Diabetologia (1999) 42: 55–59

Diabetologia

Nicotinamide protects human beta cells against chemically-induced necrosis, but not against cytokine-induced apoptosis

A. Hoorens, D. Pipeleers

Diabetes Research Center, Vrije Universiteit Brussel, Brussels, Belgium

Summary Nicotinamide intervention trials are presently undertaken to prevent Type I (insulin-dependent) diabetes in high risk subjects. They are based on studies in rodents reporting nicotinamide protection against beta-cell injury in vitro and in vivo. This study examines whether nicotinamide can protect human beta cells in vitro. At concentrations (2 and 5 mmol/l) to protect rat beta cells against necrosis by streptozotocin or hydrogen peroxide, nicotinamide prevents hydrogen peroxide-induced necrosis of human beta cells. As with rat beta cells, nicotinamide fails to protect human beta cells against apoptosis induced by a combination of the cytokines interleukin-1β, interferon-γ and tumour necrosis factor-α. In rat beta cells, nicotinamide (2 to 20 mmol/l) was also found to induce apoptosis, in particular during the days following its protection against necrosis; this cytotoxic effect was not observed with human beta cells. These data demonstrate that nicotinamide can protect human beta cells against radical-induced necrosis, but not against cytokine-induced apoptosis. This effect is not associated with a delayed apoptosis as in rat beta cells. [Diabetologia (1999) 42: 55–59]

Keywords Nicotinamide, cytokine, islet, insulin, apoptosis, diabetes.

Nicotinamide is presently tested for its potential benefit in preventing Type I (insulin-dependent) diabetes [1]. This is based on the observation that nicotinamide prevents losses in rodent beta-cell numbers or functions following exposure to toxic agents. Treatment of prediabetic non-obese diabetic (NOD) mice with nicotinamide was found to delay onset of diabetes [2]. Doses in these laboratory models have been consistently higher than those currently applied in man [3]. It is also not known whether the effects of nicotinamide on rodent beta cells can be reproduced in human beta cells. In rat beta cells, protection was induced by giving the agent after the cells had been exposed to cytotoxic compounds such as streptozotocin or hydrogen peroxide [4]. It is therefore attributed to a nicotinamide-induced defence reaction which can be operative during different mechanisms of beta-cell death [4]. The cytotoxic actions of streptozotocin and hydrogen peroxide result in death of isolated rat beta cells within one day [4]. Consequently, cytoprotective effects of nicotinamide have been studied within this short time period. By extending the culture period, we noticed that the initial protection by nicotinamide was partly or entirely lost during the following days. In view of the clinical use of nicotinamide we examined whether this was also the case for human beta cells which had been exposed to peroxide radicals or to cytokines.

Received: 7 July 1998 and in revised form: 10 September 1998

Corresponding author: D. Pipeleers, Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium

Abbreviations: NOD, Non-obese diabetic; BSA, bovine serum albumin; STZ, streptozotocin; H_2O_2, hydrogen peroxide; HCI, hydrochloric acid; IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α; IFN-γ, interferon-γ; HO 342, Hoechst 33342; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase

Materials and methods

Preparation and culture of rat and human beta-cells. Pancreatic islets were isolated from adult male Wistar rats, dissociated and sorted into pure single beta cells (purity > 92 %) [4]. The purified cells were seeded in polylysine-coated microtitre cups (3000 cells in 200 µl medium) and cultured in serum-free Ham's F10 medium (Gibco Laboratories, Grand Island, N. Y., USA) with 2 mmol/l L-glutamine, 10 mmol/l glucose and 10 g/l charcoal-treated bovine serum albumin (BSA) (factor V, RIA grade; Sigma, St. Louis, Mo., USA) [4]. Human beta-cell preparations were obtained within the framework of a multicentre programme on islet transplantation in diabetes [5]. Donor pancreata were made available by European transplantation centres in collaboration with Eurotransplant Foundation (Leiden, The Netherlands). The methods for isolation and culture have been described previously [5]. After 6 days culture the islets were dissociated in calcium-free medium containing trypsin and then sorted into viable single cell preparations enriched in beta cells (purity 69 ± 5 %) [4]. One experiment correlates with cells from one rat isolation or cells from one human donor.

Exposure to cytotoxic agents. Stock solutions of streptozotocin (STZ; Sigma) were prepared in 1 mmol/l HCl, hydrogen peroxide (H_2O_2; Merck, Darmstadt, Germany) was dissolved in culture medium. Beta cells were exposed for 15 min to STZ (5 mmol/l) or to H_2O_2 (100–500 µmol/l) in medium containing 10 mmol/l glucose for rat beta cells and 7.5 mmol/l glucose for human beta cells. They were then washed and cultured on polylysine-coated microtitre cups in medium with or without nicotinamide (Merck and Sigma), 3-aminobenzamide (Sigma) or nicotinic acid (Sigma) (0.2 to 20 mmol/l) for 1, 6 or 10 days. Exposure to the cytokines occurred during 3, 6 or 9 days of culture. Recombinant human IL-1β (200 U/ng; Genzyme, Cambridge, Mass., USA), recombinant murine TNF-α (200 U/ng; Innogenetics, Gent, Belgium), recombinant murine IFN-γ (for rat beta-cells; 10 U/ng; Holland Biotechnology, Leiden, The Netherlands) or recombinant human IFN-γ (for human beta cells; 48 U/ng; Genzyme) were used at concentrations (50 U/ml IL-1β, 1000 U/ml TNF-α and 1000 U/ml IFN-γ) that were previously found to induce apoptosis in human beta cells [6].

Assessment of necrosis and apoptosis. Under the selected culture and assay conditions, the number of plated cells remained constant between day 1 and day 10 [7]. Dead cells were identified as neutral red negative [7], as propidium iodide positive or through their fragmented nuclei [8]. In control conditions (i.e. in control cultures in the absence of nicotinamide or cytotoxic agents) the percent of living cells varied, in the rat, from 87 ± 1 % on day 2 till 78 ± 3 % on day 10, and in human preparations, from 83 ± 2 % on day 2 till 62 ± 4 % on day 10. The numbers of viable cells and of cells in necrosis and apoptosis were quantified as described previously [8]. Briefly, cell nuclei were stained simultaneously with the DNA binding dyes Hoechst 33342 (HO 342; Calbiochem Behring, La Jolla, Calif., USA) and propidium iodide (PI; Sigma) [8]. Viable cells were identified by their intact nuclei with blue fluorescence (HO 342), necrotic cells by their intact nuclei with yellow fluorescence (positivity for both HO 342 and PI) and apoptotic cells by their fragmented nuclei, fluorescing either blue (HO 342; early phase of apoptosis) or yellow (HO 342 + PI; late phase of apoptosis with secondary necrosis). At least 500 cells were counted for each condition and in each experiment. For each experiment the necrosis index was calculated with this formula [4, 6]:

$$\text{necrosis index} = \frac{\%\ \text{necrotic cells in test} - \%\ \text{necrotic cells in control condition}}{100 - \%\ \text{dead cells in control condition}} \times 100\,\%$$

The apoptosis index was obtained by replacing the percentage of necrotic cells by the percentage of apoptotic cells. Data express means ± standard error (SEM). Statistical analysis used the StatWorks computer programme (Cricket Software, Inc., Philadelphia, PA, USA) with a two sided paired *t*-test.

Results

Effect of nicotinamide on beta cell survival during culture. When rat beta cells were cultured in the presence of different concentrations (0.2, 2, 5 and 20 mmol/l) of nicotinamide, no cytotoxicity was observed after 1 day, but cell death by apoptosis occurred after prolonged exposure to high concentrations (20 mmol/l): after 5 days the apoptosis index was 31 ± 5 % ($n = 9$, $p < 0.001$ vs control condition), while the necrosis index was less than 5 %. At lower concentrations ($\leq$ 5 mmol/l) both indices remained under 5 % ($n = 9$ for 0.2 and 2 mmol/l, $n = 10$ for 5 mmol/l). Apoptosis was also induced by 20 mmol/l 3-aminobenzamide (apoptosis index > 95 % after 5 days, $n = 6$, $p < 0.001$ vs control condition) but not by 20 mmol/l nicotinic acid ($n = 6$). In human beta cells, no cytotoxicity was observed after 10 days culture with 20 mmol/l nicotinamide ($n = 4$) or 20 mmol/l nicotinic acid ($n = 4$), but apoptosis was induced by 20 mmol/l 3-aminobenzamide (apoptosis index 84 ± 8 %; $n = 4$, $p < 0.01$ vs control condition); at 5 mmol/l 3-aminobenzamide ($n = 4$) no cytotoxicity was noticed. All further experiments were carried out with nicotinamide concentrations up to 5 mmol/l.

Nicotinamide protection against streptozotocin-induced necrosis. Streptozotocin causes necrosis of rat beta cells within 1 day (Table 1). When the STZ-exposed cells are cultured with nicotinamide, the percentage of necrotic cells is greatly reduced; this cytoprotective effect is stronger at 5 mmol/l nicotinamide than at 2 mmol/l ($n = 4$, $p < 0.01$). A similar effect was seen with 3-aminobenzamide but not with nicotinic acid (data not shown). It was, however, noticed that protection was not maintained. During the following days nicotinamide (Table 1) as well as 3-aminobenzamide (data not shown) induced apoptosis of the cells that were protected from streptozotocin-induced necrosis.

Nicotinamide protection against H_2O_2-induced necrosis. Exposure of rat and human beta cells for 15 min to H_2O_2 resulted in necrosis of the majority of cells within one day (Table 1). Culture of the H_2O_2-treated cells in a medium with nicotinamide markedly reduced this cytotoxic effect. Protection against necro-

Table 1. Effect of nicotinamide on cell survival following exposure to streptozotocin or hydrogen peroxide

Day 1 Cytotoxic Agent		Culture Nicotinamide (mmol/l)	Day 2		Day 7		*n*
			Necrosis Index (%)	Apoptosis Index (%)	Necrosis Index (%)	Apoptosis Index (%)	
Rat beta cells							
STZ	5 mmol/l	0	84 ± 3	< 5	89 ± 2	< 5	9
		0.2	80 ± 3	< 5	79 ± 10	5 ± 3	4
		2	26 ± 3[a]	< 5	17 ± 11[a]	69 ± 12[a]	4
		5	6 ± 2[b]	< 5	< 5[b]	85 ± 9[b]	9
H_2O_2	100 μmol/l	0	77 ± 10	< 5	89 ± 8	< 5	5
		5	13 ± 3[a]	< 5	26 ± 4[b]	10 ± 2[a]	5
	200 μmol/l	0	> 95	< 5	> 95	< 5	9
		5	32 ± 5[b]	5 ± 1[b]	35 ± 5[b]	33 ± 6[b]	9
Human beta cells							
H_2O_2	200 μmol/l	0	65 ± 6	< 5	85 ± 6	< 5	9
		5	6 ± 3[b]	< 5	7 ± 2[b]	< 5	9
	500 μmol/l	0	73 ± 7	< 5	94 ± 6	< 5	6
		5	< 5[b]	< 5	9 ± 2[b]	6 ± 2[a]	6

Data are expressed as means ± SEM of 4 to 9 independent experiments. Statistical significance of differences with condition without nicotinamide was calculated by two-sided paired *t*-test. [a] $p < 0.01$; [b] $p < 0.001$. For each experiment the necrosis and apoptosis index was calculated as described in Methods.

sis was maintained over the subsequent 6 day culture period. The longer culture period with nicotinamide, however, led to the appearance of apoptotic cells in the H_2O_2 treated rat preparations, in particular following the higher H_2O_2 concentrations (Table 1). Total beta-cell death in nicotinamide-supplemented media remained lower than in media without nicotinamide. In human beta-cell preparations, the protective effect of nicotinamide was not associated with delayed appearance of apoptotic cells; use of 5 mmol/l 3-aminobenzamide instead of nicotinamide resulted in similar protection without induction of apoptosis (data not shown). Protection decreased with the nicotinamide concentrations, but was still detectable at 0.2 mmol/l nicotinamide (necrosis index after exposure of human beta cells to 200 μmol/l H_2O_2 in the presence of 5 mmol/l nicotinamide 7 ± 2 % and with 0.2 mmol/l nicotinamide 66 ± 12 %, $p < 0.001$ and respectively $p < 0.01$ vs 85 ± 6 % in control conditions without nicotinamide, $n = 3$).

Failure of nicotinamide to protect against cytokine-induced apoptosis. Culture in the presence of a combination of the three cytokines IL-1β plus TNF-α plus IFN-γ induces apoptosis of both rat and human beta cells (Fig. 1, $n = 4$). The percentage of apoptotic cells increases with the duration of culture. Addition of 5 mmol/l nicotinamide to the medium did not influence this cytotoxic effect (Fig. 1). Cytokine exposure of rat beta cells also resulted in necrosis of 20 % of the cells, whether nicotinamide was present or not (Fig. 1). No necrosis was induced in human beta-cell preparations (Fig. 1).

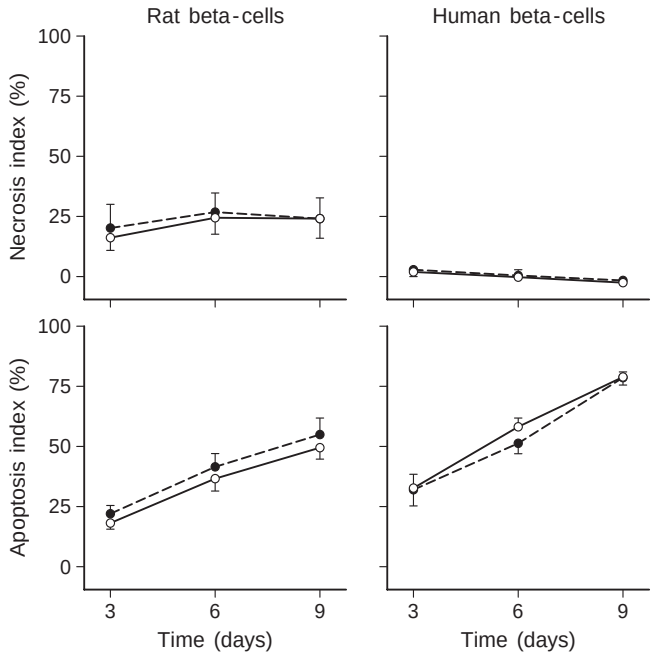

Fig. 1. Effect of 5 mmol/l nicotinamide (- - -) on the necrosis and apoptosis index in rat beta-cells (left panels) and in human beta-cells (right panels) cultured in the presence of three cytokines (50 U/ml IL-1β + 1000 U/ml IFN-γ + 1000 U/ml TNF-α). The solid lines represent the conditions of cytokines without nicotinamide. Data express means ± SEM of four independent experiments. The apoptosis index, but not the necrosis index, was significantly increased by the cytokines at the three time points in both rat ($p < 0.01$) and human ($p < 0.05$ at day 3 and $p < 0.001$ at days 6 and 9) beta-cell preparations. The presence of nicotinamide did not protect against cytokine-induced apoptosis ($p > 0.05$). For each experiment the necrosis and apoptosis index was calculated as described in Methods.

Discussion

This study confirms that nicotinamide can protect rat beta cells which have been exposed to streptozotocin or to hydrogen peroxide. In its absence the majority of cells die by necrosis within 1 day. At 2 and 5 mmol/l nicotinamide dose-dependently prevents this rapid cell death. In this concentration range nicotinamide is a poor radical scavenger but an inhibitor of poly (ADP-ribose) polymerase (PARP) [9]. A similar protection was obtained with 3-aminobenzamide but not with nicotinic acid, supporting an action through inhibiting this nuclear enzyme. It is assumed that DNA damage by streptozotocin or by hydrogen peroxide leads to PARP-activation which will increase nicotinamide adenine dinucleotide (NAD) consumption; rapid depletion in NAD is expected to compromise ATP production to such an extent that membrane functions cease and cells lyse [9]. Preservation of cellular NAD is thought to prevent cell lysis. Both nicotinamide and 3-aminobenzamide were found to inhibit PARP with a 50 % effect at, respectively, 100–200 and 5–30 μmol/l [9]. In our experiments, 10 to 50-fold higher nicotinamide concentrations were needed (2 and 5 mmol/l) to prevent necrosis of rat beta cells suggesting that this also depends on additional mechanisms. Since intracellular concentrations of the benzamides are close to the extracellular ones, it is conceivable that their protective effect also requires inhibition of mono(ADP-ribosyl) transferases, for which 100-fold higher concentrations are needed. The latter enzymes interact with mitochondrial channel proteins and may therefore influence mitochondrial functions. Addition of nicotinamide to insulted beta cells could thus prevent their rapid necrosis through preservation of the cellular NAD and ATP levels, but the surviving cells might be suppressed both in their DNA repair mechanisms and in their mitochondrial functions and could therefore become more susceptible to apoptosis. It was noted that culture of control cells with high nicotinamide concentrations (20 mmol/l) resulted in the appearance of apoptotic rat beta cells. Since many reported effects on rodent islets were obtained with nicotinamide concentrations of 10 to 50 mmol/l, it seems likely that they also include induction of apoptosis [10, 11].

Nicotinamide also protects human beta cells against necrosis induced by hydrogen peroxide. Complete protection was achieved at 5 mmol/l nicotinamide, a concentration which did not cause a delayed apoptosis as with rat beta cells. Furthermore, untreated human beta cells did not become apoptotic when exposed to high (20 mmol/l) nicotinamide concentrations. Only a 10 day exposure to the more potent 3-aminobenzamide compound resulted in apoptosis. Human beta cells seem thus more resistant than rat beta cells to apoptosis induction by nicotinamide. The reasons for this difference are still not known.

We previously reported that a combination of three cytokines, namely IL-1β plus TNF-α plus IFN-γ, induced apoptosis of human beta cells over a 9 day culture period [6]. It is now known that nicotinamide does not protect against cytokine-induced apoptosis of rat or human beta cells. This finding is consistent with the observation that nicotinamide did not prevent the reduction in DNA content of human islets exposed for 6 days to the combination of IL-1β, TNF-α and IFN-γ [11]. In rat beta cells exposure to the combination of IL-1β plus TNF-α plus IFN-γ also induced necrosis. The percent of necrotic cells was the same, whether nicotinamide was present or absent from the culture medium. In animal models of autoimmune diabetes beta cells seem to disappear through apoptosis [12]. If cytokines are directly responsible for this process, then intervention treatment with nicotinamide cannot be supported by the present data. If other factors cause beta-cell apoptosis in vivo, it will be necessary to examine whether nicotinamide has any protective effect against their destructive nature.

Preliminary data in man were indicative for a protective effect in individuals at high risk for developing the disease. The doses used in current prevention trials reach circulating concentrations of nicotinamide of 0.1 to 0.3 mmol/l [3]. At these concentrations only a 20 % protection was seen in vitro against hydrogen peroxide induced necrosis of human beta cells. Tenfold higher concentrations were needed to achieve over 50 % protection. It is, however, not excluded that the lower nicotinamide concentrations are more effective in vivo where beta-cell necrosis might occur less acutely than during in vitro exposure to hydrogen peroxide. In more general terms, the present data should be interpreted with caution as to their relevance to prevention trials in human diabetes. While in vitro studies on dispersed and purified beta-cell preparations can help clarify effects of single interactions with the beta cells, they are less informative for the in vivo process occurring in intact islet tissue. The structural organisation of the islet tissue as well as the multiplicity of interacting factors can indeed modify the relative importance of single in vitro events. A beter understanding of mechanisms at the cellular level can, however, help the dissection and understanding of in situ processes which are difficult to investigate in vivo.

Acknowledgements. The authors thank the technical staff for providing the rat and human beta-cell preparations and G. Stangé for his assistance in the present project. This study was supported by grants from the Juvenile Diabetes Foundation International (JDF-DIRP 95-97), the European Community (BMH-CT 95-1561), the Belgian Fonds voor Wetenschappelijk Onderzoek (7.0036.96) and the Interuniversity Attraction Pole P4(2). A. Hoorens is a research fellow of the Belgian Fonds voor Wetenschappelijk Onderzoek.

References

1. Elliott RB, Chase HP (1991) Prevention or delay of Type 1 (insulin-dependent) diabetes mellitus in children using nicotinamide. Diabetologia 34: 362–365
2. Yamada K, Nonaka K, Hanafusa T, Miyazaki A, Toyoshima H, Tarui S (1982) Preventive and therapeutic effects of large-dose nicotinamide injections on diabetes associated with insulitis: an observation in nonobese diabetic (NOD) mice. Diabetes 31: 749–753
3. Petley A, Macklin B, Renwick AG, Wilkin TJ (1995) The pharmacokinetics of nicotinamide in humans and rodents. Diabetes 44: 152–155
4. Pipeleers D, Van de Winkel M (1986) Pancreatic B cells possess defence mechanisms against cell-specific toxicity. Proc Natl Acad Sci USA 83: 5267–5271
5. Keymeulen B, Ling Z, Gorus FK et al. (1998) Implantation of standardized beta-cell grafts in a liver segment of IDDM patients: graft and recipient characteristics in two cases of insulin-independence under maintenance immunosuppression for prior kidney graft. Diabetologia 41: 452–459
6. Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL (1997) Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. Endocrinology 138: 2610–2614
7. Ling Z, Hannaert JC, Pipeleers D (1994) Effect of nutrients, hormones and serum on survival of rat islet beta cells in culture. Diabetologia 37: 15–21
8. Hoorens A, Van de Casteele M, Klöppel G, Pipeleers D (1996) Glucose promotes survival of rat pancreatic β cells by activating synthesis of proteins which suppress a constitutive apoptotic program. J Clin Invest 98: 1568–1574
9. Uchigata Y, Yamamoto H, Kawamura A, Okamoto H (1982) Protection by superoxide dismutase, catalase, and poly(ADP-ribose) synthetase inhibitors against alloxan- and streptozotocin-induced islet DNA strand breaks and against the inhibition of proinsulin synthesis. J Biol Chem 257: 6084–6088
10. Reddy S, Salari-Lak N, Sandler S (1995) Long-term effects of nicotinamide-induced inhibition of poly(adenosine diphosphate-ribose) polymerase activity in rat pancreatic islets exposed to interleukin-1β. Endocrinology 136: 1907–1912
11. Eizirik DI, Sandler S, Welsh N, Bendtzen K, Hellerström C (1994) Nicotinamide decreases nitric oxide production and partially protects human pancreatic islets against the suppressive effects of combinations of cytokines. Autoimmunity 19: 193–198
12. O'Brien BA, Harmon BV, Cameron DP, Allan DJ (1997) Apoptosis is the mode of β-cell death responsible for the development of IDDM in the nonobese diabetic (NOD) mouse. Diabetes 46: 750–757