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Biochimica et Biophysica Acta 1768 (2007) 2222–2234
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbamem

Cell-permeable peptides induce dose- and length-dependent cytotoxic effects

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Received 28 December 2006; received in revised form 4 June 2007; accepted 5 June 2007
Available online 14 June 2007

Abstract

We have explored the threshold of tolerance of three unrelated cell types to treatments with potential cytoprotective peptides bound to $Tat_{48–57}$ and $Antp_{43–58}$ cell-permeable peptide carriers. Both $Tat_{48–57}$ and $Antp_{43–58}$ are well known for their good efficacy at crossing membranes of different cell types, their overall low toxicity, and their absence of leakage once internalised. Here, we show that concentrations of up to 100 μM of $Tat_{48–57}$ were essentially harmless in all cells tested, whereas $Antp_{43–58}$ was significantly more toxic. Moreover, all peptides bound to $Tat_{48–57}$ and $Antp_{43–58}$ triggered significant and length-dependent cytotoxicity when used at concentrations above 10 μM in all but one cell types (208F rat fibroblasts), irrespective of the sequence of the cargo. Absence of cytotoxicity in 208F fibroblasts correlated with poor intracellular peptide uptake, as monitored by confocal laser scanning fluorescence microscopy. Our data further suggest that the onset of cytotoxicity correlates with the activation of two intracellular stress signalling pathways, namely those involving JNK, and to a lesser extent p38 mitogen-activated protein kinases. These responses are of particular concern for cells that are especially sensitive to the activation of stress kinases. Collectively, these results indicate that in order to avoid unwanted and unspecific cytotoxicity, effector molecules bound to $Tat_{48–57}$ should be designed with the shortest possible sequence and the highest possible affinity for their binding partners or targets, so that concentrations below 10 μM can be successfully applied to cells without harm. Considering that cytotoxicity associated to $Tat_{48–57}$- and $Antp_{43–58}$ bound peptide conjugates was not restricted to a particular type of cells, our data provide a general framework for the design of cell-penetrating peptides that may apply to broader uses of intracellular peptide and drug delivery.

Keywords: Cell-penetrating peptide; Tat; Intracellular delivery; Pancreatic beta-cell; HeLa cell

1. Introduction

Cell-permeable peptides (CPP) have emerged as attractive drug delivery tools. Transplantation of cells and tissues, and in particular transplantation of pancreatic islets may greatly benefit from the transducing properties of CPP as these may facilitate the uptake of otherwise non-permeable cytoprotective molecules such as peptides, proteins, or chemical compounds. CPP may thus contribute to the overall improvement of donor cells and tissues, both in yield and in quality.

Pancreatic islet transplantation has shown promise to restore glucose homeostasis in type 1 diabetic patients [1,2]. Yet, its effective success is limited by the poor availability of donors and by the significant loss of donor islets during the early steps of purification and immediately following grafting. Typically, up to 50% of the infused islet mass is destroyed by apoptosis and non-immune inflammatory responses in the early days post-transplantation. Because this process may ultimately lead to early graft failure, it is one of the critical factors that impinges on the success of islet transplantation. Furthermore, the minimal number of functional islets required for a successful graft, i.e. ca 30–50% of the mean islet content of an intact pancreas, is difficult to retrieve from a single donor,

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0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.06.003

making transplants from multiple donors a prerequisite to successful grafting [1,3].

Because of its short length, its good efficacy at crossing cell membranes of different cell types, its overall low toxicity [4], and the fact that it does not leak out from cells once internalised [5], $Tat_{48–57}$ cell-permeable peptide has generated a lot of attention for intracellular delivery of peptides, proteins, or chemical compounds [6–10]. Several studies have further shown that it could efficiently deliver peptides and proteins into insulin-secreting cells without obvious changes in biological function, either in culture, in isolated islets, or in a whole pancreas [11–13]. Moreover, retro-inverso (D-) enantiomers of Tat-conjugated peptides might provide molecules with longer-lasting biological activities, presumably because of their better resistance against proteases [11].

$Tat_{48–57}$-bound anti-apoptotic peptides are of particular interest for the field of pancreatic islet transplantation because such inhibitors could be specifically delivered to donor islets before grafting and retain their activity as long as they are not degraded by proteolysis or inactivated by other processes. In other words, they would help donor islets resist the first wave of pro-apoptotic signals without affecting the recipient's tissues.

At least 5 cell-permeable proteins or peptides have shown promising anti-apoptotic potential in insulin-secreting cells [11,14–19]. Yet, it is remarkable that whereas it seems relatively easy to block death of β-cell lines with inhibitors of either JNK, NO or NF-κB, treatments with individual peptides are unable to fully prevent human islet loss following islet isolation. The pro-apoptotic pathways that need to be blocked during the course of human islet preparation are complex [20–22], suggesting that it might be necessary to treat islets before transplantation with a cocktail of strong anti-apoptotic agents in order to succeed at preventing early graft failure.

Here, we have selected several peptides for their ability at blocking some of the main pro-apoptotic pathways induced by islet isolation and purification and/or cytokines in pancreatic β-cells, namely JNK, NF-κB, and STAT-1 [20,22,23]. We have subsequently linked these sequences to the $Tat_{48–57}$ transporter peptide, with the long-term aim of assessing their ability at protecting stressed pancreatic islets from apoptosis. Most of these conjugates triggered unexpected but significant cytotoxicity when used at concentrations above 10 μM, both in rat primary and INS-1E β-cells. In contrast, concentrations of up to 100 μM $Tat_{48–57}$ alone were essentially harmless. Interestingly, the ubiquitous $Antp_{43–59}$ CPP [24] exhibited intrinsic cytotoxic properties in the same cells, and Antp-conjugates were also more toxic than their Tat-bound counterparts. For all Tat- and Antp-peptide conjugates, cytotoxicity occurred irrespective of the sequence of the cargo. A similar trend was observed in HeLa cells, but not in 208F rat fibroblasts. Yet, the absence of cytotoxicity in the latter correlated with a reduced ability at internalising CPP.

Although the mechanisms triggering cell death have not been investigated in details, they seem to depend on the efficacy of intracellular peptide uptake, as well as on the length of the peptidic cargo. Furthermore, they may involve the activation of intracellular stress signalling pathways. These responses are of particular concern for highly sensitive cells like pancreatic β-cells.

Our results therefore indicate that in order to avoid unwanted and unspecific cytotoxicity, effector molecules bound to $Tat_{48–57}$ or $Antp_{43–58}$ transporters should be designed with the shortest possible sequence and the highest possible affinity for their intracellular binding partners, substrates, or targets. These data may provide a framework for the design of cell-penetrating conjugates, not only in the field of pancreatic islet transplantation, but also in broader applications of intracellular peptide and drug delivery.

2. Experimental procedures

2.1. Peptides

Peptides are listed in Table 1. Unless indicated otherwise, they were synthesised manually on methoxybenzhydrylamine (MBHA) resin (Novabiochem, Merck) by Nα-butyloxy (Boc) solid phase peptide synthesis (SPPS) [25]. Couplings were monitored by the trinitrobenzenesulfonic acid (TNBSA) colour test (Fluka). Peptides were subsequently cleaved and simultaneously deprotected from the resin by treatment with 90% hydrofluoridric acid (HF) and appropriate scavengers for 1 h at 0 °C [25]. Crude peptides were taken up in a 20% aqueous acetic acid solution, diluted to 5% acetic acid with distilled water and remaining scavengers were extracted by a diethylether wash. Peptides were further lyophilised to remove acetic acid, purified by preparative reverse phase HPLC on an Atlantis dC_{18} column (Waters) in 10–60% buffer B (0.1% trifluoroacetic acid (TFA) in acetonitrile) over 45 min at 15 ml/min and further characterised by ESI mass spectrometry (Thermo LTQ, Finnigan). Phosphorylated STAT1i peptide was synthesised manually on NovaSyn TGR resin (Novabiochem, Merck) by standard Nα-fluorenylmethoxycarbonyl (Fmoc) SPPS [26] and using preformed protected phospho-tyrosine [27]. Coupling reactions were monitored by the ninhydrin colour test (Kaiser test kit, Fluka) using a 10-fold excess of Fmoc-amino acid activated with a mixture of uronium-based hydroxybenzotriazol coupling reagents in the presence of diisopropylethylamine (DIPEA). STAT1i was cleaved and simultaneously deprotected from the resin by treatment with TFA/triisopropylsilane/water (95:2.5:2.5). The crude peptide was further extracted with diethylether after concentration of the cleavage mixture, lyophilised, purified by preparative reverse phase HPLC as above in 5–50% buffer B over 45 min at 15 ml/min, and characterised by ESI mass spectrometry. Antp, $dJBD_{19}$, $dJBD_{19}$-mut, JBD_{19}, NBD, NBD_{scr}, NBD_{scr}-Tat, and dJNKi were custom-synthesised by Fmoc-SPPS followed by TFA → acetate salt exchange after reverse phase HPLC purification (NeoMPS, France). FITC-labelled dJNKi was custom prepared by Auspep, Australia. Tat-BH4-Bcl-X_L was purchased from Calbiochem. All stock solutions of peptides were prepared in water except NBD, NBD_{scr}, Antp, and Antp-conjugates that were resuspended in the presence of DMSO. The maximal concentration of DMSO that was added to cells does not affect INS-1E, HeLa, or 208F cell viability (data not shown and [28]).

2.2. Rat primary β-cells

Rat pancreatic islets were isolated from adult Wistar rats by collagenase digestion and primary β-cells were purified by auto-fluorescence-activated cell sorting (FACS, FACStar, Becton-Dickinson and Co., Sunnyvale, CA, USA) [29,30]. FACS-purified β-cell preparations, ca 90% pure [30,31] or whole rat islets, were cultured in HAM's F-10 and RPMI-1640 mediums, respectively [31,32].

2.3. Cell culture

Rat insulin-secreting INS-1E cells [33], kindly provided by Prof. C. Wollheim (University Medical Centre, Geneva, Switzerland), were cultured in RPMI-1640 medium with Glutamax I (Gibco) supplemented with 5% foetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM 2-

Table 1
Peptides

Name	Amino-acid sequence	Length (aa)	Net charge	References
dTat	dRdRdRdQdRdRdKdKdRG	10	+8	[11]
L-Tat	GRKKRRQRRR	10	+8	[6]
Antp	RQIKIWFQNRRMKWKK	16	+7	
$dJBD_{19}$	dDdQdSdRdPdVdQdPdFdLdNdLdTdTdPdRdKdPdR	19	+3	[11]
$dJBD_{19}$-mut	dDdQdSdRdPdVdQdPdFdAdNdAdAdAdAdKdPdR	19	+3	[38]
JBD_{19}	RPKRPTTLNLFPQVPRSQD	19	+3	[11]
NBD	TALDWSWLQTE	11	−2	[34]
NBD_{scr}	WELDTSTQLWA	11	−2	this work
β-arrestin 2	HFLMSDRRSLHLEASLDKEL	20	−1	[44]
dJNKi	dDdQdSdRdPdVdQdPdFdLdNdLdTdTdPdRdKdPdR·***dPdP***·dR dRdRdQdRdRdKdKdRG	31	+11	[11,38]
dJNKi-mut	dDdQdSdRdPdVdQdPdFdAdNdAdAdAdAdKdPdR·***dPdP***·dR dRdRdQdRdRdKdKdRG	31	+10	[38]
dTat-βarr2	dRdRdRdQdRdRdKdKdR·***GG***·HFLMSDRRSLHLEASLDKEL	31	+7	[44]
L-JNKi	GRKKRRQRRR·***PP***·RPKRPTTLNLFPQVPRSQD	31	+11	[11,38]
Tat-BH4-Bcl-X_L	GRKKRRQRRR-βA-SNRELVVDFLSYKLSQKGYS	31	+9	[41–43]
STAT1i	KGTG[Y*]IKTELGGRKKRRQRRR	21	+9	[35]
STAT1 control	KGTGYIKTELGGRKKRRQRRR	21	+9	[35]
NBD-Tat	TALDWSWLQTE·***G***·GRKKRRQRRR	22	+6	[34]
NBD_{scr}-Tat	WELDTSTQLWA ***G***·GRKKRRQRRR	22	+6	this work
Tat-NBD_{scr}	GRKKRRQRRR·***G***·WELDTSTQLWA	23	+6	this work
Tat-RDM	GRKKRRQRRR·***GG***·AVFRFIPRHPDELELDVDD	31	+4	this work
Antp-JNKi	DRQIKIWFQNRRMKWKK·***PP***·RPKRPTTLNLFPQVPRSQD	38	+9	this work and [11]
Antp-NBD	DRQIKIWFQNRRMKWKKTALDWSWLQTE	28	+4	[34,39,40]

dTat, L-Tat, and Antp are peptide carriers; $dJBD_{19}$, $dJBD_{19}$-mut, JBD_{19}, NBD, NBD_{scr}, and β-arrestin 2 are non permeable cargos corresponding to, respectively, (i) dJNKi, (ii) dJNKi-mut, (iii) L-JNKi and Antp-JNKi, (iv) NBD-Tat and Antp-NBD, (v) NBD_{scr}-Tat and Tat-NBD_{scr}, and (vi) dTat-βarr2 conjugated peptides. Tat-BH4-Bcl-X_L, STAT1i, STAT1 control, and Tat-RDM have been used in Tat-conjugated forms only. For CPP-conjugates, the transport sequence is underlined. dX residues indicate retro-inverso (D-) amino acids; [Y*] stands for phosphorylated tyrosine; 1- or 2 aa-long linkers between peptide carrier and cargo are shown in bold and italic letters.

mercaptoethanol. HeLa cells were cultured in RPMI-1640 medium with Glutamax I medium supplemented with 10% foetal bovine serum and 1 mM sodium pyruvate. 208F rat fibroblasts (ECACC, Salisbury, UK) were cultured in DMEM medium with Glutamax I and 4.5 g/l glucose (Gibco) and supplemented with 10% foetal bovine serum.

2.4. Cytotoxicity

Adherent FACS-purified rat β-cells (10,000 cells/condition) and rat fibroblasts (6000 cells/condition) were cultured in 96-well dishes. Rat islets (10 islets/condition) were cultured free-floating in 96-well dishes. Adherent INS-1E (30–40,000 cells/condition) and HeLa (20–30,000 cells/condition) cells were cultured in 3 cm tissue culture plates. Primary β-cells and rat islets were exposed to cell-permeable peptides (see Table 1) for 24 h. INS-1E cells, HeLa cells, and rat fibroblasts were exposed to the peptides for 48 h. The percentage of dead cells was determined by inverted fluorescence microscopy using the DNA dyes Hoechst 342 (20 μg/ml) and propidium iodide (PI; 10 μg/ml) [11]. A minimum of 500 cells was counted in duplicate for each experiment.

2.5. Cell peptide uptake and confocal laser scanning microscopy

5 μM of FITC-labelled dJNKi peptide was added to the culture medium of 40–50%-confluent cells for 1 h at 37 °C. Cells were rinsed three times in PBS and fed with fresh medium. Peptide internalisation was monitored by fluorescence in live cells using a Leica SP5 AOBS confocal laser scanning microscope equipped with 10 and 63× water submersible objectives. Excitation was performed at 488 nm and emission was detected between 500 and 650 nm. Free FITC was used as a control for background signals.

2.6. MAPK assays and immunoblotting

Cellular extracts were prepared by scraping control and CPP-treated cells in lysis buffer containing 20 mM Tris-acetate, 0.27 M sucrose, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 10 mM p-nitrophenyl-phosphate, and 1× antiprotease mix (Roche). Insoluble material was removed by a 5-min centrifugation at 14000 rpm and protein contents were measured by the Bradford procedure (Bio-Rad).

For JNK and ERK solid phase activity tests, 10 μg of protein extracts were incubated for 2 h at 4 °C in the presence of 1 μg of either glutathione S-transferase (GST)-$cJun_{1-79}$ or GST-Elk1 beads as respective JNK- and ERK-specific substrates. Beads were subsequently washed 3 times in scraping buffer containing 0.1% Triton X-100 and rinsed twice in a buffer containing 20 mM HEPES pH 7.5, 20 mM β-glycerophosphate, 10 mM $MgCl_2$, and 1 mM DTT. Kinase reactions were initiated by the addition of 5 μCi 5′-[γ-^{33}P]ATP (3000 Ci/mmol, Amersham Biosciences) and incubated for 30 min at 30 °C. Reaction products were resolved by 10–12% SDS-PAGE analysis, gels were dried, and phosphorylation signals were analysed by autoradiography (Kodak) and phosphorimaging using an Instant Imager system.

p38 MAPK phosphorylation levels were assessed by western blotting. 20 μg of total proteins were separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in 1× TBS (pH 7.6), 0.1% Tween 20, 5% non-fat dry milk, transferred into in 1× TBS, 0.1% Tween 20, 4% non-fat dry milk, and probed overnight at 4 °C with 1:2000 or 1:1000 respective dilutions of anti-phospho-p38 MAPK or anti-p38 MAPK antibodies (Cell Signaling). After 4 to 5 washes in 1× TBS, 0.1% Tween 20, membranes were further exposed for 1 h at room temperature to a 1:3500 dilution of horseradish peroxidase-linked secondary anti-rabbit antibody (Cell Signaling), and washed again in TBS-Tween buffer. Peroxidase activity was detected by enhanced chemiluminescence (Amersham Biosciences).

3. Results

3.1. Tat_{48-57}-bound cell-permeable peptides induce cytotoxicity in primary pancreatic β-cells

We have selected peptides to block three of the main pro-apoptotic pathways induced by islet isolation and purification

and/or cytokines in pancreatic β-cells [20–22] namely, JNK (L- and dJNKi peptides [11]), NF-κB (NEMO-binding domain (NBD) peptide [34]) and STAT-1 (STAT1i [35]). These peptides were linked to the $Tat_{48–57}$ transporter sequence of HIV-1 virus [36]. As controls, we prepared L- and D (retro-inverso)-forms of the $Tat_{48–57}$ sequence alone, a scrambled NBD sequence bound to $Tat_{48–57}$ in two orientations, as well as non-permeable NBD and NBD_{scr} cargos (Table 1). As pancreatic β-cells are known for their marked sensitivity to a variety of compounds such as transfecting agents (data not shown), we first determined the threshold of cytotoxicity induced by these peptides in primary β-cells (Fig. 1). Briefly, FACS-purified primary rat β-cells were exposed to increasing doses of peptides for 24 h and cytotoxicity was measured by counting the percentage of Hoechst/PI-positive cells under a fluorescence microscope. As observed in Fig. 1, the basal level of primary β-cell death was ca. 11%. Exposure to up to 50 μM of either the carriers alone (i.e. L- or dTat) or the non permeable cargos (i.e. NBD and NBD_{scr}) did not induce any cytotoxicity. In contrast, L-JNKi, NBD-Tat, and NBD_{scr}-Tat induced significant cytotoxicity at concentrations starting from 10 μM and all Tat-conjugates were toxic at 50 μM. At least for NBD-Tat, cytotoxicity does not seem to be related to its inhibitory function since NBD_{scr}-Tat and Tat-NBD_{scr} control peptides were both toxic. In these peptides, the position of the carrier does not seem to affect peptide toxicity in a significant fashion.

Similarly to what was observed in primary β-cells, exposure of rat islets to 50 μM of either NBD-Tat or NBD_{scr}-Tat induced significant cytotoxicity (control 17±5, NBD-Tat 29±10, Tat-NBD_{scr} 61±4% of dead cells; data are means±SEM, n=2–5), whereas Tat transporters alone were harmless (data not shown). Clear signs of cytotoxicity were also seen in human islets exposed to 30 and 100 μM dJNKi for 24 h, but in this case, dTat induced some cytotoxicity at 50 μM (data not shown and [37]). Taken together, these results show that pancreatic islets and β-cells are highly sensitive to cell-permeable peptide conjugates at concentrations above 10 μM.

3.2. $Tat_{48–57}$- and $Antp_{43–58}$-bound cell-permeable peptides induce cytotoxicity in INS-1E and HeLa cells

Due to the difficulties in obtaining primary β-cells, we further investigated CPP-induced cytotoxicity in the well differentiated INS-1E pancreatic cell line [33]. Several peptides were used for these experiments (Table 1): L- and $dTat_{48–57}$ carriers as above, non-permeable JBD_{19}, $dJBD_{19}$, $dJBD_{19}$-mut, NBD, NBD_{scr}, and β-arrestin-2 (βarr2) peptides as controls, and a series of $Tat_{48–57}$-bound peptide conjugates. The latter include IB1/JIP-1-based inhibitors of the JNK pathway (i.e. L- and dJNKi [11]), a non-inhibitory dJNKi-mut peptide [38], inhibitors of the NF-κB (i.e. NBD-Tat and NBD_{scr}-Tat as a non-functional control [34,39,40]) and STAT-1 pathways (i.e. phosphorylated STAT1i and non-phosphorylated STAT1 control [35]), a Bcl-X_L blocker (i.e. Tat-BH4-Bcl-X_L [41–43]), a putative inhibitor of JNK3 (i.e. dTat-βarr2 [44]) and an unrelated Tat-RDM control peptide. To assess whether cytotoxicity was specific of the Tat system, we also tested $Antp_{43–58}$ peptide carrier [24] and two conjugates, Antp-JNKi and Antp-NBD [34,39,40]. Both JNKi and Bcl-X_L inhibitors have been shown previously to promote cytoprotection against IL-1β-induced apoptosis in pancreatic β-cells at concentrations ranging between 0.3 and 1 μM [11,18].

Cytotoxicity was measured 48 h after peptide addition, as described for primary cells. Cell death reached 2.3% on average in untreated INS-1E cells (Fig. 2A). This percentage was not significantly increased in the presence of up to 100 μM of either L- or dTat, which is in agreement with previous observations of poor Tat-associated cellular toxicity [4]. Concentrations of 100 μM of non-permeable peptides were also harmless. In contrast, exposure to Tat-conjugates led to severe cytotoxic effects at concentrations above 10 to 30 μM for all peptides tested except for dJNKi-mut, that only triggered mild toxicity (i.e. 7–8% cell death at 10 and 30 μM). Cytotoxicity is unlikely to be due to the inhibition of

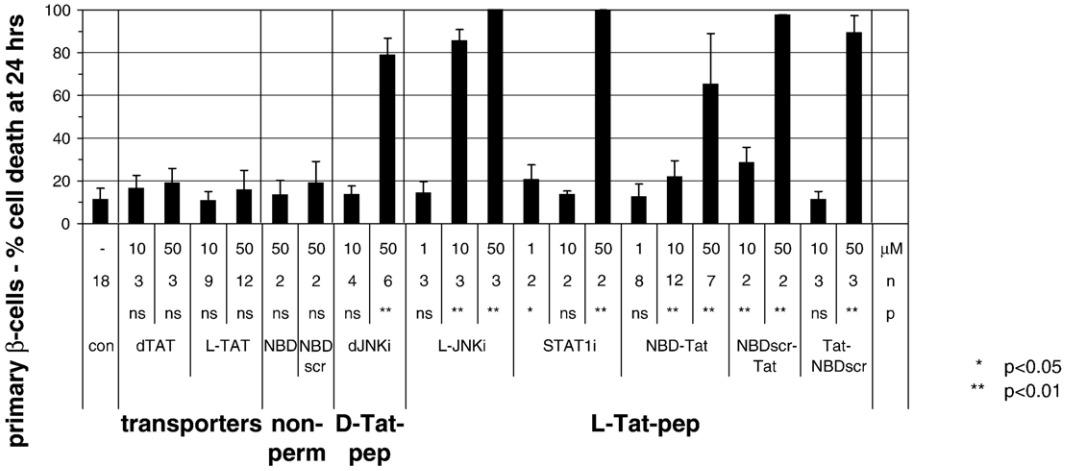

Fig. 1. CPP-induced cell death in primary pancreatic β-cells. FACS-purified rat β-cells were isolated and pre-cultured for 48 h prior to adding peptides at the indicated concentrations in the culture medium. Cells were kept in culture for another 24 h and cell death was measured by counting the percentage of Hoechst-PI-positive cells under a fluorescence microscope. Data are shown as means±SD (n=2 to 18). Non-perm: non-permeable peptides. * and **: $p \leq 0.05$ and $p \leq 0.01$ versus control, t-test; ns: non-significant.

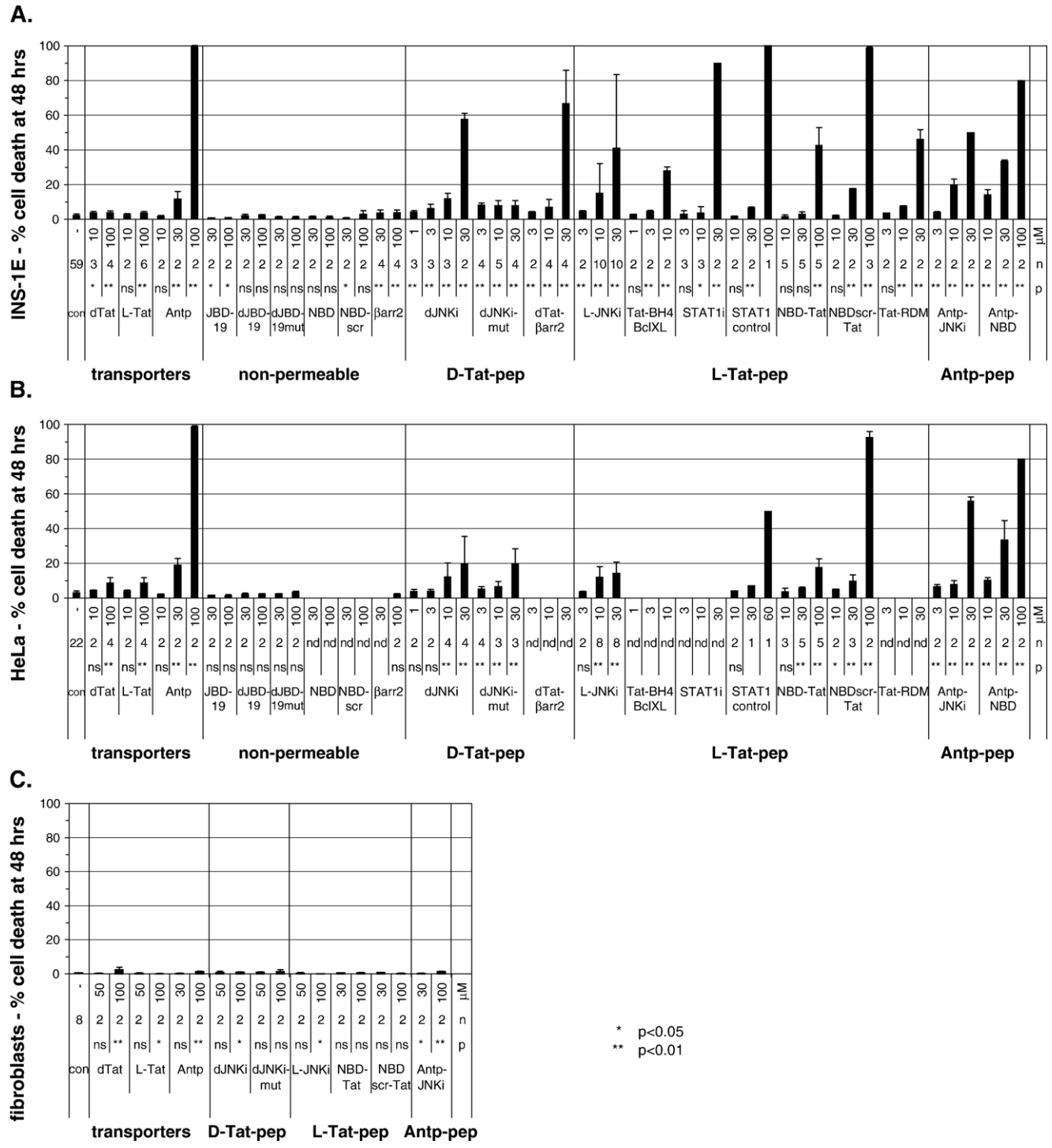

Fig. 2. CPP-induced cytotoxicity in (A) rat pancreatic INS-1E cells, (B) human HeLa cells, and (C) rat 208F fibroblasts. Peptides were added to the culture medium at the indicated concentrations and cells were cultured for 48 h at 37 °C. Cell death was measured by counting the percentage of Hoechst-PI-positive cells under a fluorescence microscope. Data are shown as means ± SD ($n = 1$ to 59). * and **: $p \leq 0.05$ and $p \leq 0.01$ versus control, *t*-test; ns: non-significant; nd: not done.

JNK, NF-κB or STAT-1 signalling cascades as NBD_{scr}-Tat, Tat-RDM and STAT1 control peptides all caused cellular damage.

Antp carrier alone was significantly more toxic that Tat, triggering cell death above 10 to 30 μM, whereas Antp-NBD and Antp-JNKi conjugates induced comparable effects above 3 to 10 μM. Thus the toxic response elicited by cell-permeable peptides in these cells is not restricted to the Tat transporter system.

Typically, toxic peptides induced the formation of intracellular vesicles in INS-1E cells, ultimately leading to cell detachment and death (Fig. 3). In cases of strong cytotoxicity (i.e. above 30 to 40%), these signs became visible as early as 2 to 6 h following peptide addition (data not shown).

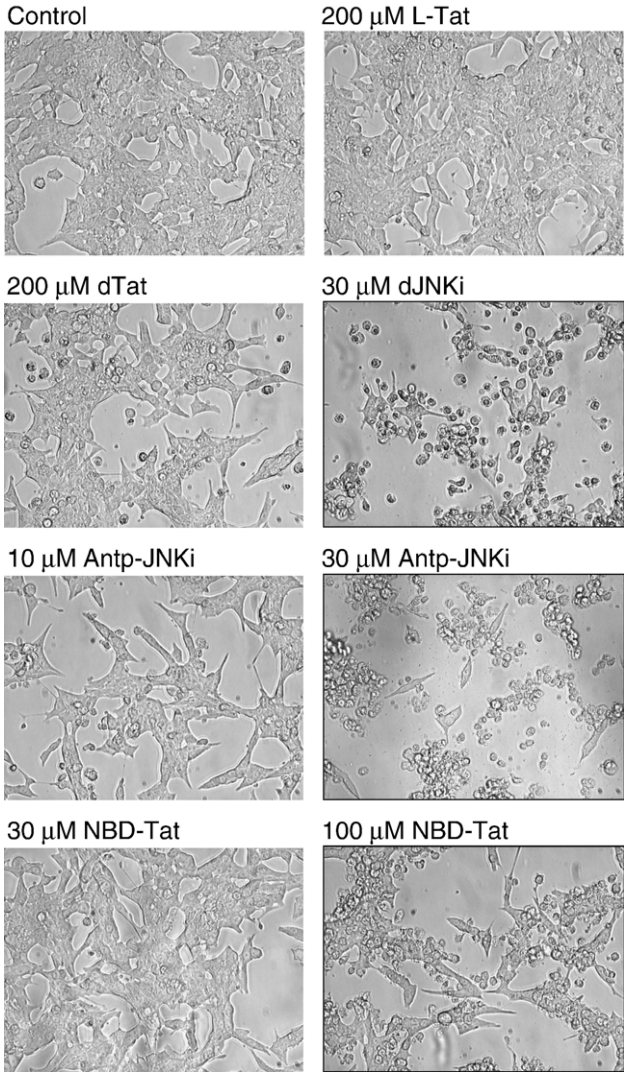

Fig. 3. CPP-induced cytotoxicity in rat pancreatic INS-1E cells. Cells were treated as in Fig. 2 and pictures were taken 48 h after incubation with the indicated peptides. Major signs of cytotoxicity can be observed in the presence of 30 μM dJNKi, 30 μM Antp-JNKi, or 100 μM NBD-Tat (boxed pictures), whereas there is limited toxic effect in the presence of 200 μM of Tat transporters alone. Pictures are representative of 4 independent experiments.

To assess whether peptide-induced toxicity was specific of β-cells, we ran similar experiments in non pancreatic human HeLa cells and rat 208F fibroblasts (Fig. 2B and C). Cytotoxicity was generally slightly reduced in HeLa compared to INS-1E cells, but followed a similar trend otherwise. L- and dTat transporters alone triggered some toxicity above 100 μM in this cell line, whereas the effects of Antp and Antp-conjugates were similar to those seen in pancreatic cells. In contrast, no cytotoxicity was observed in 208F fibroblasts, even in the presence of up to 100 μM of CPP-conjugates (Fig. 2C).

3.3. Absence of cytotoxicity correlates with poor cell-permeable peptide uptake in 208F rat fibroblasts

We further investigated whether there was a correlation between the extent of cytotoxicity and the efficacy of CPP transduction by comparing the uptake of FITC-labelled dJNKi in the 3 abovementioned cell lines. Briefly, cells were exposed to 5 μM of FITC-labelled dJNKi for 1 h at 37 °C. They were then thoroughly rinsed with PBS, fed with fresh medium and analysed by confocal laser scanning fluorescence microscopy. As shown in Fig. 4, the uptake of FITC-dJNKi was evident in both INS-1E and HeLa cells, with a percentage of fluorescent cells close to 100%. Internalised peptide was detected both in the cytoplasm and in the nucleus where it tended to accumulate into relatively large granules. Punctuate fluorescent patterns in the perinuclear space of HeLa cells (Fig. 4B) are suggestive of an accumulation within endosomal vesicles, as reported in the literature [10,45]. It is more difficult to draw conclusions for INS-1E cells as these are small, tend to grow in aggregates and exhibit limited cytoplasmic spreading. It seems nonetheless that the fluorescence of internalised dJNKi is evenly distributed throughout the cytoplasm in these cells. In contrast, we could not detect any dJNKi uptake in 208F cells. In other words, the better tolerance of 208F fibroblasts towards CPP-conjugates correlates with their higher resistance to peptide entry. Along these lines, we also noted that these fibroblasts were considerably more resistant to detergent-free proteinase K treatment than INS-1E and HeLa cells, suggesting that membrane compositions might significantly differ between these cell lines (data not shown).

3.4. Effect of cell-permeable peptides on MAPK activity

The mechanisms by which CPP-bound peptides induce cell death have not been investigated in details. Yet, most of the Hoechst/PI-positive cells exposed to peptidic conjugates displayed clear signs of apoptosis such as nuclear condensation and late fragmentation. Therefore, we sought to determine whether cytotoxicity correlated with the activation of two well-known pro-apoptotic stress signalling pathways in β-cells, namely JNK and p38 MAPK. In parallel, we also assessed ERK activity.

In pilot time-course assays performed in INS-1E and HeLa cells exposed to 30 μM of Tat-conjugates, we detected a peak of JNK activation within 2 to 6 h post peptide addition (data not shown). In subsequent experiments, we measured JNK activities in total protein extracts prepared from cells treated for 3 h with several of the peptides described above (Fig. 5). A series of observations can be drawn from these results. First, neither treatment with 200 μM of L- or dTat alone resulted in a marked activation of JNK in INS-1E cells. In contrast, all treatments with cytotoxic conjugates correlated with a clear activation of JNK in these cells (Fig. 5A and B). The fold-increase of JNK activity was the highest in the presence of NBD-Tat (25-fold at 100 μM), Tat-BH_4-Bcl_{XL} (22 fold at 10 μM), Antp-JNKi (13 fold at 10 μM), and STAT-1i (5 fold at 30 μM) and a more moderate increase of JNK activity was detected in the presence of L- and dJNKi (2.5 and 4 fold at 30 μM, respectively). The reduced activation of JNK in the presence of JNKi can be explained by the known inhibitory effect that these peptides exert on JNK activation when used

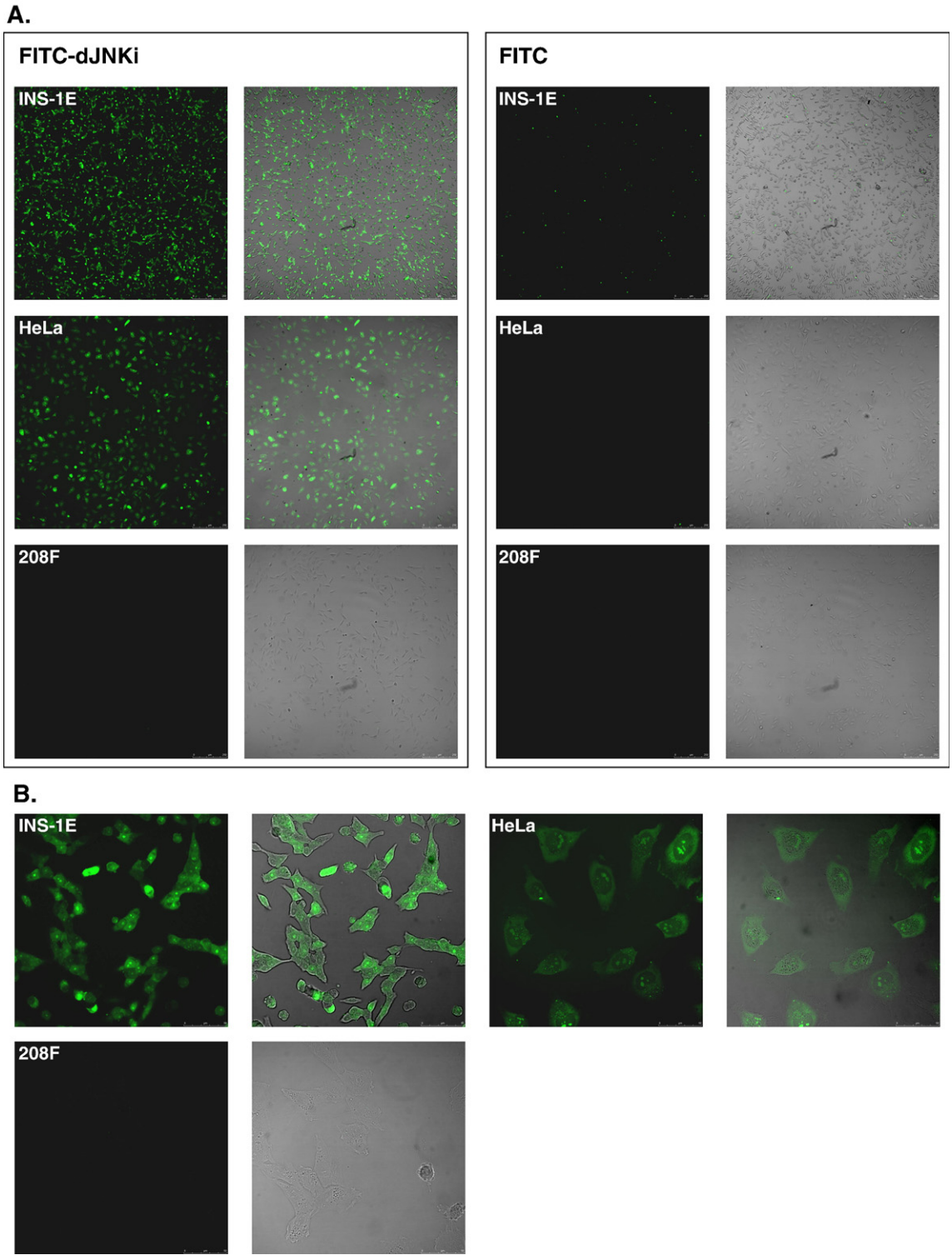

Fig. 4. Efficacy of peptide transduction into INS-1E, HeLa, or 208F cells. Cells were incubated for 1 h in the presence of 5 μM FITC-labelled dJNKi or FITC alone. Peptide internalisation was visualised in live cells by confocal scanning microscopy. Results of fluorescence microscopy are presented alone or in superposition with bright field images at 100-fold (A) and 630-fold (B) magnifications. Peptide uptake is significantly stronger in INS-1E and HeLa cells than in fibroblasts. Pictures are representative of 3 independent experiments.

above 10 μM [11]. Yet and despite the blockade of JNK activity, L- and dJNKi both promoted significant cell death above 10 μM, suggesting that other and/or additional death pathways were involved. High levels of JNK activity observed in the presence of 10 and 30 μM Antp-JNKi may relate to the intrinsic toxicity of the Antp carrier.

JNK activity was also measured in proteins extracted from HeLa and 208F cells exposed to CPP and CPP-conjugates. In

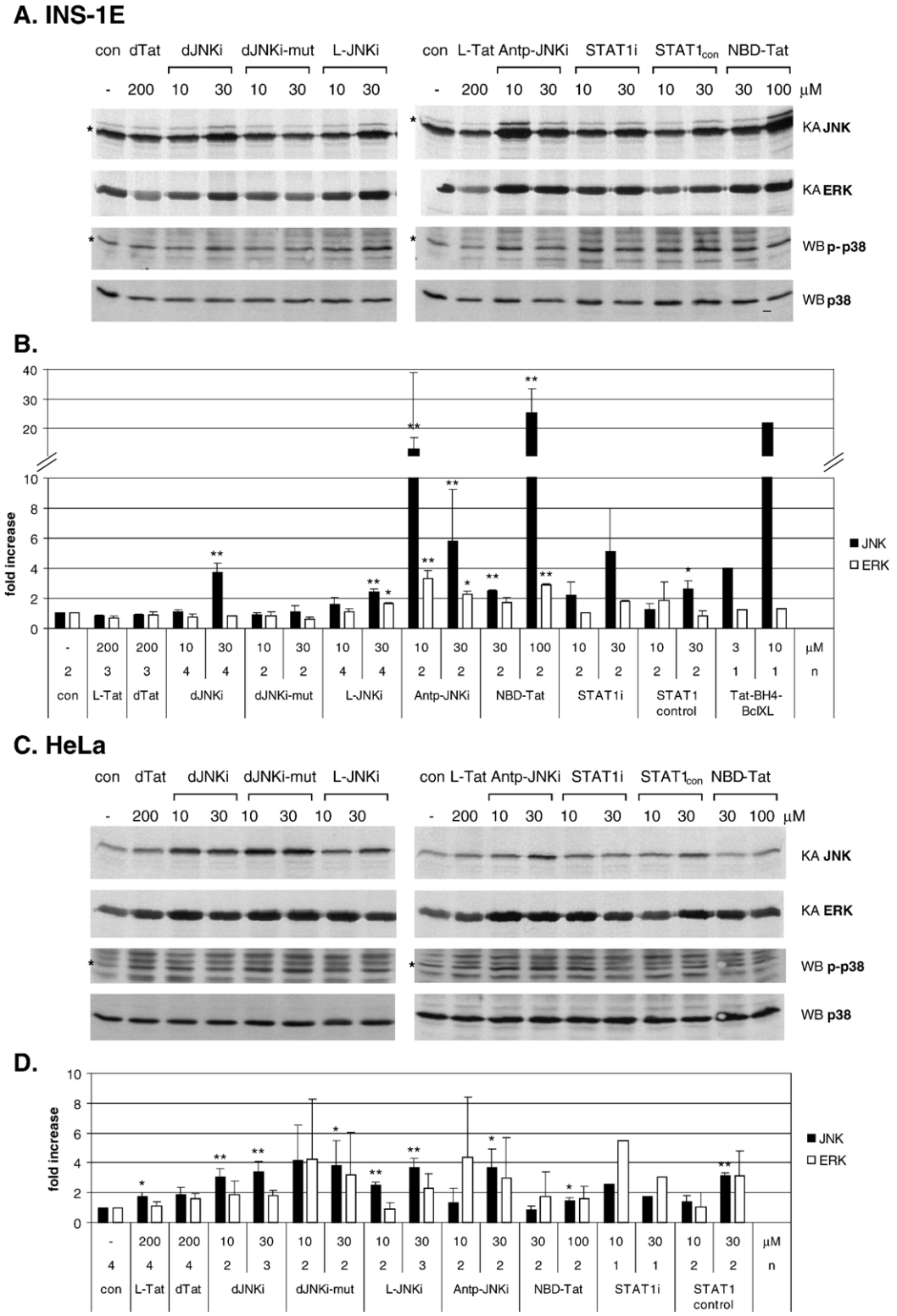

Fig. 5. JNK, ERK, and p38 MAPK activities in INS-1E and HeLa cells incubated in the presence of Tat-conjugates. (A and C) Typical results of JNK and ERK kinase activity tests (KA) and anti-phospho-p38 MAPK western blots in total protein extracts of (A) INS-1E and (C) HeLa cells treated for 3 h with the indicated peptides. Bands of interest are indicated by asterisks. (B and D) Intensities of c-Jun and Elk-1 substrate phosphorylation were quantified by phosphor-imaging and normalised to those measured in (B) non-treated INS-1E and (D) HeLa cells. Data are shown as means±SEM ($n=2$ to 4). * and **: $p \leq 0.05$ and $p \leq 0.01$ versus control, *t*-test.

both cell lines, basal levels of active JNK were significantly lower than in INS-1E cells, which is in agreement with previous reports about elevated basal levels of JNK activity in pancreatic β-cells [46]. In HeLa cells, the patterns of peptide-induced JNK activation correlated well with those of cytotoxicity, although the fold-increase in activity was generally more moderate than

in INS-1E cells (Fig. 5C and D). In contrast, only marginal effects were detected in fibroblasts (data not shown), which was consistent with their reduced ability at internalising Tat peptides.

Extracts used to probe JNK activity were tested in parallel for the levels of p38 MAPK phosphorylation by western blotting, and for those of ERK activity by kinase activity assays using GST-Elk-1 as a substrate. As shown in Fig. 5A and C, the most potent peptides, including 30 μM of L- or dJNKi, induced a slight increase in p38 MAPK phosphorylation in INS-1E and HeLa cells. Yet, the overall fold-augmentation of p38 phosphorylation was lower than that of JNK activation, at least in INS-1E cells. Similarly to the effect on JNK activity, exposure to Tat transporters alone produced no effect on p38 phosphorylation in INS-1E cells and induced a moderate increase in phosphorylation in HeLa cells. In contrast, ERK activities were not significantly changed by any treatment in all 3 cell lines (Fig. 5 for INS-1E and HeLa cells, data not shown for 208F fibroblasts).

Taken together, these results indicate that cell-permeable peptides may induce cytotoxicity when used at concentrations above 3 to 10 μM. Remarkably, cytotoxicity is not restricted to β cells but is likely to affect broader types of cells, depending on their ability at internalising CPP. Moreover, the cytotoxic response is not restricted to one type of transporters, as both Tat- and Antp-conjugates lead to major deleterious effects in INS-1E and HeLa cells. CPP-induced cytotoxicity in INS-1E cells correlated with a significant activation of JNK and a moderate phosphorylation of p38 MAPK, two of the best known pro-apoptotic pathways in pancreatic β-cells. Unexpectedly, high doses of JNK peptide inhibitors also lead to β-cell death, suggesting that JNK-independent death pathways are recruited by these peptides. Slightly reduced levels of peptide-induced cytotoxicity correlated with a more moderate activation of JNK and p38 MAPK pathways in HeLa cells, and minor JNK activation was paralleled by negligible cytotoxicity and poor CPP uptake in 208F fibroblasts.

4. Discussion

To date, many investigations about the biology of cell-permeable peptide transporters have focused at elucidating their respective mechanism of membrane translocation [47]. Yet, only a few reports addressing possible CPP toxicity have been published. Most of them, by studying transducing peptide sequences in various cell types, have basically found limited if no toxicity associated to concentrations of up to 10 to 100 μM $Tat_{48–57}$ and $Antp_{43–58}$ transporters and for exposure of up to 24 h [4,36,48]. Trehin et al only noted significant cytotoxicity for both CPP when applied at 1 mM on canine kidney MDCK cells [48], but the ability of Tat peptides to transduce this cell line is debatable [49]. Recently, Saar et al. have reported very little if no cytotoxicity associated to the uptake of 10 μM of $Tat_{48–60}$ or $Antp_{43–58}$ in 3 human cell lines [4]. They also inferred very little cytotoxicity on erythrocytes as 50 μM of $Tat_{48–60}$ did not induce hemolysis and $Antp_{43–58}$ produced only a marginal detrimental activity. The low intrinsic toxicity of $Tat_{48–57}$ in INS-1E and HeLa cells is consistent with these previous reports. In contrast, we detected clear cytotoxicity in the presence of 30 μM $Antp_{43–58}$ alone in these cells. Drin et al similarly reported notable cell lysis in K562 cells exposed to increasing concentrations of $Antp_{43–58}$ (typically 5 μM and above), whereas Tat produced no such effect [50]. These differences were attributed to the elevated lipid-binding affinity of Antp (related to its hydrophobicity and amphipathicity), that could account for plasma membrane destabilisation and permeabilisation. Such a process might also explain the intrinsic cytotoxicity of Antp in INS-1E and HeLa cells.

At least 5 cell-permeable proteins or peptides had shown promising anti-apoptotic potential in insulin-secreting cells [11,14–19]. Yet, we have provided evidence here that when effector sequences, including anti-apoptotic peptides, are linked to $Tat_{48–57}$- or $Antp_{43–58}$, the resulting conjugates induce cytotoxic responses in pancreatic β-cells, irrespective of the sequence of the cargo. Although unexpected, these results do not contradict the reports mentioned above, but rather point at the importance of the dose of peptide delivered. Specifically, most of the initial experiments were performed in the presence of relatively low concentrations of CPP-conjugates. The cytoprotective effects of heme-oxygenase-1 and neuroglobin Tat-fusion proteins against oxidative stress were obtained in the nM range [16,17]. Also, 1 μM of Tat-bound JNKi peptides conferred good protection against IL-1β-induced apoptosis in β-TC3, RIN-5AH-T_2B, and INS-1 cells [11,46,51]. Cytoprotection was further accompanied by JNK inhibition, as illustrated by the significant reduction of both c-Jun phosphorylation and c-*fos* mRNA expression. In addition, pre-treating rat pancreatic explants with 1 μM dJNKi markedly inhibited JNK activity (ca 60%) and c-*fos* mRNA expression (ca 70%) upon islets isolation, thereby conferring enhanced viability to purified islets [37]. The same treatment also conveyed mild protection against IL-1β-induced apoptosis. In contrast, exposure to 5 μM of dJNKi induced marked apoptosis in rat islets [37], whereas 10 μM of L- and dJNKi produced serious damage in primary and INS-1E pancreatic cells (Figs. 1 and 2). In other words, 5 μM stands around the limit of toxicity for $Tat_{48–57}$-JNKi peptides in rat islets and β-cells, but partial cytoprotection can be attained below this concentration. This threshold of toxicity appears to vary between species. We have indeed recently shown that concentrations of up to 30 μM of dJNKi produced limited cytotoxicity in human islets [37], whereas others reported that 10 μM of 11-Arg-bound JNKi significantly improved pig and mouse islets recovery and function for transplantation [14,52]. These discrepancies may reflect species-associated differences in the efficiency of peptide uptake or in the sensitivity to intracellular death signals triggered by these peptides. In the latter case, they might also result from distinct intrinsic properties of $Tat_{48–57}$ and 11-Arg carriers, although data comparing both carriers in the same experimental model are currently lacking. Along these lines, it has also been documented that rat islets were usually significantly more sensitive to toxic stimuli than mouse or human islets [53].

A concentration of 200 nM Tat-BH4-Bcl-X_L was sufficient to decrease caspase 6 activation by approximately 50% in non-

human primate islets [18]. Presumably, the same concentration was used to obtain partial protection from IL-1β-induced apoptosis in human islets, although not clearly specified in the original report [18]. In our hands however, the same dose did not seem to promote INS-1E cell survival against IL-1β-induced apoptosis (% apoptosis at 48 h: control 1.4±0.2, 2 ng/ml IL-1β 7.6±1.1, 200 nM Tat-BH4-Bcl-X_L 8.4±1.4; data are means±SD, n=3–4). At this stage, it is not clear whether these results denote the need for a higher dose of peptide to reach protection, or whether pathways blocked by Tat-BH4-BclX_L are not sufficient to block IL-1β-induced apoptosis in this cell line.

The comparison between our results and data published previously for NBD peptides is less trivial. Rehman et al. showed that in vitro transduction of isolated mouse islets with 200 μM of a PTD_5-NBD peptide for 2 h prevented NF-κB activation by IL-1β [19]. In addition, injection of 200 nmol PTD_5-NBD into the common bile duct prior to pancreatic islet isolation, followed by 12 to 18 h of culture in the presence of 200 μM peptide improved islets viability by 10 to 15% and glucose-stimulated insulin release by 10 to 30% [19]. As the efficiency of peptide uptake mediated by PTD_5 versus Tat and Antp carriers has not been compared in pancreatic β-cells and islets, it is difficult to extrapolate whether the apparent conflict between these data and ours are the consequence of differences in peptide entry or carrier-dependent levels of toxicity. Interestingly, Tat appears ca 3-fold more efficient than PTD_5 to transduce small cargo sizes (60 kDa) in HeLa cells whereas both carriers seem equally effective at internalising large proteins (500 kDa) [54]. Similarly to the data obtained with dJNKi, it is also conceivable that the threshold of NBD-induced cytotoxicity is lower in rat than in mouse β-cells and islets. Of note, cytotoxicity of Tat-bound NBD peptides does not appear to be related to the long-term inhibition of NF-κB activity since NBD_{scr}-Tat and Tat-NBD_{scr} control peptides were equally toxic (Figs. 1 and 2). This is in agreement with independent data showing that long-term specific inhibition of NF-κB activity in adult mouse β-cells does not affect insulin release or glucose tolerance in vivo [55].

We have presently shown that the INS-1E cytotoxic response correlated with a significant activation of JNK-, and to a lesser extent p38 MAPK. These stress signalling pathways are well known for their potent pro-apoptotic function in pancreatic β-cells and are likely to contribute to INS-1E cell death [11,20–22,46]. A less pronounced JNK and p38 MAPK activation was detected in HeLa cells. At this stage, we do not have indications about the internalisation process of Tat- or Antp-conjugates in INS-1E or HeLa cells. It is therefore difficult to speculate as to how these cells sense exposure to CPP conjugates as toxic and how this relates to the activation of downstream stress signalling pathways. Yet, cell entry appears mandatory for the onset of toxicity as high concentrations of non permeable peptides were harmless and cells resistant to dTat-mediated peptide internalisation were also resistant to cytotoxicity. In other words, cytotoxicity is determined by the intracellular concentrations of CPP-conjugates.

The poor capability of 208F fibroblasts at internalising FITC-labelled dJNKi is intriguing. As reported in other experimental models, $Tat_{48–57}$ peptide internalisation appears to be modulated by specific plasma membrane contents and organisation. In particular, differences in cell surface heparan sulfate proteoglycans [10,56,57] and cholesterol contents [58] have been shown to affect Tat-mediated peptide uptake. We have not compared the levels of cell surface heparan sulfate or cholesterol in 208F versus INS-1E and HeLa cells, but we suspect that differences in cell surface composition (i.e. types and contents of proteoglycans, glycoproteins, or phospholipids) are likely to account for the differences in CPP uptake, stress pathways activation and onset of toxicity.

Addressing or comparing the mechanisms of $Tat_{48–57}$- and $Antp_{43–58}$-mediated internalisation was beyond the scope of the present work. Yet, it is interesting to note that FITC-labelled dJNKi was detected both in the cytoplasm and in the nucleus of INS-1E and HeLa cells, and that it tended to accumulate into relatively large nuclear granules in both cell lines. Similar nuclear accumulation of CPP has been described in several other experimental models [45,50,59]. In addition, punctuate fluorescent patterns in the perinuclear space of HeLa cells (Fig. 4B) might be suggestive of an accumulation within endosomal vesicles, as reported in the literature for Tat and Antp [10,45,50,59]. Conversely, the resistance of 208F fibroblasts to Antp treatment may suggest that 208F are also poorly efficient at internalising Antp. In the literature, data on Tat and Antp internalisation processes are somehow conflicting, and several, partially overlapping mechanisms have been proposed [10]. It is conceivable that a process common and necessary to the entry of both types CPP is missing or altered in these cells.

Recently, Ziegler et al have reported that short exposure (<4 min) of NIH-3T3 fibroblasts to 50 and 500 μM $Tat_{47–57}$ peptide was accompanied by a rapid, transient, and dose-dependent decrease in the rate of extracellular acidification [60]. The authors further suggested that these pH modifications might result from a direct effect of the CPP on intracellular activities such as glycolysis, proton pumps performance, or endosomal proton flux. We have not presently investigated local pH changes upon peptide addition, but we have observed that several of the peptides leading to β-cell toxicity also caused severe inhibition of glucose oxidation in whole rat islets (data not shown). Thus, cytotoxicity correlates with both the activation of stress pathways and the impairment of essential cellular functions.

A major outcome of this study is that Tat-conjugates are significantly more toxic than Tat transporters alone. As shown in Fig. 6, increased cytotoxicity correlates with longer peptides, at least in INS-1E cells. Specifically, 31 aa Tat-conjugates, namely L- and dJNKi, Tat-BH4-Bcl-X_L and Tat-RDM were all toxic at 10 μM, 21–22 aa STAT-1i and NBD-Tat became toxic at 30 to 100 μM, and 10 aa Tat transporters were harmless up to 100 μM. A possible link between CPP toxicity and peptide length had already been suggested by others who showed that stretches of ca 75 arginine residues induced cytotoxicity at concentrations ranging from 0.8 to 50 μM in Jurkat T-cells, whereas tracts of 7 to 30 arginines were essentially harmless [61]. In these experiments, it was however not possible to discriminate whether cytotoxicity related to the length or to the

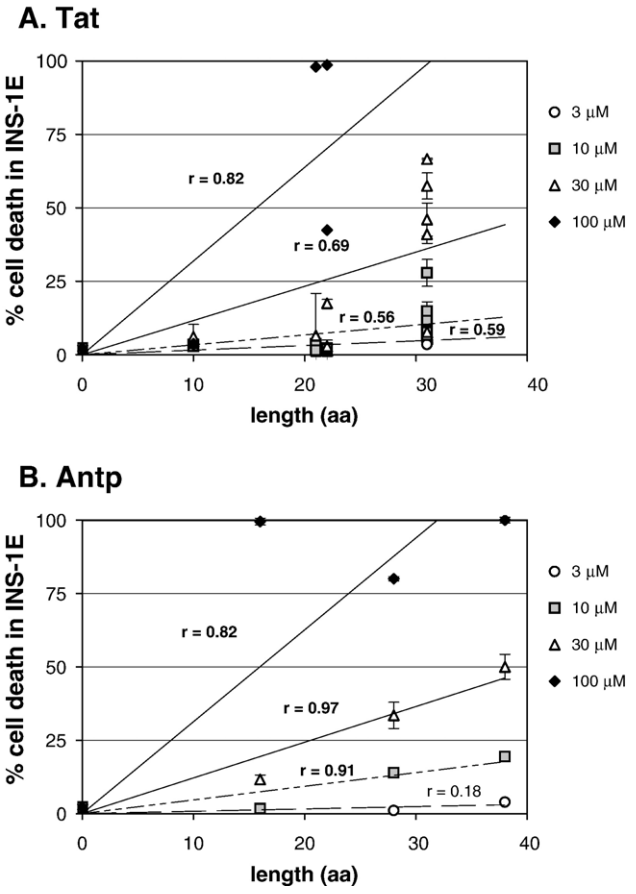

Fig. 6. Cytotoxicity increases with peptide length and concentration in INS-1E cells. The percentage of cytotoxicity induced by cell-permeable peptides and conjugates in INS-1E cells is presented relative to their length in amino acids (aa). Data are shown as means±SD for (A) Tat-, and (B) Antp peptides and conjugates. *r*: coefficient of correlation, Pearson.

net charge of the peptides, as both factors were indissociable. In contrast, our results suggest that the potency of CPP depends on their size rather than on their charge, as we were not able to detect any correlation between the levels of cytotoxicity and the net charge of the peptides (Fig. 1 and Table 1).

Data obtained recently in mouse myoblasts have suggested that the mechanisms of cellular uptake of Tat-conjugates might also depend on the size of the cargo. In particular, whereas Tat fusion proteins were mostly internalised in cytoplasmic vesicles, peptides less than 50 aa long seemed to rapidly enter cells in a manner that was dependent on the membrane potential [45]. Evidence obtained in neuronal PC12 cells had also suggested that the Tat peptide might induce temporary cell membrane perturbations by causing transient phosphatidyl-serine redistribution, in a process described as "membrane-flip" [62]. This flip together with Tat-transduction could be blocked by pre-treating PC12 cells with an excess of poly-lysine [62]. Although we did not have data indicative of distinct modes of entry for the peptides tested here, we reasoned that if membrane reorganisation occurred in INS-1E (or HeLa) cells, it would presumably be accompanied by a (transient) dose- and cell type-dependent shift in membrane potential that, at its extreme, might lead to irreversible cellular damage. To investigate this possibility, we treated INS-1E cells for 30 to 60 min with concentrations of L-JNKi ranging from 1 to 30 µM (i.e. from non toxic to toxic doses) and monitored the kinetics of membrane potential modifications by patch-clamp analysis (data not shown). As these assays failed to show any significant difference between untreated and treated cells, we concluded that perturbations of membrane potential were unlikely to contribute to CPP-dependent cytotoxicity. In this context, one may add that the possibility of a direct penetration mechanism across the lipid bilayer has been recently debated, several experimental data arguing against such a possibility [57,58,63].

In summary, we have shown that $Tat_{48–57}$-bound CPP produced significant dose- and size-dependent cytotoxicity in insulin-secreting cells and in HeLa cells. Cytotoxicity also occurred when replacing $Tat_{48–57}$ by $Antp_{43–58}$. The mechanisms that trigger cell death seem to depend on the efficacy of peptide entry and correlate with the length of the peptidic cargo. Furthermore, they may involve the activation of intracellular JNK, and to a lesser extent p38, stress signalling pathways, two of the major pro-apoptotic cascades in pancreatic β-cells. Taken together, these results indicate that in order to avoid unwanted and unspecific cytotoxicity, effector molecules bound to $Tat_{48–57}$ or $Antp_{43–58}$ should be designed with the shortest possible sequence and the highest possible affinity for their binding partners or targets, so that concentrations below 10 µM may be successfully applied to cells. These data provide a framework for the design of effective cell-penetrating peptides not only for the field of pancreatic islet studies, but also for broader applications of intracellular drug delivery.

Acknowledgments

We wish to thank Christelle Bielmann, Adrien Manighetti, and Lamia Mellaim Quaresma for their excellent technical assistance, and Yannick Krempp of the Cellular Imaging Facility of the University of Lausanne for his help with confocal microscopy. This work was supported by the JDRF Center for Prevention of Beta-Cell Destruction in Europe (grant no. 4-2002-457) and by the Swiss National Foundation for Scientific Research (grant nos. 3200-065139 and 3200B0-105595). AKC is a research associate of the Belgian National Foundation for Scientific Research.

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