cells inhibits the process of insulin exocytosis. It is the toxicity of the hydrogen peroxide which is generated during redox cycling of HC with Alx at a low micromolar concentration and not a specific inhibitory interaction of Alx with the SH groups of the glucokinase molecule, for which exposure of cells to millimolar concentrations of Alx would be required in the absence of a thiol without the requirement of a redox-cycling reaction to take place (Lenzen 2008b).

The observation of an increased hexokinase activity after exposure of the cells to HC independent from the presence of Alx can be interpreted as the de-differentiating effect of HC, explaining why the BRIN-BD11 cells become less responsive to stimulation of insulin release at physiological glucose concentrations. Higher hexokinase activity allows survival of the cells of this tissue culture line.

The insulin secretory potential of all amino acids tested was reduced following chronic culture of cells with HC or Alx. However, it was the 72-h culture of HC plus Alx that resulted in the most damaging influence on the insulin secretory function. ROS generated during culture of cells with HC plus Alx damaged the cells, thereby disturbing the insulin exocytotic machinery of the insulin-producing cells. To obtain direct evidence for redox cycling of Alx with the thiols CE and HC, the initial oxygen uptake was determined. Oxygen was consumed in a solution of Alx with both thiols. The combination of Alx with HC resulted in a significantly higher rate of oxygen uptake compared with the rate of oxygen consumption with CE (Elsner *et al.* 2006), showing

that HC redox cycles with Alx much more vigorously than CE. This can explain the greater toxicity of Alx in the presence of HC, even though the mechanism of Alx toxicity is the same in the presence of different monothiods. In a complex chain of chemical reactions (Lenzen 2008b), Alx redox cycles with GSH, CE and HC in the same fashion. In this reaction, which takes place extracellularly, the membrane-permeable reactive species hydrogen peroxide is generated. After gaining access to the intracellular space, hydrogen peroxide and ROS derived from it, in particular the hydroxyl radical, cause the damage to the beta cells (Lenzen 2008b). The insulin-secretory effect of the sulphonylurea, Tol, which closes the K_{ATP} channel by direct interaction with the SUR1 subunit (Ashcroft & Gribble 1999), and the membrane depolarisation-mediated insulin-secretory effects of KCl (Roefeldt *et al.* 1992) were also significantly reduced, as were the insulinotropic effects of raised (7.68 mM) Ca^{2+} (Ammon *et al.* 1989). Chronic culture of these cells with HC resulted in detrimental effects on the subsequent responses to all secretagogues tested. Furthermore, this reduction in insulin output was sustained after an 18-h recovery period during which cells had been returned to normal culture conditions without HC. This would suggest permanent damage to the cellular stimulus-secretion coupling mechanisms induced by the 72-h HC exposure.

In conclusion, the present data indicate that HC results in a decline of insulin secretory cell function due to the toxicity of ROS, effects that were considerably worsened in the presence of Alx.

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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