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Dose-dependent cysteine-mediated protection of insulin-producing cells from damage by hydrogen peroxide

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Abstract

Aims/Hypothesis: Oxidative damage is believed to play a key role in the process of pancreatic beta cell destruction leading to type 1 diabetes. The beta cells are sensitive to oxidative stress because their intracellular anti-oxidative defence mechanisms are weak. The defence mechanisms depend heavily on glutathione, the synthesis of which is dependent on the availability of cysteine. We investigated whether an increased amount of cysteine available could protect beta cells from oxidative damage. *Methods*: Rat insulinoma cells (RINm5F) were exposed to 50 or 100 μM hydrogen peroxide in the presence of three different cysteine concentrations (0.1, 1 and 5 mM). Cell viability was analyzed by vital staining and the cellular metabolic status by C,*N*-diphenyl-*N*′-4,5-dimethyl thiazol-2-yl tetrazolium bromide (MTT) analysis. Intracellular insulin, DNA and glutathione contents were measured. The mechanism of death was further clarified by gel electrophoretic DNA fragmentation analysis. *Results*: Hydrogen peroxide decreased cell viability and induced functional impairment. Vital staining indicated that 1 mM cysteine effectively protected the cells. The protective effect was confirmed by the MTT assay showing preserved metabolic integrity, and by measurements of intact intracellular insulin and DNA content. Cysteine increased intracellular glutathione. Gel electrophoretic analysis of DNA revealed hydrogen peroxide-induced apoptotic fragmentation. This was also abolished by 1 mM cysteine. The therapeutic window of cysteine was narrow: 0.1 mM cysteine provided inadequate protection, and 5 mM cysteine was already toxic in this setting. *Conclusion*: A proper dose of cysteine could provide a safe and effective means to protect beta cells from oxidative damage.

Keywords: Anti-oxidant; Cysteine; Oxidative stress; Pancreatic beta cell; Pathogenesis; Cell death

1. Introduction

Incomplete reduction of molecular oxygen by the mitochondrial respiratory chain and some reactions of cellular metabolism can generate ROS including H_2O_2, superoxide anion and hydroxyl radical. Further sources of ROS include leukocytes activated in inflammation. These metabolites react with biological molecules, such as proteins, lipids, carbohydrates and DNA. Living organisms are constantly exposed to oxidative stress which may increase dramatically in inflammation, by exogenous toxins, etc. [1,2]. Cells have an extensive endogenous defence system against this hazard. It consists of anti-oxidative enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and of small anti-oxidants, such as GSH and Vitamins C and E [3,4].

GSH is of particular importance because of its high intracellular concentration and its dual role both in anti-oxidation and in detoxification [5]. GSH is synthesized in two steps: first cysteine is linked with glutamate in an ATP-dependent reaction catalyzed by γ-glutamylcysteine synthetase and the product, γ-glutamylcysteine, is further linked with glycine in another ATP-dependent reaction

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Abbreviations: ADP, adenosinediphosphate; ATP, adenosinetriphosphate; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; H_2O_2, hydrogen peroxide; HO, Hoechst 33342; HEPES, (*N*[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); IDDM, insulin dependent diabetes mellitus; INS-1, cells mouse insulinoma cell line; KRB, Krebs–Ringer buffer; MTT, C,*N*-diphenyl-*N*′-4,5-dimethyl thiazol-2-yl tetrazolium bromide; NAC, *N*-acetylcysteine; PBS, phosphate-buffered saline; PI, propidium iodide; RINm5F cells, rat insulinoma cell line; ROS, reactive oxygen species; RT, room temperature.

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catalyzed by glutathione synthetase. Peroxides are removed with GSH in reactions which lead to the formation of oxidized glutathione (GSSG). GSSG can be converted back to the reduced form by glutathione reductase. Under oxidative conditions, the level of GSH may decrease temporarily but should be rapidly restored by increased synthesis and reduction. GSH also reacts with hydroxyl radical, peroxynitrite, and hydroperoxides, as well as with reactive electrophiles, and maintains thiol-dependent enzymes and Vitamins C and E in their active forms [6,7].

IDDM is believed to result from a chronic cell-mediated immune response towards the insulin-producing pancreatic beta cells. During the prediabetic period pancreatic islets are invaded by lymphocytes and macrophages. The infiltrating cells, mostly T-cells and macrophages, produce and secrete ROS and cytokines (interferon-γ, interleukine-1, tumor necrosis factor-α) which damage beta cells by inducing intracellular ROS-production [8,9]. ROS can, for example, activate the nuclear enzyme poly(ADP-ribose) polymerase (PARP) leading to excessive formation of poly(ADP-ribose), depleting cells of NAD^+ (nicotinamide adenine dinucleotide), and triggering cell death [10]. The continuous ROS-mediated stress leads to reduced insulin gene transcription and induction of beta cell apoptosis [11,12].

The aim of this study was to find out whether H_2O_2-induced stress in insulinoma cells could be inhibited by simultaneous supply of cysteine, providing reductive power and extra resources for intracellular GSH synthesis. Mechanisms of oxidative damage were investigated in order to understand the role of apoptosis under oxidative conditions and to reveal whether cysteine supplementation was anti-apoptotic.

Our results show a concentration-dependent protective effect of extracellular cysteine against H_2O_2-induced oxidative stress on insulinoma cells. This anti-oxidative treatment maintains a higher cellular viability as well as integrity of nuclear DNA.

2. Materials and methods

2.1. Chemicals

Hydrogen peroxide was purchased from Merck, and its concentration was checked with a spectrophotometer at 240 nm. All other chemicals, unless otherwise stated, were from Sigma, and were stored and used according to the manufacturer's advice.

Cysteine was used fresh and its quality was checked with HPLC using thiol specific labelling and a fluorescence detector.

2.2. Cell cultures

RINm5F cells (kindly provided by Prof. Claes Wollheim, University of Geneva) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin and 100 μg/mL streptomycin, pH 7.0–7.4. Tissue culture flasks and petri-dishes were from Greiner Labortechnic. INS-1 cells (also from Prof. Wollheim) were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin pH 7.0–7.4. For assays the cells were detached by a 10–15-min incubation with trypsin/EDTA (GIBCO).

2.3. Nuclear staining with Hoechst 33342 and propidium iodide

RINm5F cells suspended in 500 μL of medium were plated on eight-chamber tissue culture slides (Falcon) and allowed to attach overnight. The medium was removed and replaced with KRB (10 mM HEPES, 2 g/L BSA (bovine serun albumin), pH 7.4). H_2O_2 (50 and 100 μM) and cysteine (0.1, 1 and 5 mM) were added into the KRB and allowed to affect the cells for 60 min at 37°. KRB was then removed and the cells were incubated in RPMI 1640 (supplements as before) for the next 24 hr. DNA binding dyes HO and PI (Calbiochem) were added to the medium at 5 and 1 mg/L concentrations, respectively. The cells were exposed to the dyes for 30 min, the culture medium was removed, and the cells analyzed immediately under a fluorescence microscope with ultraviolet excitation at 340–380 nm (a dual DAPI/PI filter). The number of viable, apoptotic and necrotic cells were scored as described previously [15]. Briefly, HO freely passes cell membranes and stains nuclear DNA blue. PI is impermeable to intact membranes and only enters necrotic cells and late-phase apoptotic cells, inducing red fluorescent staining of DNA. Viable or necrotic cells were identified by intact nuclei with either blue (HO) or yellow (HO + PI) fluorescence, respectively. Apoptotic cells were detected by their fragmented nuclei which exhibited either a blue (HO) or yellow (HO + PI) fluorescence depending on the stage of the process.

2.4. DNA fragmentation analysis

To harvest DNA, a commercial kit was used according to the manufacturers instructions (Apoptotic DNA Ladder Kit, Boehringer Mannheim). Harvested DNA samples were treated with 500 μg/mL Rnase A. DNA isolated from apoptotic human testis was used as a positive control and a 100 bp DNA marker was used for sizing linear fragments. The exact DNA content was measured spectrophotometrically and 3 μg of DNA per sample was precipitated and resuspended in 10 μL of sterile water. The samples were 3′-end labelled with digoxigenin-dideoxy-UTP and fractionated through a 2% SeAKem Agarose gel (containing 1/20,000 ethidium bromide) in 1× Tris–acetate (TAE) buffer

(0.04 M Tris–acetate, 1 mM EDTA, pH 8.0). The gel was denatured by 0.4 M NaOH/1.5 M NaCl (30 min in RT) and neutralised by 1.5 M NaCl/1.0 M Tris, pH 8.0 (40 min in RT), and blotted onto a positively charged nylon hybridization transfer membrane (Amersham) overnight. The filter was autocrosslinked next day by UV light followed by exposure to a blocking solution (30 min in RT) and anti-digoxigenin antibody (30 min in RT). The filter was rinsed twice with washing buffer (0.1 M Maleic acid, 0.03% Tween 20) and finally labelled with CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3.3.1.1 3,7]decan}-4-yl) phenyl phosphate, Boehringer Mannheim) yielding a chemiluminescent signal. Film exposures of 3–5 min were sufficient. The information (O.D.) given by the X-ray films was digitized by a tabletop scanner (Microtec ScanMaker, Microtec International, Inc.), and the data were analyzed with NIH image (1.61) analysis software (NIH).

2.5. Measurement of intracellular insulin and DNA contents

For determination of the intracellular insulin content, the cells were plated on six-well plates and allowed to attach in RPMI 1640 (supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin and 100 μg/mL streptomycin, pH 7.0–7.4) overnight. The stimulations were performed in KRB as above following the 24 hr incubation in RPMI 1640 with supplements. Thereafter the cells were harvested, rinsed once with PBS and resuspended in 300 μL of distilled water followed by homogenization by ultrasonic disruption of cell membranes (10 s per sample). Cellular insulin content was measured by a commercial radioactive insulin assay (RIA) in dilutions (1:20) of acid ethanol extracts [13]. DNA content was fluorometrically measured from the sonicates [4,14].

2.6. MTT (C,N-diphenyl-N′-4,5-dimethyl thiazol-2-yl tetrazolium bromide) colorimetric assay

INS-1 cells were cultured on 96-well microplates for 3–4 days. The MTT colorimetric assay was performed as previously described [16]. Briefly, the cells were preincubated for 60 min at 37° in KRB in the presence of hydrogen peroxide and/or cysteine. The buffer was then removed and the cells were incubated for 120 min at 37° in KRB supplemented with 0.5 mg/mL MTT and 16.7 mM glucose after which the formazan crystals were solubilized with 0.04 N HCl in isopropanol. The O.D. was recorded at 550 nm.

2.7. Measurement of glutathione

RINm5F cells were cultured on six-well cell culture plates. Confluent cells were washed with PBS and exposed to the stimulants in KRB for 60 min at 37°. After incubation the cells were once rinsed with PBS followed by exposure to 15 mM monobromobimane in 50 mM *N*-ethylmorpholine (100 μL) and PBS (100 μL) for 5 min in dark at 37°. The reaction was terminated by addition of 100% trichloroacetic acid. The cells were collected and the protein was pelleted at 14,000 *g* for 5 min. One microliter of supernatant was injected onto a Waters Novapak C-18 HPLC column (4 μm, 3.9 mm × 150 mm) running an isocratic mobile phase consisting of 7% acetonitrile, 0.25% acetic acid and 0.25% perchloric acid, pH 3.7. The fluorescent product was detected using Shimadzu RF-10AxL spectrofluorometer with excitation and emission wavelengths at 394 and 480 nm. The amounts of glutathione and cysteine were calculated with standards derivatized and analyzed as above.

2.8. Statistical analysis

Differences between multiple groups were counted with StatView 5.0 software for the Macintosh, using one-way ANOVA followed by Fischer's protected least significant difference test taking 95% level as the limit of significance.

3. Results

3.1. Cell viability and apoptosis

Based on nuclear double staining with PI and HO, $89 \pm 5\%$ of the control RINm5F cells were scored viable. Exposure to 50 or 100 μM H_2O_2 reduced the amount of viable cells to $60 \pm 10\%$ ($P < 0.005$) and $28 \pm 8\%$ ($P < 0.0001$), respectively. Cysteine was used in three different concentrations (0.1, 1 and 5 mM) to study the possible protective effect against damage caused by H_2O_2. One micromolar cysteine was protective and significantly increased the amount of intact cells after exposure to either of the H_2O_2 concentrations (to $80 \pm 5\%$, $P < 0.05$ in 50 μM H_2O_2 and to $65 \pm 9\%$, $P < 0.005$ in 100 μM H_2O_2 treated cells). However, 0.1 mM cysteine was ineffective and 5 mM cysteine was found to be highly toxic, resulting in nearly 100% PI positivity (Figs. 1 and 2).

Nuclear fragmentation (apoptosis) of RINm5F cells was studied in more detail by gel electrophoresis. Control DNA showed an intact lane with no detectable fragmentation. A total of 50 μM H_2O_2 induced unambiguous apoptotic DNA fragmentation. Similarly, 100 μM H_2O_2 increased apoptotic activity but simultaneously apparently induced necrotic cell death, suggested by the increased background noise signal of degraded DNA. Again, 1 mM cysteine effectively inhibited DNA fragmentation induced by 50 μM H_2O_2, but could not decrease the more extensive damage induced by the 100 μM concentration. This was further evidenced by densitometry. Optical densities of the band characterizing the intact DNA and a band with molecular weight of

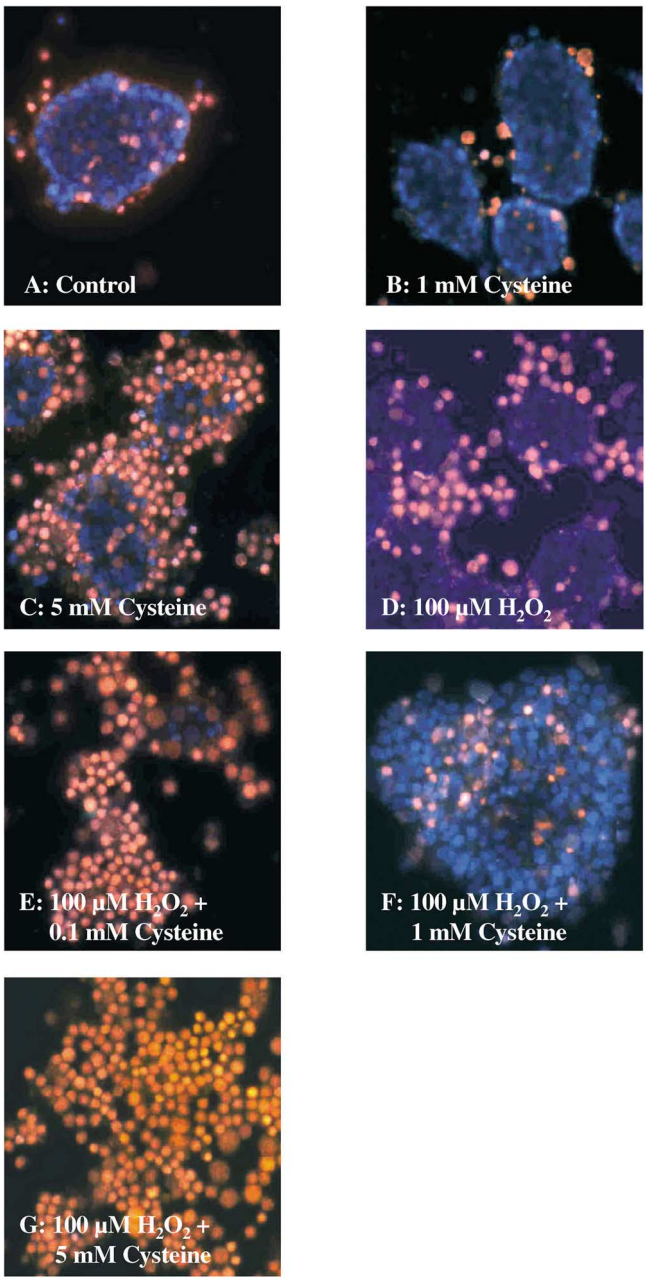

Fig. 1. Viability analysis in RINm5F cells based on nuclear staining with HO(5 μg/mL) and PI (1 μg/mL). HO freely passes plasma membrane and enters cells with intact membranes staining DNA blue. PI only penetrates cells with damaged membranes staining DNA red. Viable nuclei are detected in blue (HO), necrotic nuclei in red/yellow (HO + PI). (A–B) Untreated cells and cells treated with 1 mM cysteine are highly viable; (C) 5 mM cysteine dramatically increases the number of dead cells; (D–G) only 1 mM cysteine is able to prevent cell death induced by 100 μM H_2O_2. Objective magnification 20×.

400 bp characterizing fragmented DNA were measured and their ratios were calculated. After exposure to 50 μM H_2O_2 1 mM cysteine decreased DNA fragmentation shown by a 4-fold increase in this ratio in comparison to 50 μM H_2O_2. Five micrormolar cysteine provided only slight protection (21% increase) and 0.1 mM concentration did not protect at all (Fig. 3). In cells treated with 100 μM H_2O_2, 1 mM cysteine had a minimal protective effect (<20% reduction in DNA fragmentation).

3.2. Intracellular insulin and DNA contents

Exposure to 50 or 100 μM H_2O_2 decreased both the amount of cellular insulin and DNA. The significant protective effect of 1 mM cysteine against H_2O_2 toxicity was evident also with these assays (Fig. 4A and B). The insulin content increased from 82 ± 4 to $98 \pm 9\%$ of control (insignificant) in 50 μM H_2O_2 treated cells and from 42 ± 5 to $70 \pm 14\%$ of control ($P < 0.01$) in 100 μM H_2O_2 treated cells. Similarly, the DNA content increased from 71 ± 8 to $93 \pm 5\%$ ($P < 0.03$) of control after exposure to 50 μM H_2O_2 and from 44 ± 3 to $69 \pm 4\%$ ($P < 0.012$) of control after exposure to 100 μM H_2O_2. The ratio of insulin per DNA remained intact after treatment with either of the H_2O_2 concentrations, indicating that the insulinoma cells have a constitutive rate of insulin synthesis which is not significantly modified by the oxidative challenge as long as the cell remains viable. However, the highest concentration of cysteine (5 mM) decreased the insulin content per DNA significantly, suggesting that excessive cysteine concentrations interfere with insulin biosynthesis (Fig. 4C).

3.3. MTT (C,N-diphenyl-N′-4,5-dimethyl thiazol-2-yl tetrazolium bromide) assay

Cell viability and functional capacity were further measured by the MTT colorimetric assay, which is a convenient tool for rapidly assessing the metabolism of beta cells [16]. Exposure to 100 μM H_2O_2 decreased the reduction of MTT significantly ($50 \pm 14\%$ of control, $P < 0.01$). A dose-dependent protection against H_2O_2-induced cellular damage was obtained with cysteine. Already 0.5 mM cysteine provided significant protection ($P < 0.005$) and the effect was maximal with 1 mM cysteine ($P < 0.0005$). However 5 mM cysteine potentiated the toxicity of H_2O_2 (Fig. 5).

3.4. Glutathione content

Exposure to 100 μM H_2O_2 decreased the intracellular glutathione level as expected (to $46 \pm 15\%$ of control). Cysteine supplementation restored the level of glutathione to $84 \pm 8\%$ of control ($P < 0.05$) (Fig. 6).

4. Discussion

Both pancreatic islets and insulin-producing cell lines have been shown to express substantially lower levels of anti-oxidant enzyme genes in comparison with most other cell types [17,18]. In clinical studies, patients with IDDM and their first degree relatives at risk of developing the

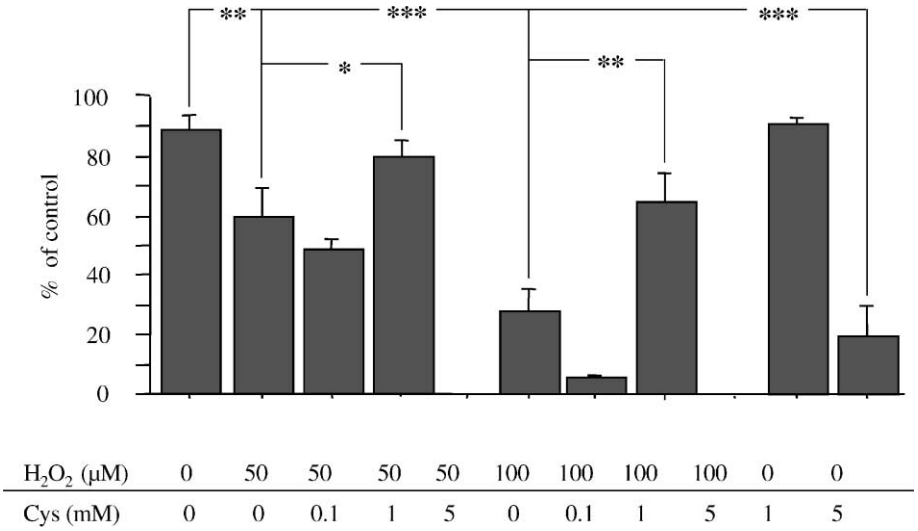

Fig. 2. RINm5F cell viability quantitated by nuclear double staining with HO and PI. Results are presented as value ± SEM from 3 to 4 individual experiments ($^{*}P < 0.05$; $^{**}P < 0.005$, $^{***}P < 0.0001$).

disease, have been reported to have significantly reduced total anti-oxidant activity [19]. This decreased capacity of anti-oxidative defence combined with increased oxidative stress during the pre-diabetic period is likely to increase the susceptibility of beta cells to oxidative damage [20].

We show that by extracellular delivery of L-cysteine, many of the adverse effects of an experimental oxidative attack by H_2O_2 are dose-dependently inhibited. One millimolar cysteine significantly reduced the number of dying cells confirmed by the preserved intracellular insulin and DNA contents and restored the metabolic activity (ability to reduce MTT). Importantly, lower (0.1 mM) and higher (5 mM) concentrations of cysteine did not show any protection. Instead, the 5 mM concentration had an adverse effect on all tested parameters. We have observed this with other cell lines as well (data not shown). Plasma cysteine concentrations usually range from 10 to 50 μM, but in clinical trials using NAC, NAC concentrations from 100 to 1000 μM were safely achieved [22]. NAC has been used instead of cysteine because of its better stability and it is deacetylated to cysteine in many cell types. We chose to use cysteine instead of NAC in this *in vitro* study, because

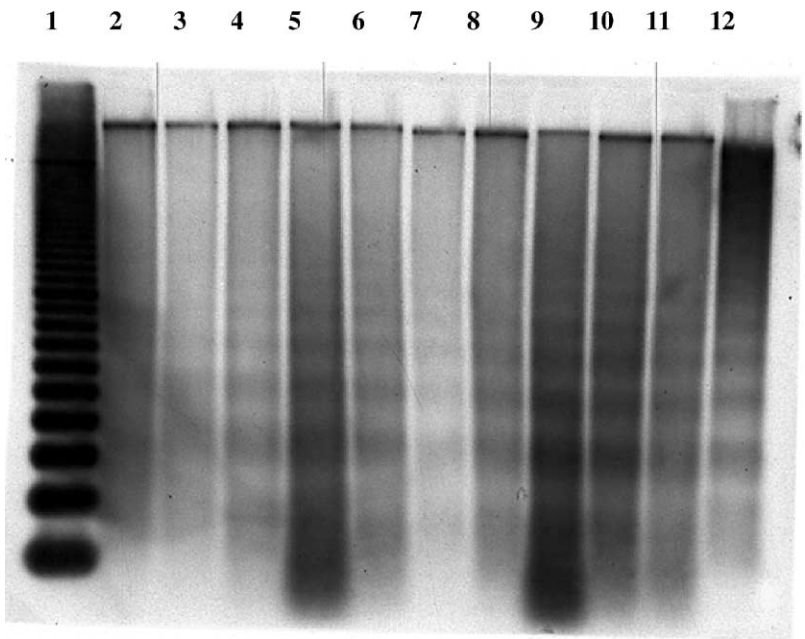

Fig. 3. Autoradiographic image of the DNA fragmentation pattern in RINm5F cells. Description of lanes: (1) 100 bp marker; (2) control; (3) 5 mM cysteine; (4) 50 μM H_2O_2; (5) 100 μM H_2O_2; (6) 50 μM H_2O_2 + 0.1 mM cysteine; (7) 50 μM H_2O_2 + 1 mM cysteine; (8) 50 μM H_2O_2 + 5 mM cysteine; (9) 100 μM H_2O_2 + 0.1 mM cysteine; (10) 100 μM H_2O_2 + 1 mM cysteine; (11) 100 μM H_2O_2 + 5 mM cysteine; (12) apoptotic testis DNA (positive control).

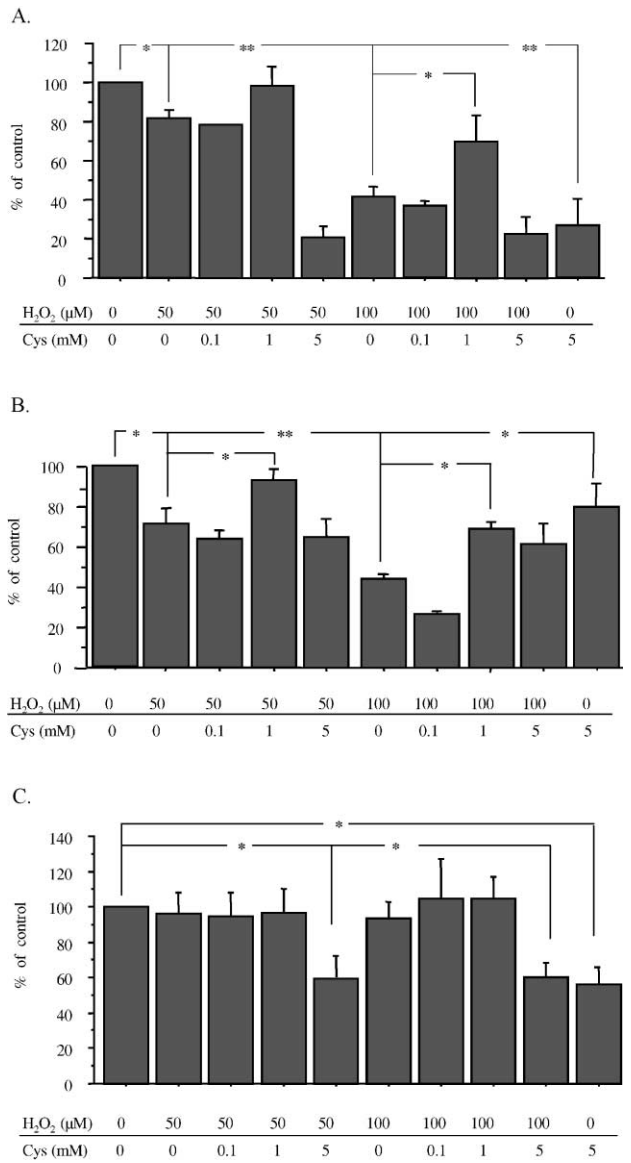

Fig. 4. Intracellular insulin content (A); DNA content (B) and their ratio (C). Results are presented as percent of experiment-specific control ± SEM from three to four individual experiments each done in duplicate ($^{*}P < 0.05$; $^{**}P < 0.0001$).

the deacetylation reaction may not be operative in cell culture models [23]. The cysteine concentrations tested in our setting were selected according to earlier studies on cysteine *in vitro* [21].

Hydrogen peroxide was chosen instead of known beta cell specific toxins (e.g. alloxan and streptozotocin) because it represents a physiological agent present during most oxidative processes independent of the trigger. The H_2O_2 concentration used is probably supranormal, but our INS-1 and RINm5F insulinoma cells are adapted to higher oxidative stress, because they have been cultured in a far higher oxygen pressure than normal beta cells. Thus, these results can not be directly applied into the situation *in vivo*.

Cysteine and other thiols are able to directly react with H_2O_2, and thus reduce the oxidative stress. The rate constants for these reactions depend on temperature, pH and p*K* of the thiol group [24]. We tested glutathione and NAC as well, but could not get positive results with concentrations varying from 0.1 to 10 mM (data not shown) although these thiols would have been able to react with H_2O_2. This suggests that cysteine also acts in some additional ways, one of them being the observed increase in intra-cellular glutathione concentration.

GSH plays a key role in the anti-oxidative defence, and its consumption increases upon oxidative stress. We have observed that H_2O_2 treatment decreases the GSH-concentration in RINm5F cells. The loss of GSH occurs through two separate mechanisms. Firstly, it is oxidized and forms disulfides with itself or with other thiols. This loss is reversible with additional reducing power. Secondly, it is conjugated with toxic compounds formed during oxidative stress and lost through excretion. Cysteine provides the cells with a rate-limiting ingredient of GSH [25], but it also helps to maintain other anti-oxidative molecules in their reduced form [6,7]. The previously described direct mechanism of protection becomes feasible in our study considering the short exposure time to H_2O_2 (1 hr) in comparison to the turnover of glutathione which is known to last several hours [26]. Nevertheless, our data indicate that the resulting improvement in beta cell viability and function is at least partly due to mechanisms which lead to an increase in cellular GSH content.

Depending on the dose, oxidative stress may induce cell death *via* different and partly overlapping mechanisms [27,28]. Exposure of NIT-1 insulinoma cells (mouse pancreatic beta cell line) to a low level of oxidative stress, comparable to the one around activated macrophages under oxidative burst (30 μM H_2O_2 for 15 min at 37°), lead to lysosomal rupture and nearly total cell death through post-apoptotic secondary necrosis [29]. Several studies have demonstrated the role of anti-oxidants in inhibiting programmed cell death. NAC was shown to inhibit NF-κB activation and tumor necrosis factor-α mRNA expression in human monocytic leukaemia cells [30,31]. In addition, NAC has been shown to decrease lipopolysaccharide-induced apoptosis through GSH-mediated mechanisms in splenocytes from p53 haploinsufficient mice [32]. In our study, both 50 and 100 μM H_2O_2 concentrations caused DNA fragmentation, but the damage in 100 μM conditions consisted of a mixture of necrosis and apoptosis. Apoptosis induced by 50 μM H_2O_2 could be abolished by 1 mM cysteine.

In conclusion, we provide experimental evidence suggesting that the anti-oxidative defence of beta cells can be improved by providing an appropriate amount of extracellular cysteine. Cysteine provides reducing (anti-oxidative) power and supports GSH synthesis. The appropriate amount depends on experimental conditions. However, this study shows that the correct dose of cysteine needs to be carefully evaluated in order to obtain the therapeutic effect.

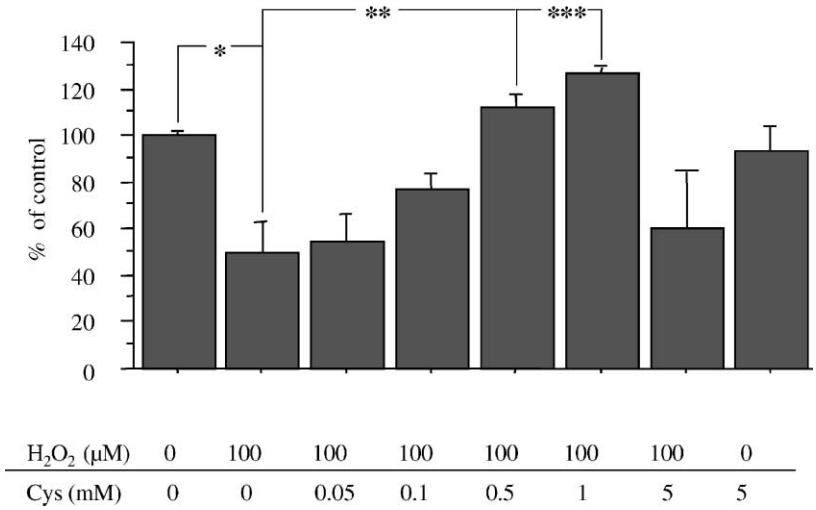

Fig. 5. Cellular metabolic status of INS-1 cells studied by the MTT assay. Results are presented as percent of experiment-specific control ± SEM from three individual experiments each done with six replicates ($^{*}P < 0.01$; $^{**}P < 0.005$; $^{***}P < 0.0005$).

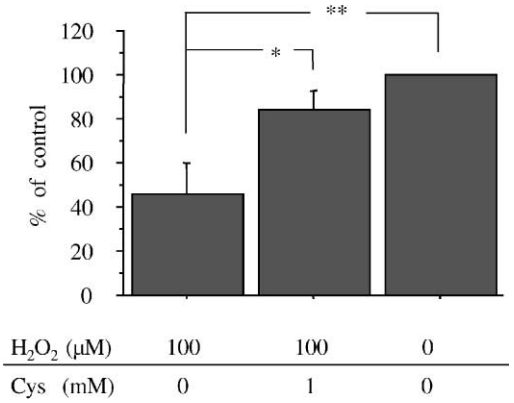

Fig. 6. Measurement of intracellular glutathione concentrations in RINm5F cells. Results are presented as percent of experiment-specific control (±SEM) from four individual experiments ($^{*}P < 0.05$, $^{**}P < 0.005$).

Acknowledgments

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