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NICOTINAMIDE DECREASES NITRIC OXIDE PRODUCTION AND PARTIALLY PROTECTS HUMAN PANCREATIC ISLETS AGAINST THE SUPPRESSIVE EFFECTS OF COMBINATIONS OF CYTOKINES

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It has been recently reported that human pancreatic islets in tissue culture produce nitric oxide (NO) and show a decreased function when exposed for 6 days to combinations of cytokines (interleukin-1β (IL-1β) + tumor necrosis factor-α (TNF-α) + interferon-γ (IFN-γ). Here we study the effects of nicotinamide (Nic; 10 or 20 mmol/l) on these deleterious effects of cytokines (50 U/ml IL-1β + 1000 U/ml TNF-α + 1000 U/ml IFN-γ). Islets were isolated from 8 human pancreata at the Central Unit of the β-Cell Transplant, Brussels, sent to Uppsala and, after 3–5 days in culture, exposed for 6 additional days to the cytokines and/or Nic. The cytokines induced a 6-fold increase in islet NO production ($P < 0.001$), and this effect was partially counteracted by Nic (50-60% decrease in NO production; $P < 0.001$). The cytokines severely decreased the islet insulin content and glucose-induced insulin release (16.7 mmol/l glucose; 90% decrease; $P < 0.001$). Both these effects of cytokines were partially counteracted by Nic, especially at the highest concentration (20 mmol/l; 2–4-fold increase compared to islets exposed to cytokines alone; $P < 0.01$). Nic by itself did not affect the insulin content or insulin release by control islets. In conclusion, the present data indicate that Nic counteracts the deleterious effects of cytokines on human pancreatic islets. This effect of Nic may be relevant for the beneficial effects of the drug in early IDDM.

KEY WORDS: Pancreatic islets, nitric oxide, nicotinamide, interleukin-1β, tumor necrosis factor-α, interferon-γ, insulin release.

INTRODUCTION

Cytokines may be effectors of β-cell destruction during the early stages of IDDM[1,2]. *In vitro* exposure of rodent pancreatic islets to cytokines, especially IL-1β, impairs islet function and may lead to islet cell death[1–3]. These deleterious effects of cytokines are related to increased production of nitric oxide (NO)[1,3,4]. Although IL-1β by itself neither inhibits human islet function nor induces NO production[5], combinations of cytokines (IL-1β + TNF-α + IFN-γ) impair human islet function[6,7], induce expression of mRNA for the inducible form of nitric oxide synthase (iNOS)[7] and increase medium nitrite accumulation (nitrite is a stable product of NO oxidation)[6,7]. It is noteworthy that while in rodent islets inhibition of NO production prevents most of the deleterious effects of cytokines[1,3,4], data obtained using human islets are conflicting. Thus, while Corbett and co-workers described protection by inhibitors of NO generation against the suppressive effects of cytokines[6], we did not observed a similar protection by such agents in a larger series of experiments[7]. Note that Corbett and co-workers experiments[6] with inhibitors of NO synthesis were performed with a limited number of human islet preparations, and that the protection observed was just partial.

The nicotinic acid derivative nicotinamide has received considerable attention as a putative therapeutic agent against β-cell damage in early IDDM[8]. Nicotinamide protects against both streptozotocin-induced diabetes in rodents[9] and spontaneous diabetes in NOD mice[10]. The drug promotes islet regeneration following partial pancreatectomy[11] and it may prevent development of hyperglycemia in "prediabetic" pateints[12,13]. Presently, a large multicenter study is under way to test if nicotinamide can prevent the outbreak of diabetes in patients at risk to develop IDDM[8]. *In vitro* experiments indicate that high concentrations of nicotinamide block rodent islet cell lysis induced by

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macrophages[14], partially protect rat islets against suppressive effects of IL-1[15,16] and inhibit cytokine-induced NO production by a rat insulinoma cell line[17].

It is noteworthy that nicotinamide (10 mmol/l) partly inhibits rodent islet function *in vitro*[18], but it fails to impair human islet function[19]. This, and the above described difference between human and rodent islets in their responses to cytokines, emphasize the need for detailed studies on the effects of nicotinamide and/or cytokines on human pancreatic islets. Moreover, it is crucial that such studies are performed in well characterized human islet preparations[7,20], using enough number of different human islet preparations (i.e., islolated from different donors) to allow adequate statistical treatment of the data. In the present study, islets isolated from eight human organ donors were used to assess the effects of nicotinamide on cytokine-induced β-cell dysfunction.

MATERIALS AND METHODS

Cytokines

Human recombinant interleukin-1β (IL-1β) was kindly donated by Dr K. Hejnaes (Novo-Nordisk, Bagsvaerd, Denmark), and had a bioactivity of 50 U/ng. Human recombinant tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were provided by Dr G.R. Adolf (Ernst-Boehringer Institute, Vienna, Austria), and had a bioactivity of respectively 50 U/ng and 10 U/ng. The cytokines were tested for bioactivity as previously described[7].

Islet isolation and culture

Eight pancreata were excised from adult organ donors, transported to the Central Unit of the β-Cell Transplant (Medical Campus, Vrije Universiteit Brussels, Brussels, Belgium) and the islets were isolated as recently described[20]. The mean ± SEM age of the donors was 41 ± 5 years (range of 25–63 years; n = 8). Aliquots of the islet enriched fraction were routinely examined by electron microscopy in Brussels, showing 5.5 ± 0.7% dead cells and 2.0 ± 1.2% exocrine cells in the preparations. Light microscopical examination of immunocytochemically stained islets indicated a prevalence of 48 ± 4% insulin positive cells and 8 ± 1% glucagon positive cells. The islet insulin content was 0.235 ± 0.024 pmol insulin/ng DNA.

Following isolation and culture in Brussels (5.0 ± 0.7 days) the islets were subsequently sent by air from Brussels to Uppsala, Sweden[20]. The human islets were then cultured free-floating in groups of 100–150 islets/dish in medium RPMI 1640 containing 5.6 mmol/l glucose and supplemented with 10% fetal calf serum, and the medium was changed every 2 days[20]. After 5–6 days in culture, the medium was supplemented with a combination of human cytokines (IFN-γ, 1000 U/ml; TNF-α, 1000 U/ml; IL-1β, 50 U/ml) and/or nicotinamide (10 or 20 mmol/l) and the culture was continued for 6 days. This time course was selected in light of our previous data[7] showing that cytokine-induced human islet suppression only occurs after several days of exposure. Medium was changed after 3 days, and fresh cytokines were added to the culture.

Islet insulin release, DNA and insulin and NAD + NADH contents, and nitrite determination

Following 6 days exposure in culture to cytokines and/or nicotinamide, samples from the medium were taken for assessment of insulin by RIA[20] or nitrite concentrations[7]. The inter-assay variation of the RIA is 7% and the intra-assay variation is 4%. The detection limit for nitrite in our assay is 5 pmol/80 μl. Islets were then recovered and utilized for determination of acute glucose-stimulated insulin release and insulin and DNA contents[20]. In brief, triplicate groups of 7–10 islets each were incubated in sealed glass vials containing 0.25 ml Krebs-Ringer bicarbonate buffer supplemented with 10 mmol/l HEPES and 2 mg/ml BSA (KRBH). During the first hour of incubation at 37°C ($O_2 : CO_2$; 95 : 5) the KRBH contained 1.7 mmol/l glucose. The buffer was then carefully removed and replaced by KRBH containing 16.7 mmol/l glucose, and the islets were incubated for another hour. In some experiments, groups of 50–100 islets were incubated for 60 min in plastic tubes containing 100 μl KRBH and then retrieved for NAD + NADH determination[21].

Statistical analysis

Data are presented as means ± SEM, and groups of data were analyzed by ANOVA, followed either by Fisher's test or by Student's paired t-test. When the t-test was used, P values were corrected for multiple comparisons using the Bonferroni method[22]. In all experimental series each islet preparation (i.e., islets obtained from one donor) was considered as one individual observation. When experiments were performed in triplicate, a mean was calculated and considered as one separate observation. Coefficients of correlation (r) were obtained by linear regression. Considering the limitations in obtaining adequate numbers of human islet preparations to perform correlation analysis, in some of these calculations we pooled the present data with previous observations, performed in a similar way[7].

RESULTS

Islet recovery (i.e. % islets remaining in culture) following 6-days exposure to the different combinations of cytokines was > 90% in all experimental groups, and this was not affected by the presence of cytokines and/or nicotinamide (data not shown). Cytokine exposure induced a 25% decrease in islet DNA content ($P < 0.05$), and this effect was not counteracted by nicotinamide (Table 1). A decrease in islet DNA content may indicate islet cell death, and thus toxic effects by cytokines on human islets. To evaluate if this was the case in a larger series of experiments, we pooled the present data (Table 1) with our previous observations on the effects of combinations of cytokines on human islets[7]. The DNA content (ng/10 islets) were 403 ± 33 ($n = 17$) in the control group, and 320 ± 41 ($n = 17$; $P < 0.05$) in the cytokine-exposed islets, confirming that combinations of cytokines induce a loss of $\approx 20\%$ of islet cells. In this combined series we observed a significant correlation between cytokine-induced decrease in islet DNA and insulin content ($P < 0.001$; $r = 0.73$), suggesting that at least part of the cell loss may be due to β-cell death. Considering that these data were obtained from two separate series of observations (ref. 7 and present data), these conclusions must be interpreted with caution.

Islet insulin content was decreased by 90% in the cytokine-treated group ($P < 0.001$ when compared to the "None" group; Table 1), and this effect was partially counteracted by 10 or 20 mmol/l nicotinamide (Table 1). However, the insulin content of islets exposed to cytokines in the presence of nicotinamide remained significantly lower ($P < 0.001$) than the values observed in the non-cytokine exposed corresponding controls. Nicotinamide by itself did not affect islet DNA or insulin content. Islet NAD + NADH content was not affected by prolonged exposure to cytokines (Table 1). Nicotinamide induced a trend ($P < 0.1$) for an increase in islet NAD + NADH content, which was independent of the concomitant presence of cytokines.

As positive controls, in parallel experiments rat pancreatic islets were acutely exposed to streptozotocin (0.55 mmol/l), and presented a 50% decrease in islet NAD + NADH content ($P < 0.05$; data not shown), as previously described[21].

The cytokine combination induced an almost 6-fold increase in medium nitrite accumulation over the 6-days of exposure ($P < 0.001$ when compared to the "None" group; Table 1). The stimulatory effect of cytokines on nitrite accumulation was similar between days 1–3 and 4–6 of culture (data not shown); the data shown in Table 1 are therefore presented as the sum of days 1–3 + 4–6. Both concentrations of nicotinamide partially prevented cytokine-induced NO production (Table 1), but the production of nitrite by these islets was still higher than that observed in the respective controls ($P < 0.05$). Interestingly, when pooling the present and previous data[7], we observed a trend for a correlation between cytokine-induced NO increase and decrease in islet DNA content ($n = 17$; $P = 0.056$; $r = 0.52$).

As previously observed[7], there was a 4-fold increase in medium insulin accumulation ($P < 0.05$) during the first three days of culture in the presence of cytokines (Table 2). Nicotinamide neither modified medium insulin accumulation nor prevented the early stimulatory effects of cytokines. During the last three days of observation the medium insulin accumulation returned to basal levels in the cytokine-treated group.

Glucose (16.7 mmol/l)-induced insulin release was inhibited by 90% by the combination of cytokines ($P < 0.001$; Table 2). Moreover, while control islets presented a 5-fold increase in insulin release in response to 16.7 mmol/l glucose, as compared to release at 1.7 mmol/l glucose, cytokine-treated islets barely responded to 16.7 mmol/l glucose. Nicotinamide did not affect the basal or glucose-induced insulin release (Table 1), but the drug partially prevented the suppressive effects of cytokines ($P < 0.05$ vs Cyt group and $P < 0.01$ vs. respective control groups).

Finally, we evaluated possible correlations between

Table 1 Effects of 6 days exposure to cytokines and/or nicotinamide on insulin and DNA contents and nitrite production of human islets

Treatment	Insulin content (pmol/10 islet)	DNA content (ng/10 islets)	NAD + NADH content (pmol/10 islets)	Medium nitrite (pmol/islet × 6 d)
None	224 ± 33	402 ± 34	4.6 ± 1.5	20 ± 4
Nic 10	253 ± 23	393 ± 51	6.6 ± 1.7	$5 \pm 2^{*}$
Nic 20	227 ± 28	368 ± 55	8.7 ± 1.1	18 ± 4
Cyt	24 ± 5	303 ± 48	4.4 ± 1.0	115 ± 17^{b}
Cyt + Nic 10	32 ± 5^{b}	330 ± 48	7.0 ± 1.5	$62 \pm 12^{**b}$
Cyt + Nic 20	$56 \pm 10^{*b}$	282 ± 36	6.7 ± 1.8	$51 \pm 13^{**a}$

Groups of islets were isolated and exposed to cytokines (Cyt; IL-1β; 50 U/ml + TNF-α; 1000 U/ml + IFN-γ; 1000 U/ml) and/or nicotinamide (Nic; 10 or 20 mmol/l) for 6 days. The results are means ± SEM of 7–8 experiments $^{*}P < 0.05$; $^{**}P < 0.001$ when comparing Nic (10 or 20) vs. None, or Cyt + Nic (10 or 20) vs. Cyt; $^{a}P < 0.05$; $^{b}P < 0.001$ when comparing Cyt or Cyt + Nic (10 or 20) vs respective controls (i.e. None, Nic 10 or Nic 20).

Table 2 Effects of 6 days exposure to cytokines and/or nicotinamide on medum insulin accumulation and acute glucose-induced insulin release of human islets

Treatment	Medium insulin (pmol/10 islets × 3 days)		Acute insulin release (pmol/10 islets × 1 h)	
	Days 1–3	Days 4–6	1.7 mmol/l glucose	16.7 mmol/l glucose
None	63 ± 13	64 ± 17	2.7 ± 0.6	14.7 ± 2.2
Nic 10	44 ± 11	33 ± 13	1.8 ± 0.6	16.1 ± 2.3
Nic 20	60 ± 21	26 ± 6	1.8 ± 0.4	14.2 ± 2.8
Cyt	236 ± 40[a]	53 ± 17	1.2 ± 0.3	1.7 ± 0.3[c]
Cyt + Nic 10	288 ± 57[b]	63 ± 16	1.8 ± 0.4	3.3 ± 0.6**[C]
Cyt + Nic 20	346 ± 93[c]	89 ± 22	2.1 ± 0.4	7.2 ± 0.8**[b]

Groups of islets were isolated and exposed to cytokines (Cyt; IL-1β; 50 U/ml + TNF-α; 1000 U/ml + IFN-γ; 1000 U/ml) and/or nicotinamide (Nic; 10 or 20 mmol/l) for 6 days. Media were collected for insulin determinations at 72-h intervals. For acute insulin release, islets were incubated in triplicate groups of 10 in KRBH buffer and sequentially exposed to 1.7 or 16.7 mmol/l glucose. The results are means ± SEM of 7–8 experiments. *$P < 0.05$; **$P < 0.02$ when comparing Cyt + Nic (10 or 20) vs. Cyt; [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$ when comparing Cyt or Cyt + Nic (10 or 20) vs respective controls (i.e., None, Nic 10 or Nic 20).

gender or age of the islet donors and effects of cytokines on medium nitrite, islet DNA and insulin content and insulin release in response to glucose. In this small series (n = 17) we did not find any significant effect of age or gender of the donor on cytokine effects. The only exception was a trend for positive correlation between age and nitrite production ($P = 0.063$; $r = 0.51$). To further evaluate this issue, the donors were divided in two groups: donors < 40 years (age: 26 ± 2 years; n = 11) and donors > 40 years (age: 52 ± 3 years; n = 6). Islets obtained from the older donors showed higher levels of nitrite in the medium (pmol/islet × 6 days; 127 ± 16) than islets obtained from the younger donors (77 ± 14; $P < 0.05$).

DISCUSSION

The present data confirm previous observations that combinations of cytokines induce NO production and β-cell dysfunction in human pancreatic islets *in vitro*[6,7]. Moreover, it shows that cytokines may also induce human islet cell death, as judged by the observed decrease in islet DNA content. The suppressive effects of cytokines are characterized by a profound decrease in islet insulin content and insulin release in response to glucose, and does not seem to be affected by age and sex of the islet donor.

The observed discrepancies between cytokine effects on rodent[1–4] and human pancreatic islets (5–7, present data) make it important to emphasise some methodological points regarding the human islet preparations. The β-cell content in the presently used human preparations varies between 45–60%, as compared to 60–75% in rodent islets. In the different human islet preparations 15–30% of the cells can not be properly identified, and a large fraction of these are probably severely degranulated endocrine cells. Note that studies from other groups which report "islet purity of 90–95%" base this affirmation only on dithizone staining, without performing detailed morphological examination. Although dithizone staining can be helpful during their procedure of islet isolation, it does not provide reliable information on the actual number of β-cells per islet (even fetal porcine islet preparations, containing as little as 3–5% β-cells stain positively with dithizone (Sandler *et al.*, unpublished data) and could thus be considered as a "very pure preparation"). A second relevant methodological issue is that transfer of isolated human pancreatic islets by air does not induce significant changes in human islet cell survival nor islet cell composition, as judged by islet morphology, function, insulin and DNA content determinations (20; Eizirik, unpublished data). Moreover, when similar experiments regarding human islet sensitivity to toxic agents were performed in Brussels (i.e. without transport) and in Uppsala (after transport), similar results were observed[23]. Finally, we observed equal stimulatory effects of IL-1 on human islets[5] in the presence of 1% or 10% fetal calf serum (FCS) (Eizirik, unpublished data). Furthermore, concentrations of cytokines (IL-1β + TNF-α + IFN-γ) similar to the ones used in the present study induced severe toxicity to mouse pancreatic islets after 48 h in the presence of 10% FCS[24].

It is noteworthy that human pancreatic islets show nitrite production even in the absence of cytokines (7 and present data). This suggest that, as described for rat pancreatic islets[25], human islets also express a constitutive form of NOS. However, the present observation that 10 mmol/l nicotinamide reduced basal nitrite production by 75% without affecting islet insulin content or insulin release, suggest that constitutive NOS is not essential for human islet insulin synthesis and release. The finding that 20 mmol/l nicotinamide failed to affect basal nitrite production is unexpected, and further dose-response studies might be necessary to clarify this issue.

Nicotinamide partially prevented cytokine-induced

NO production by human pancreatic islets. Similar observations were made in mouse fibroblasts[26], rat pancreatic islets[16] and in a rat insulinoma cell line (RINm5F)[17], but to our knowledge this is the first report of such an effect in human tissue. NO may be a mediator of β-cell damage in IDDM[4,27], and potential sources of NO in this context are the β-cells themselves[28], stationary and infiltrating macrophages and endothelial cells[27]. If the presently observed inhibitory effect of nicotinamide on NO production can be reproduced in human macrophages and endothelial cells, it can help to explain some of the beneficial effects of the drug on human and experimental IDDM[12,13]. The mechanisms behind nicotinamide-induced inhibition of NO production remains unclear. Recent data obtained in RINm5F cells suggest that lower concentrations of the drug (10–20 mmol/l) prevent NO accumulation by posttranscriptional mechanisms, while higher concentrations of nicotinamide (50 mmol/l) also inhibits iNOS mRNA expression[17].

Besides inhibiting NO production, nicotinamide also partially protected human islets against cytokine-induced decrease in islet insulin content and glucose-induced insulin release. As previously described for 10 mmol/l nicotinamide[19], 10 or 20 mmol/l of the drug did not modify the function of control islets. The beneficial effects of nicotinamide could be due to: *i*: inhibition of NO synthesis (see above); *ii*: inhibition of the enzyme poly(ADP-ribose) polymerase (PARP), and consequent prevention of cellular NAD depletion[29]; *iii*: scavenging of oxygen free radicals[30]. Our previous observations that inhibitors of NO production do not protect human islets against cytokines[7] argue against nicotinamide-induced inhibition of NO production as a single explanation for the present findings. Previous data suggest that effective inhibition of PARP is already achieved at low concentrations of nicotinamide (1 mmol/l or less)[30,31]. Since 10 mmol/l nicotinamide induced only a minor protection against cytokines (present data), it is unlikely that inhibition of PARP plays a major role in this context. However, it can not be excluded that cytokine-induced β-cell dysfunction affected nicotinamide uptake. Finally, the present observation that cytokines did not decrease NAD + NADH content argues against PARP activation as a primary mechanism for cytokine-induced human β-cell dysfunction.

High concentrations of nicotinamide (> 10 mmol/l) can scavenge toxic oxygen free radicals[30], and it has been shown that oxygen free radical scavengers protect rodent islet cells against combinations of cytokines[32]. Interestingly, NO may interact with the oxygen radical superoxide, forming the highly toxic radical peroxynitrite[33]. Since nicotinamide may prevent both cytokine-induced NO formation (present data) and production of oxygen free radicals[30], it is conceivable that the combination of these two effects may explain the beneficial effects of the drug against cytokine-induced impairment of β-cell function. Considering that nicotinamide is presently being tested as a therapeutic agent in early IDDM[8,12,13], it is crucial to understand the cellular mechanisms by which this agent may prevent human β-cell damage. Although the present scarcity of human material prevent us to perform all the experiments needed to clarify this issue, the present findings of a partial protective action of nicotinamide against cytokines may provide the much needed model to solve this problem under controlled *in vitro* conditions.

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