INTRODUCTION

Gliclazide, a second-generation sulfonylurea, is an oral hypoglycemic drug used in the treatment of type 2 diabetes. It has been reported that gliclazide has free radical scavenging properties at pharmacological concentrations. This property is not shared by other sulfonylureas (glibenclamide, glipizide, tolbutamide), which do not have the azabicyclo-octyl ring (1). The antioxidant effect of gliclazide is thought to be of clinical importance. Monami et al. have demonstrated in a retrospective observational cohort study that mortality for cardiovascular diseases and malignancies of type 2 diabetic patients treated with gliclazide was lower than in glibenclamide group (2). Monami et al. have also found in a case-control study that exposure of type 2 diabetes mellitus patients to metformin and gliclazide for more than 36 months was associated with a significant reduction in the risk of cancer (3).

It is well documented that type 2 diabetes mellitus patients have increased risk of cancer development due to elevated oxidative stress. It was shown that type 2 diabetes mellitus patients had increased level of oxidative DNA damage and decreased efficacy of DNA repair (4, 5). DNA damage and repair play a crucial role in neoplastic transformation (6, 7). The oxidative stress is a source of reactive oxygen species which create various type of DNA damage, including all base modifications, which are primarily repaired by BER pathway (8). UV radiation introduces predominantly to DNA two kinds of products: cyclobutane pyrimidine dimmers and pyrimidine-pyrimidine (6-4) photoproducts. Both lesions are repaired by a common pathway, nucleotide excision repair (NER) (9). Single strand breaks (SSB) are repaired by sequential action of 3'-phosphodiesterase, DNA polymerase β and a DNA ligase (10). DNA double-strand breaks (DSBs) are the most pronounced DNA damage induced by a variety of different mechanisms including exposure to ionizing radiation and a number of chemicals. DSBs, if not repaired or misrepaired, may lead to the mutations and cell death. Two pathways remove DSBs: homologous recombination (HR) repair and non-homologous DNA end joining (NHEJ) (11, 12).

The anticancer action of gliclazide is still unclear. It has been postulated that it may be connected with its antioxidant properties. Recently, we have found that gliclazide may protect DNA against damage introduced by the oxidative stress, but its action on the DNA repair mechanisms is unclear. Therefore, the aim of this study was to assess whether gliclazide has any effect on the DNA repair pathways, e.g. nucleotide excision repair (NER) and non-homologous end joining (NHEJ). NER activity was assessed in the extract of human lymphocytes and pancreatic cancer cells (PANC-1) treated or not with gliclazide by use of an UV-irradiated plasmid as a substrate and by quantitative PCR performed to evaluate the efficacy of the removal of UV-induced lesions from the p53 gene by intact cells. The efficacy of NHEJ pathway was examined by a simple and rapid in vitro assay based on fluorescent detection of repair products. We did not observe significant differences between the efficiency of NER and NHEJ for extracts of lymphocytes alone and lymphocytes treated with gliclazide. Contrary, gliclazide increased the efficacy of NER (46.0% vs. 84.0%, p<0.01) and NHEJ (58.0% vs. 66.0%, p<0.05) in PANC-1 cells. In conclusion, the present study showed that gliclazide did not affect NER and NHEJ in human normal cells, but it may stimulate DNA repair in cancer cells.

Key words: gliclazide, nucleotide excision repair, non-homologous end joining, diabetes, lymphocytes, pancreatic cancer cells, hyperglycemia
assume these actions of gliclazide may arise as a result of its free radical scavenger properties rather than interaction with DNA repair. However, it cannot be excluded that gliclazide may interact with DNA repair mechanism(s). Therefore, the aim of the present study was to check whether gliclazide exerts any effect on DNA repair pathways, e.g. nucleotide excision repair (NER) and non-homologues end joining (NHEJ) in human normal lymphocytes and human pancreatic carcinoma of ductal cells (PANC-1).

MATERIAL AND METHODS

Cells

Blood was obtained from young (18-31 years), male, healthy, non-smoking donors. Peripheral blood lymphocytes were isolated by centrifugation in a density gradient of Gradisol L (15 min, 280 g). The viability of the cells was measured by trypan blue exclusion and was found to be about 99%. Lymphocytes accounted for about 92% of leukocytes in the obtained cell suspension as judged by the characteristic shape of nucleus. An epithelioid cell line, started from a human pancreatic carcinoma of ductal cell origin, PANC-1, were purchased from the American Tissue Culture Collection (ATCC) and were cultured in T-75 flasks in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose (ATCC) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma Co St. Louis, MO, USA). Cells were maintained at 37°C in a 5% CO2 atmosphere. The cells were detached from the flask by trypsinization.

Chemicals and reagents

Gliclazide was kindly provided by Servier, France. Phosphate buffered saline (PBS), penicillin and streptomycin were purchased from Sigma. QIAamp DNA Blood Mini Kit for isolation of high-molecular-weight DNA was obtained from QIAGEN (Chatsworth, CA, USA). [γ-32P]-dATP deoxyadenosine triphosphate was purchased from Amersham Biosciences (Munich, Germany). Plasmids were prepared by alkaline lysis from QIAprep Spin Miniprep Kit (Qiagen, Germany). DNA polymerase LA Taq was purchased from TaKaRa Bio INC (Otsu, Japan). All other chemicals were of the highest commercial grade available.

Nucleotide excision repair

1. Cell extract preparation

Cell extracts were prepared according to the previously described protocol (15). Briefly, after incubation with gliclazide at 20 µM, were washed with ice-cold PBS and harvested by centrifugation, and the cell pellet was suspended in hypotonic buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 10 mM MgCl2, 1 mM DTT) containing protease inhibitors. The cells were disrupted with a Dounce homogenizer on ice. Nuclei were harvested by centrifugation, and the nuclear proteins were extracted in hypotonic buffer containing 150 mM NaCl. Cytosolic and nuclear extract proteins were precipitated by addition of ammonium sulfate. The precipitates were resuspended in dialysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA), 100 mM mono-K glutamic acid, and 10% glycerol and dialyzed for 2 hours at 4°C. The extracts were frozen in liquid nitrogen and stored at -70°C.

2. Preparation of plasmids and treatment with UV

The 2961-bp PBS (pBluescriptII KS-; Stratagene, La Jolla, CA, USA) and the 4361-bp pBR322 (Fermentas, Burlington, Ontario, Canada) plasmids were prepared by alkaline lysis from Escherichia coli XL1 Blue strain. Both plasmids were obtained from 1% agarose gel after electrophoresis and purified by gel extraction kit (Qiagen). pBluescriptII KS-plasmid was treated with UV light (300 J/m2) at 254 nm (PBS-UV) producing about 10 UV lesions per plasmid (16).

3. In vitro nucleotide excision repair assay

The repair synthesis assay was performed as described elsewhere with some modifications (17). The reaction mixture (50 µL), containing 250 ng damaged pBluescriptII KS- and untreated pBR322 closed circular plasmids, 0.37 MBq (4.5 µCi) [γ-32P]-dATP deoxyadenosine triphosphate (4500 Ci/mmol), 200 µg cell extract proteins and 70 mM potassium glutamate, was added to the reaction buffer (45 mM HEPES-KOH [pH 7.8]; 7.4 mM MgCl2; 0.9 mM DTT; 0.4 mM EDTA; 2 mM [ATP]; 20 µM each [dTTP], [dGTP], and [dCTP]; 4 µM [dTTP]; 40 mM phosphocreatine; 2.5 µg phosphocreatine kinase, 3.4% glycerol; and 18 µg bovine serum albumin) for 3 hours at 30°C. Before electrophoresis on 1% agarose gel containing 0.5 µg/mL ethidium bromide, plasmid DNA was purified from reaction mixtures and linearized with EcoRI. Gels were dried onto Whatman 3MM paper under vacuum at 80°C for about 1.5 h. Dried gel was put into a cassette with intensifying screens and exposed to preflashed X-ray film and placed at -80°C for 6 hours. Photographs of the ethidium bromide-stained gels were scanned. Bands from the exposed gel was excised, together with the attached filter paper, under UV light, and the value of the cpm was calculated in a scintillation counter (TRI-CARB-2900TR Liquid Scintillation Analyzer; PerkinElmer, Dovners Grove, IL, USA). The relative density of the bands of both plasmids on the positive of the photograph of the ethidium bromide-stained gel was quantified by densitometry (in Genius Bio Imaging; Syngene, Cambridge, UK). Specific incorporation of [γ-32P]-dAMP (deoxyadenosine monophosphate) was expressed as radiolabel incorporation in the damaged versus undamaged plasmid. The value of [γ-32P]-dAMP incorporation was normalized to the same amount of total DNA.

Q-PCR

1. Cell treatment and preparation of genomic DNA

The cells before UV irradiation were incubated for 24 hours with gliclazide at 20 µM in 5% CO2 atmosphere at 37°C. The cells were then washed twice with phosphate buffered saline (PBS) and irradiated with UV light at 254 nm emitted by EMITA model VP-60 lamp (FAMED, Lodz, Poland). After UV irradiation PBS was replaced with the complete medium and the cells were incubated in a 5% CO2 atmosphere at 37°C for indicated time and then washed with PBS prior to DNA isolation. High-molecular-weight DNA, suitable for QPCR, was isolated using the QIAamp DNA isolation kit.

2. Q-PCR analysis

Q-PCR analysis was performed as described elsewhere with some modifications (18). Amplification reaction was carried out in a volume of 50 µl (MJ Research INC, model PTC-100, Waltham, MA, USA). Each reaction consisted of 150 ng of total genomic DNA, primers to coamplify a 500-bp fragment of the p53 gene (0.1 µM each) for the control of amplification, the deoxyribonucleotides (0.35 mM each) and two units of Taq DNA polymerase (TaKaRa LA Taq). The primer sequences were for the p53 gene, from exon 4 to exon 11, sense-5' TGAGGAACCTG and 3'-CTGACGCAGTCA.

\[
\text{p53 forward: 5'-TGAGGAACCTG-3'}
\]

\[
\text{p53 reverse: 3'-CTGACGCAGTCA-5'}
\]
GTCCTCTGAC3' and antisense 5'TGACGCACACCTATTGC
AAG3'; for the IFNβ1 gene, sense-5'ATGAGCTACAACTTGCT
TTGGA3' and antisense 5'TCAGTTTCGGAGGTAACCTGT3'.
The cycling profile for the p53 gene consisted of an initial
denaturation at 95°C for 5 min, 14 cycles at 95°C for 1 min and at
67°C for 7 min, 16 cycles at 95°C for 1 min and at 67°C for 8 min,
72°C for 5 min. The cycling profile for the fragment of the IFNβ1
gene included an initial denaturation at 95°C for 5 min, 30 cycles
at 95°C for 1 min and at 68°C for 2 min and 72°C for 10 min.
QPCR was performed in a MJ Research PTC-200 Peltier thermal
cycler. An aliquot of an equal amount of PCR product for the p53
and the IFNβ1 genes was mixed and resolved on a 1.5% gel and
electrophoresed in 0.5 × Tris-borate-EDTA buffer at 5 V/cm for 1
hour. The gels were stained with ethidium bromide for a
quantitative analysis.

3. Data analysis

Densitometric quantification of DNA repair was performed
as described elsewhere (18). To quantify the repair rate of the
damage in the QPCR assay, amounts of PCR products were
measured with an In Genius Bio Imaging analysis system
(SynGene, Cambridge, UK). The amount of amplified product of
the target p53 gene was normalized by the coamplified control
template (IFNβ1 sequence). This normalized value for each
treated sample was then used to calculate the lesion frequency
(S) per genomic strand at each time point, according to the
following equation:

\[ S = -\ln \frac{A_D}{A_C} \]

where \( A_D \) is the amount of normalized value of amplified
product of damaged p53 DNA template and \( A_C \) is the amount of
normalized value of coamplified product of the control IFNβ1
DNA template. The repair efficiency (RE) of each time point was
measured as 1 minus the ratio of the lesion frequencies between
time t (S(t)) and zero (S(0)) (initial damage) RE (%) = (1 - S(t)/S(0)) ×
100%.

Non-homologous end joining

1. Preparation of cellular extracts

The cells were incubated for 24 hours with gliclazide at 20
µM in 5% CO₂ atmosphere at 37°C. The cells were then
washed twice with phosphate buffered saline (PBS) in order to
obtain whole cell extract the cells were pelleted twice in ice-
cold PBS (900x g) and resuspended in the hypotonic lysis
buffer [10 mM Heps (4-(2-hydroxylethyl)piperazine-1-
ethanesulfonic acid), pH 7.9, 60 mM KCl, 1 mM EDTA, pH
8.0, 1 mM DTT and protease inhibitors cocktail according to
the manufacturer's instructions] (min. 4-6 x 10⁷ cells/0.5 mL.
extraction buffer). Then the cells were lysed by three cycles of
freeze-thawing in a bath of dry ice and ethanol and in a 37°C
water bath. After the final thawing the extract was clarified by
centrifugation at 15,000 x g for 30 min, removed as supernatant
and stored at -70°C until needed. Protein determinations were

Fig. 1. Analysis of NER activity by in vitro
repair synthesis assay in extracts from human
lymphocytes and PANC-1 cells pretreated or
not with gliclazide. NER synthesis activity in
UV-damaged plasmid was examined for 3
hours at 30°C with the extracts from human
lymphocytes and PANC-1 cells pretreated
(grey bars) or not (black bars) with 20 M
gliclazide for 24 hours at 37°C. Cell extracts
were incubated with damaged (pBS-UV) or
undamaged (pBR322) plasmids. Ethidium
bromide-stained gel and autoradiography of
the dried gel (upper panels), quantification of
the DNA repair activity expressed as the ratio
of radiolabel incorporation in the damaged
dna vs. undamaged plasmid (lower panels). The
figure shows mean results from three
independent experiments. Error bars denote
S.E.M. * p <0.05.
made according to the method of Bradford (19), using bovine albumin as the standard.

2. DNA preparation

DNA substrate with either 5' complementary or blunt ends was produced by complete digestion of the pUC19 plasmid with EcoRI restriction endonuclease. Protein was removed by phenol/chloroform extraction and the plasmid DNA was recovered in TE buffer, pH 8.0.

3. End joining assay

The end joining assay was performed as described previously by Pastwa et al. (20). The repair reactions were conducted in total volume of 50 l. The reaction medium contained 50 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, pH 8.0, 5 mM MgCl2, 1 mM ATP, 1 mM DTT, 5% polyethylene glycol (PEG) 8000, protease inhibitors cocktail according to the manufacturer's instructions, 100 ng substrate DNA and lymphocytes or PANC-1 whole cell extract. The repair was stopped by adding of 0.4% SDS and incubation at 65°C with phenol/chloroform (1:1 v/v) and ethanol precipitation using 0.5 µg RNA as a carrier. The repair products were identified by gel shift following 1% agarose electrophoresis and staining for 1 hour with Vista Green according to the manufacturer's instructions. The images were digitized with a Gel Doc 2000 system and quantified densitometrically using Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The mean value and the standard error were calculated for at least three independent assays. The significance of differences between experimental variables was determined using the Student's t-test. If no differences between variations were found, the differences between means were evaluated by applying the ANOVA test.

RESULTS

We compared impact of gliclazide on the efficiency of nucleotide excision repair and non-homologous end joining in cell extracts prepared from human lymphocytes and PANC-1 cells. We observed that gliclazide did not exert any effect on nucleotide excision repair in human lymphocytes. However,
cancer cells-PANC-1 incubated with gliclazide more effectively removed UV induced DNA damage. Using NER functional assay we observed significant increase of the efficacy of DNA synthesis in nucleotide excision repair in PANC-1 cells treated with gliclazide (46.0% vs. 84.0%, \( p < 0.01 \)). We also revealed that the efficacy of NER was significantly higher in PANC-1 cells compared to lymphocytes (Fig. 1). Fig. 2 shows UV damage repair efficiency in the \( p53 \) gene at 2, 4, 8 and 24 hours after UV-irradiation at 0.5 J/m\(^2\). The efficiency in PANC-1 cells treated with gliclazide increased up to 24.7% after 2 hours of repair incubation (\( p < 0.01 \)), 18.7% after 4 hours of repair incubation (\( p < 0.05 \)), 14.7% after 8 hours of repair incubation (\( p < 0.05 \)).

Fig. 3 presents effect of gliclazide on the effectiveness of NHEJ. There was no significant difference between the efficiency of DNA end joining for extracts of lymphocytes alone and lymphocytes treated with gliclazide. However, treatment of PANC-1 cells with gliclazide increased the efficiency of DNA end joining (8%, \( p < 0.05 \)). We also observed that the efficacy of NHEJ was significantly lower in PANC-1 cells compared to lymphocytes.

DISCUSSION

The cellular and molecular mechanisms of diabetic complications are still not fully recognized. However, it has been suggested that chronic hyperglycemia, insulin resistance and secondary hyperinsulinism, and oxidative stress are key players. These pathological changes may affect numbers of intracellular signaling pathways leading to the morphological damage and dysfunction of various tissues and organs including cardiovascular and nervous system, and gastrointestinal tract (21-23).

Chronic hyperglycemia, a typical feature of type 2 diabetes mellitus, generate an oxidative stress in which genome stability is threatened. In such condition the risk of DNA damage, mutations, and genomic instability increase. It is well recognized that unrepaired or improperly repaired DNA damage may have serious consequences, including neoplastic transformation. To protect against genome instability and its dangerous consequences organisms have developed several DNA repair pathways (24). Nucleotide excision repair (NER) is the most versatile mechanism of DNA repair, recognizing and dealing with a variety of helix-distorting lesions, such as bulky DNA adducts.
UV-induced photoproducts cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4 PPs) (9, 25). NER operates through two sub-pathways: global genome repair (GGR) and transcription-coupled repair (TCR). GGR repairs the DNA damage throughout the entire genome and is initiated by the HR23B/XPC complex, while the CSB protein-governed TCR process removes DNA lesions from the actively transcribed strand (26). The efficiency of these pathways is important in avoiding cancer and genomic instability (27).

One of the major pathways for the repair of ionizing radiation-induced DSBs (double-strand breaks) in mammalian cells is NHEJ (non-homologous end-joining). DNA DSBs are considered the most cytotoxic type of DNA lesion. The main proteins required for NHEJ in mammalian cells are the Ku heterodimer (Ku70/80 heterodimer), DNA-PKcs (the catalytic subunit of DNA-PK (DNA-dependent protein kinase)), Artemis, XRCC4 (X-ray-complementing Chinese hamster gene 4), DNA ligase IV and XLF (XRCC4-like factor; also called Cernunnos). Additional proteins, including DNA polymerases mu and lambda, PNK (polynucleotide kinase) and WRN (Werner's Syndrome helicase), may also play a role (28, 29).

It was found that type 2 diabetic patients had diminished the effectiveness of DNA repair (30). Both increased level of oxidative stress (oxidative DNA damage) and decreased the efficacy of DNA repair in T2DM may play a crucial role in neoplastic transformation. It is well documented that gliclazide, an oral hypoglycaemic drug used in the treatment of type 2 diabetes mellitus, is a free radical scavenger. Our previous studies confirmed antioxidant action of gliclazide and showed that gliclazide might hasten the repair of DNA damage in normal human lymphocytes and cancer cells. The results of this study indicate that the increase of DNA repair efficacy was transient. It may suggest that this effect is probably related to the drug free radical scavenger activity (13, 14). However, it cannot be excluded that other mechanisms of gliclazide action may affect DNA repair pathways. To address this problem we compared the effect of gliclazide on the efficiency of NER and NHEJ pathways in normal human lymphocytes and PANC-1 cells.

Our experiments revealed that gliclazide had no significant impact on the efficiency of DNA repair pathways, e.g., NER and NHEJ in normal human lymphocytes. These results suggest that lower risk of cancer development in type 2 diabetic patients treated with gliclazide observed by Monami et al. may be associated with its antioxidant action rather than its interaction with DNA repair. On the contrary, gliclazide altered the efficacy of analyzed DNA repair pathways in cancer cells (PANC-1). In the current report, we observed significantly higher efficacy of NER in PANC-1 cells. We speculate that it may be connected with hypertriploidy of PANC-1 cells (31). We also found that gliclazide increased the efficacy of DNA synthesis in nucleotide excision repair in these cells assessed by Q-PCR. These observations stay in agreement with our previous results that gliclazide improved repair of DNA damage induced by UV radiation in β-TC-6 cells (13). The ability of gliclazide to hasten the nucleotide excision repair has not heretofore been described. The kinetics of NER in PANC-1 cells treated with gliclazide displayed higher repair rate during the first 8 hours in comparison to PANC-1 cells without gliclazide. The kinetics was studied in the p53 gene, which is a good candidate for the examination of the transcription-coupled DNA repair in human cells (32). Therefore, we considered at least three possibilities: (1) the faster rate of DNA repair in PANC-1 may be linked with additional involvement (apart from TCR) of GGR proteins involved in the repair of the UV-induced DNA damage in the p53 gene; (2) different genetics construction (hypertriploidy of PANC-1 cells) may increase DNA repair efficacy in pancreatic carcinoma of ductal cells; and (3) which combines (1) and (2). Other scenarios are also possible.

We found that the efficacy of NHEJ was higher in lymphocytes compared to PANC-1 cells, which show a very high frequency of p53 mutation (33). Wild-type p53 tumor suppressor protein is involved in DSBs repair. Normal human lymphocytes possessing p53 protein were able to rejoin 5% compatible overhang ended DNA via NHEJ more efficient than p53-mutated cell line PANC-1. This observation is consistent with several studies showing that p53 directly enhances rejoicing of DSB with cohesive ends (34, 35). The study showed that gliclazide increased the effectiveness of ligation in PANC-1 cells. The ability of the drug to improve the NHEJ has not heretofore been described. Based upon obtained results we speculate that gliclazide might stimulate the expression of NHEJ genes (Ku70, Ku80 and DNA-PKcs). Recently, it has been reported that melatonin, that possesses an antioxidant properties similarly to gliclazide, inhibited the expression of the clock gene BLAM1 that suppressed the expression of DNA repair genes (p53, Ku70) and apoptosis associated genes (36). In addition, Leja-Szpak et al. found that melatonin could protect PANC-1 cells from apoptosis by the stimulation of production and phosphorylation of heat shock protein 27 (37). Both these observations may indirectly support our suggestion that gliclazide may have a stimulatory effect on the expression of NHEJ genes.

In conclusion, the present study showed for the first time that gliclazide had no impact on NER and NHEJ in human normal lymphocytes. On the contrary obtained results suggest that gliclazide may stimulate DNA repair in PANC-1 cells. Therefore, the influence of gliclazide on the specific repair system may be determined by specific cell type. The further investigations should be done to establish a definite role of gliclazide in the efficacy of NHEJ and NER.

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