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Dihydrolipoic acid protects pancreatic islet cells from inflammatory attack

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Abstract

In vitro models of pancreatic islet cell inflammation are the lysis of isolated islet cells by activated macrophages or by oxygen radicals released by the endothelial enzyme xanthine oxidase. Dihydrolipoic acid protected islet cells in both systems by different modes of action. Macrophage cytotoxicity towards islet cells, which is nitric-oxide-mediated, was suppressed by 2 h of preincubation of macrophages with lipoic acid. Similarly, 2 h of preincubation sufficed to protect islet cells against enzymatically produced oxygen radicals. Dihydrolipoic acid was found by chemiluminescence assay to scavenge directly such radicals. In macrophages dihydrolipoic acid suppressed the production of nitrite as a measure of nitric oxide release. These results suggest that dihydrolipoic acid is an anti-inflammatory agent which at the same time interferes with nitric oxide release from inflammatory macrophages and protects target cells from oxygen radical attack.

Introduction

Previous studies *in vitro* and in animal models of human type I diabetes revealed that the loss of pancreatic islet cells is mainly mediated by the cytotoxic effector mechanisms of autoreactive immune cells. Islet cells are known to be highly susceptible to inflammatory attacks because of their reduced capacity to scavenge reactive oxygen radicals [1]. Macrophages were found to play a prominent role in the process of islet cell destruction. During *in vitro* coculture, activated macrophages rapidly lyse islet cells whereas cells isolated from other organs such as the thyroid or the liver remain unaffected [2]. The islet toxic product of macrophages was recently identified by us as nitric oxide [3]. Similarly, islet cells are rapidly lysed by oxygen radicals generated by the endothelial-cell-associated xanthine oxidase [4]. This enzyme is activated during certain inflammatory conditions such as states of ischemia/reperfusion and thought to contribute to the tissue damage observed [5, 6]. In the present study we analyzed the potential of dihydrolipoic acid, a novel antioxidant [7], to protect islet cells against inflammatory attacks. Lipoic acid or its reduced form dihydrolipoic acid is a dithiol which interacts physiologically with vitamin E, vitamin C and glutathione in the protection against peroxidation intracellularly, probably by the replenishment of reduced glutathione [8]. Recently, it was shown that the supplementation of diet with lipoic acid increased the resistance of homogenates of rat liver and heart to lipid peroxidation, whereas *in vitro* dihydrolipoic acid was more efficient in the inhibition of lipid peroxidation [9].

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To investigate the islet cell protective effect of dihydrolipoic acid, we chose *in vitro* models of inflammatory islet cell destruction in which isolated islet cells were exposed to the cytotoxic mediators released from activated macrophages or from the enzyme xanthine oxidase.

Materials and methods

Preparation of islet cells

Pancreatic islet cells were prepared from Wistar rats provided from our own breeding colony. Isolation of whole islets was performed by collagenase digestion of the pancreatic tissue (40 min, 37 °C, 0.56 U/ml collagenase, Serva GmbH, Heidelberg, FRG), followed by enrichment of the islets on a Ficoll density gradient (Ficoll 400, Pharmacia GmbH, Freiburg, FRG) with subsequent hand-picking [2]. The islets were dissociated into single cells in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS, GIBCO EUROPE, Heidelberg, FRG) including trypsin (2.5 mg/ml, Boehringer, Mannheim, FRG). Then the islet cells were labelled by incubation with 6 μCi/ml ^{3}H-leucine (specific activity 1 mCi/ml, Amersham-Buchler, Braunschweig, FRG) for 20 h (5% CO_2, 37 °C) in leucine-free RPMI 1640 medium (GIBCO) with 4.5 m*M* glucose supplemented with ampicillin 25 mg/l, penicillin 120 mg/l, streptomycin 270 mg/l (Serva GmbH, Heidelberg, FRG), sodium pyruvate 1 m*M*, L-glutamine 2 m*M*, non-essential amino acids (100×) 10 ml/l (GIBCO), $NaHCO_3$ 2 g/l, HEPES 2.38 g/l (Serva) and 10% fetal calf serum (GIBCO).

Preparation of macrophages

Macrophages were isolated from the peritoneal cavity of Wistar rats pretreated for 5 days with an intraperitoneal injection of a heat-inactivated suspension of Corynebacterium parvum (0.5 ml/animal, Wellcome, Burgwedel, FRG) as described previously [2].

Macrophage–islet-cell coculture

The effect of dihydrolipoic acid (kindly provided by Dr. Ulrich, ASTA Medica Deutschland, Frankfurt, FRG) on macrophage-mediated islet cell toxicity was determined in a coincubation assay in the absence or presence of dihydrolipoic acid. Labelled islet cells (5000 cells in 200 μl per well) were cocultured (37 °C, 5% CO_2) with macrophages at various target:effector ratios [2] in complete RPMI 1640 medium (GIBCO) with 7.5 m*M* glucose supplemented with the same constituents as the medium used for the labelling of islet cells. After 15 h of incubation, the concentration of radiolabel released from the islet cells was determined in the supernatants of the cultures in a liquid scintillation counter. The specific lysis of the islet cells was calculated from the supernatants of the macrophage–islet-cell cocultures (test cpm), from the cultures of islet cells alone (spontaneous cpm) and from samples of islet cells completely lysed with Triton X-100 (Serva) (maximum cpm):

$$\text{specific lysis (\%)} = 100 \times \frac{(\text{test cpm} - \text{spontaneous cpm})}{(\text{maximum cpm} - \text{spontaneous cpm})}.$$

Exposure of islet cells to oxygen radicals

Islet cell toxic oxygen radicals were generated by incubation of xanthine oxidase (XO, 25 mU/ml, Sigma) with its substrate hypoxanthine (HX, 0.5 m*M*, Sigma) [4]. The specific lysis of ^{3}H-leucine-labelled islet cells mediated by the xanthine-oxidase–hypoxanthine system was determined corresponding to the evaluation of the macrophage-islet-cell coculture assay described above. Dihydrolipoic acid was added into the samples in various concentrations to study its possible protective effect on islet cell toxicity mediated by oxygen radicals.

Chemiluminescence analysis

The generation of oxygen radicals from macrophages or from the xanthine-oxidase–hypoxanthine system was determined in a bioluminescence analyzer (Biolumat LB 9505C, Berthold, Wildbad, FRG) as described in [4]. Macrophage suspensions (10^6 cells/ml) or a solution of hypoxanthine (0.5 m*M*) were incubated in the presence or absence of dihydrolipoic acid. Luminol (5×10^{-4} *M*, Sigma) was used for the detection of the chemiluminescence activity of the samples. After the background chemiluminescence reached constant levels (about 10^4 cpm), the generation of oxygen radicals was initiated by the addition of

zymosan particles (100 μg/ml) to the macrophage suspension or by the addition of xanthine oxidase (25 mU/ml) to the hypoxanthine solution. The release of oxygen radicals was monitored for 50–80 min. The data obtained from the chemiluminescence activities of the various samples were processed by special software supplied by Berthold and plotted against the time.

Nitrite determination

Nitrite determination as an indirect measure of nitric oxide release from macrophages was performed by the Griess reaction as described previously [10]. Briefly, 10^5 macrophages (200 μl) were incubated per well of a 96-well flat-bottom microtiter plate in the presence of rising concentrations of dihydrolipoic acid. After 15 h, 120 μl of the supernatant were removed and mixed with 160 μl sulfanilamide (1% in 4 *N* HCl, Sigma). Then 20 μl of concentrated HCl were added and, after 10 min 120 μl of *N*-(1-naphthyl)-ethylenediamine (1% in methanol, Sigma) were added. The optical density of 100 μl of the samples was measured at 540 nm and the nitrite concentrations were calculated from a standard curve obtained with $NaNO_2$.

Test of islet cell metabolic activity

The metabolic activity of the islet cells was determined by a colorimetric assay which detects the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma) into its formazan product [11, 12]. This method was suggested to allow an indirect estimation of the intensity of mitochondrial respiratory processes. It was successfully used to determine the metabolic activity and viability of isolated islet cells [13]. After exposure to xanthine-oxidase–hypoxanthine, 100 μl of a solution of MTT (1 mg/ml phosphate-buffered saline, GIBCO) were added into each well of the islet cell culture. The resulting formazan crystals were dissolved in 50 μl isopropanol and the ΔOD of the solution was determined photometrically (measuring wavelength 540 nm, reference wavelength 690 nm).

Statistical analysis

For statistical evaluation of the data, the Student's *t*-test was used.

Results

When activated macrophages were added to ^{3}H-leucine-labelled islet cells, release of radiolabel was observed after 15 h of coculture (Fig. 1), reflecting islet cell lysis as shown previously [2]. The addition of dihydrolipoic acid to the incubation mixture protected the islet cells from lysis (Fig. 1). Almost complete inhibition was achieved at a dose of 0.25 mg/ml. Preincubation of the macrophages for 2 h with 0.1 mg/ml dihydrolipoic acid and subsequent removal of non-cell-bound thiols sufficed to reduce islet cell lysis significantly (Fig. 2). At a target : effector ratio of 1 : 10, even complete protection was noted.

As an indirect measure of nitric oxide release, the accumulation of nitrite in the supernatant of 10^5 macrophages was determined by the Griess reaction. In the presence of dihydrolipoic acid, the nitrite concentration was reduced in a dose-dependent manner from 12.3 ± 0.4 nmol in the medium control to 6.8 ± 0.6 nmol at a dose of 0.25 mg/ml dihydrolipoic acid ($p < 0.01$) (Fig. 3). To exclude a cytotoxic effect of dihydrolipoic acid, 2×10^4 macrophages per well of a microtiter plate were exposed to rising concentrations of the substance for 15 h. The MTT staining showed no significant changes in the metabolic activity of macrophages in the absence (ΔOD 0.26 ± 0.05) or in

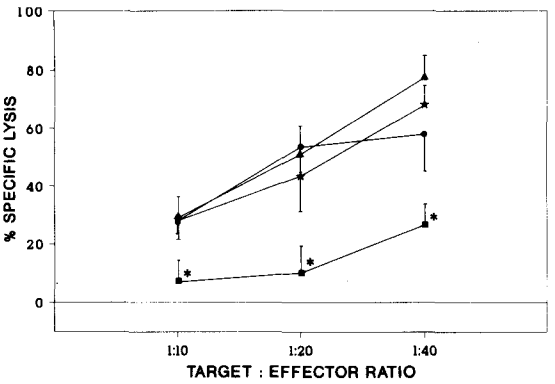

Figure 1
Inhibition of macrophage-mediated islet cell lysis by dihydrolipoic acid. The specific lysis was determined as described under Materials and Methods, after 15 h of coincubation of islet cells (targets) and macrophages (effectors) in the absence (●) or presence of dihydrolipoic acid 0.05 mg/ml (▲), 0.1 mg/ml (★), and 0.25 mg/ml (■). The data show the mean ± SD from three experiments. *$p < 0.01$ compared to the corresponding value of the control without dihydrolipoic acid.

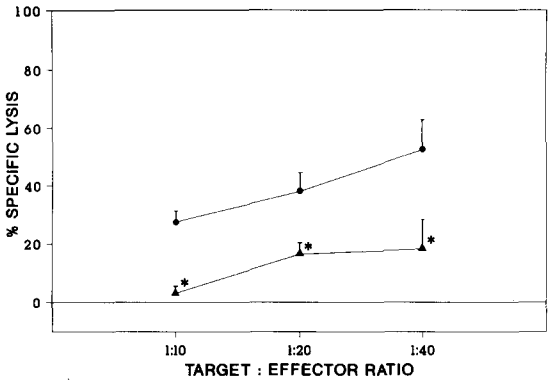

Figure 2
Suppression of islet cell lysis by pretreatment of macrophages for 2 h with 0.1 mg/ml dihydrolipoic acid (■) compared to macrophages sham-treated with medium as control (●). The specific lysis of the islet cells (targets) was determined after 15 h of cocultivation with macrophages (effectors). The data show the mean ± SD from three experiments. $*p < 0.01$ compared to the corresponding value of the control without dihydrolipoic acid.

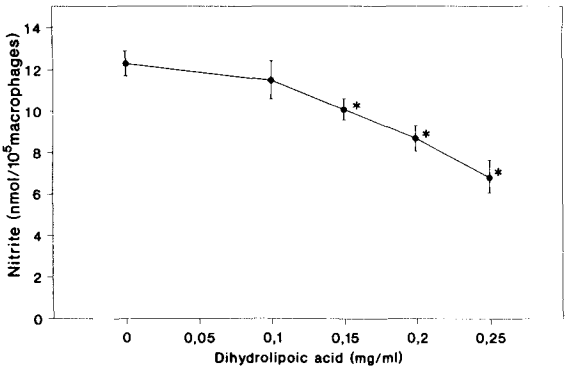

Figure 3
Reduction of the nitrite concentration as detected by the Griess method in the supernatants of 10^5 macrophages incubated for 15 h in the presence of increasing doses of dihydrolipoic acid. The data show the mean ± SD from three experiments. $*p < 0.01$ compared to the control without dihydrolipoic acid.

the presence of dihydrolipoic acid up to a concentration of 0.25 mg/ml (ΔOD 0.27 ± 0.09). These data indicate the means ± SD from three experiments. Control experiments did not show an interference of dihydrolipoic acid with the MTT staining procedure and the Griess reaction.

In a chemiluminescence analyzing system, we studied the release of oxygen radicals from macrophages in the presence of dihydrolipoic acid at concentrations which proved to be effective in the macrophage–islet-cell coculture assay. At a dose of 0.1 mg/ml dihydrolipoic acid, a strong inhibition of the release of oxygen radicals from macrophages was noted (Fig. 4).

Using the conversion of MTT into its formazan salt as a quantitative measure of islet cell metabolic activity, we tested the potential protective effect of dihydrolipoic acid against oxygen radicals generated by the enzyme xanthine oxidase. As shown in Fig. 5, a dose of 0.25 mg/ml dihydrolipoic acid,

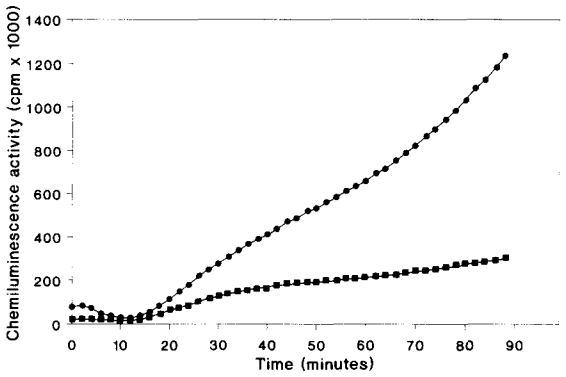

Figure 4
Suppressive effect of dihydrolipoic acid (0.1 mg/ml ■) compared to the medium control (●) on the release of oxygen radicals from macrophages as detected by luminol-enhanced chemiluminescence. Three experiments were performed. The curves show the means of a representative experiment.

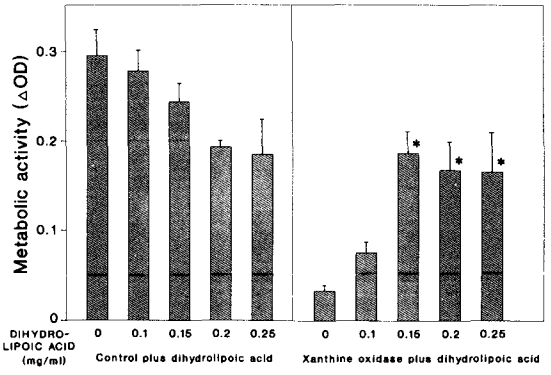

Figure 5
Preservation of islet cell metabolic activity after exposure for 15 h to the xanthine-oxidase–hypoxanthine system in the presence of dihydrolipoic acid. The data show mean ± SD from three experiments. $*p < 0.01$ compared to the control without dihydrolipoic acid.

which was protective in the macrophage–islet-cell cocultures, largely protected islet cells from oxygen radical damage. The mechanism involved is probably the direct scavenging of oxygen radicals in the presence of dihydrolipoic acid as could be demonstrated in the chemiluminescence analyzing system (Fig. 6).

Another finding from Fig. 5 is that dihydrolipoic acid in concentrations above 0.15 mg/ml is partially toxic to islet cells as evident from the decreasing capacity of the cells to reduce MTT to formazan. In this experiment islet cells were exposed to high doses of dihydrolipoic acid for 15 h. We, therefore, tested whether a shorter incubation period with dihydrolipoic acid would obliterate any toxic effects but retain its protective action against oxygen radicals. As shown in Fig. 7, a 2 h incubation period with high doses of dihydrolipoic acid was well tolerated by islet cells and even slightly improved the survival *in vitro*. When non-cell-bound dihydrolipoic acid was washed away before the islet cells were exposed to xanthine-oxidase-generated oxygen radicals, complete protection was reached by 0.25 mg/ml of dihydrolipoic acid (Fig. 7).

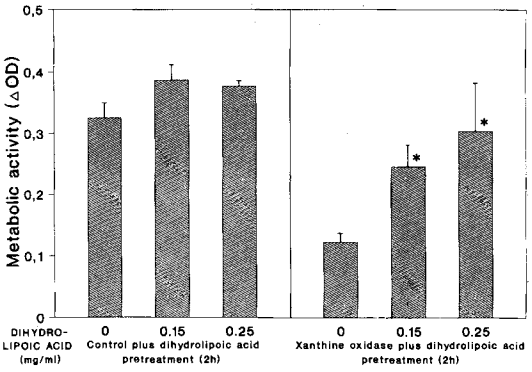

Figure 7
Preservation of islet cell metabolic activity by 2 h treatment with various doses of dihydrolipoic acid before exposure to the xanthine-oxidase–hypoxanthine system. The data show mean ± SD from three experiments. $*p < 0.01$ compared to the control without dihydrolipoic acid.

Discussion

The results of our experiments indicate that dihydrolipoic acid attenuates the cytotoxic activity of activated macrophages against pancreatic islet cells.

Since dihydrolipoic acid has antioxidant activity [8, 14], it is conceivable that the thiol scavenges oxygen radicals released from activated macrophages and, thus, prevent islet cell lysis. In fact, the results of our studies and of experiments with human mononuclear cells [15] indicate that the release of oxygen radicals from macrophages is suppressed in the presence of dihydrolipoic acid. However, we have previously shown that the toxicity of macrophages towards islet cells is not dependent on oxygen radicals but is solely mediated by the release of nitric oxide [3]. The inhibition of islet cell lysis by dihydrolipoic acid treatment of activated macrophages is, thus, probably due to the interference with the L-arginine-dependent nitric oxide cytotoxic pathway. Such an effect of dihydrolipoic acid is directly shown here by a decrease of nitrite production from activated macrophages in the presence of the drug.

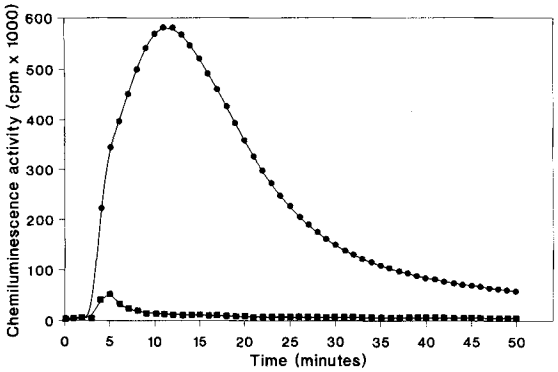

Figure 6
Suppressive effect of dihydrolipoic acid (0.25 mg/ml ■) compared to the medium control (●) on the release of oxygen radicals from the xanthine-oxidase–hypoxanthine system as detected by luminol-enhanced chemiluminescence. Three experiments were performed. The curves show the means of a representative experiment.

Another model of inflammatory attack against islet cells is the incubation of islet cells with the endothelial-cell-associated enzyme xanthine oxidase, known to be involved in tissue damage during processes of ischemia/reperfusion [5, 6]. We have recently found that such enzymatically generated oxygen radicals cause the lysis of islet cells as determined by Trypan blue exclusion, ^{3}H-leucine release and a metabolic test [4]. The mechanism of protection is probably the direct scavenging of oxygen radicals. Recently, it could be demonstrated that dihydrolipoic acid is a very effective scavenger of xanthine-oxidase-generated superoxide radicals and also of hydroxyl radicals [7], which were

shown to be highly toxic to pancreatic islet cells [17].

Interestingly, it was found that nitric oxide may rapidly interact with the superoxide anion, thereby forming the highly reactive peroxynitrite and NO_2, which in turn gives rise to hydroxyl radicals [18]. Therefore, the beneficial effect of dihydrolipoic acid may be explained by the ability of the drug to interfere with two islet cell toxic mediators, the reactive oxygen intermediates as well as the nitric oxide. A direct protection from nitric oxide on the level of the islet cells can be excluded, since recently we could demonstrate that dihydrolipoic acid is not able to reduce the deleterious effects of chemically generated nitric oxide on islet cells [16].

Our data indicate that dihydrolipoic acid concentrations above 0.15 mg/ml are harmful to islet cells. However, the potential toxicity of dihydrolipoic acid could be avoided by short-term treatment with the thiol, which nevertheless caused long-lasting anti-inflammatory protection. This indicates that for a similar effect *in vivo* effective levels of the drug need to be reached only during few hours per day.

In conclusion, the experiments show that dihydrolipoic acid protects islet cells from inflammatory attack at two different levels. For one, dihydrolipoic acid attenuates the cytotoxic activity of activated macrophages, probably at the level of both oxygen radical and nitric oxide release. Secondly, dihydrolipoic acid directly protects islet cells from the damaging effect of oxygen radicals by its radical scavenger properties.

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