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Proteínas recombinantes ligadas a TAT e sua aplicação terapêutica na reversão de dano isquêmico de ilhotas pancreáticas: impacto em transplante

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Índice Geral

Fraker C, Ricordi C, Inverardi L, Pastori RL. Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic beta-cells. Biochem Biophys Res Commun 2003;305(4):876-81..... **ANEXO 1**

8.2 Ribeiro MM, Ricordi C, Pastori RL. Removal of endotoxin from recombinant proteins after acidic pH treatment in polypropylene tubes. Manuscrito em revisão editorial *Protein Expression and Purification*…… **ANEXO 2**

8.3 Pastori RL, Klein D, **Ribeiro MM**, Ricordi C. Delivery of proteins and peptides into live cells by means of protein transduction domains: potential application to organ and cell transplantation. Transplantation 2004;77(11):1627-31..... **ANEXO 3**

8.4 Klein D, **Ribeiro MM,** Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, Molano RD, Inverardi L, Ricordi C, Pastori RL. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 2004;323(2):473-8..... **ANEXO 4**

8.5 Pastori RL, **Ribeiro MM,** Klein D, Ricordi C. Protein Transduction Domain as a Novel Tool for Delivery of Proteins, Peptides and Nucleic Acids. In*: "Biomaterials for Delivery and Targeting of Proteins* and *Nucleic Acids"* RI Mahoto Editor. CRC Press 2005..... **ANEXO 5**

8.6 Mendoza V, Klein D, Ichii H, **Ribeiro MM**, Ricordi C, Hankeln T, Burmester T, Pastori RL. Protection of islets in culture by delivery of oxygen binding neuroglobin via protein transduction**.** Transplant Proc 2005;37(1):237-40..... **ANEXO 6**

8.7 Domínguez-Bendala J, Klein D, **Ribeiro M**, Ricordi C, Inverardi L, Pastori R, Edlund H. TAT-Mediated Neurogenin 3

Protein Transduction Stimulates Pancreatic Endocrine Differentiation In Vitro. Diabetes 2005;54(3):720-6..... **ANEXO 7**

Índice de Figuras e Tabelas

Índice de abreviaturas :

AKT = PkB	Proteina Quinase B
ASK1	Sinal de Apoptose Quinase 1
7AAD	7-aminoactinomicina D
β-TC3	Insulinoma de camundongo beta TC3
Bl21	Cepa de *E. coli*
CMRL	Meio de cultura
Cy3	Corante Cyanine 3
Cy5	Corante Cyanine 5
DTZ	Ditizona
dl	Decilitro
2-DIGE	Gel de eletroforese de duas dimensões
ERK1,2	Extra cellular signal regulated kinase- sinal extracelular regulador de Quinase
ELISA	Enzyme-Linked ImmunoSorbent Assay
FCS	Fetal Calf Serum- Soro bovino fetal
FACS	Fluorescence activated cell sorting
Ga	Gauge
HO1	Heme oxigenase 1
H&E	Hematoxilina e Eosina
HIV	Vírus de imunodeficiência humana
HSV-1	Vírus da Herpes Simplex 1
h	Hora
HBSS	solução salina balanceada e tamponada Hank´s
IkB	Proteína inibidora de NkFB
IEF	Isoelectric focusing
JNK	Jun N-terminal kinase
Kg	Quilograma
LAL	Limulus Amebocyte Lysate
L.I.	Long ischemia – Isquemia longa
LPS	Lipopolisacarídeo
MAPK	Mitogen activated kinase – Quinase de ativação mitogênica
MAPK kinase	Mitogen activated kinase kinase
MAPKK kinase	Mitogen activated kinase kinase kinase
mg	Miligrama
ml	Mililitro
min	Minuto
µM	Micromolar
µm	Micrômetro
µl	Microlitro
µg	Micrograma
p38	Mitogen-activated protein kinase p38
PTD	Proteína de transdução proteica
pTAT	Vetor de expressão TAT
PNF	Primary non function – Enxerto primário não funcional
PI	propidium iodine
pI	Ponto isoelétrico
rpm	Rotações por minuto
SAPK	Stress activated protein kinase- Proteína quinase ativada por *stress*
TAT-PTD	Domínio de transdução protéica (PTD) TAT
TAT	Domínio de transdução protéica TAT

TAT-PTD-HO1	Domínio de transdução protéica ligado a HO1
TAT-D-JNKi	Versão D do inibidor da molécula JNK ligada a TAT
TAT-HO1	Heme oxigenase 1 ligada ao domínio de transdução TAT
TAT-Bcl-XL	TAT fundio a proteína reguladora de apoptose Bcl-XL
TAT-BH4	TAT fundido a porção anti-apoptótica da Bcl-XL
TAT-Ngb	TAT- fundido a neuroglobina
TAT-NgN3	TAT fundido ao fator de desenvolvimento neurogenina
TAT-HO1	Heme oxigenase 1 ligada ao domínio de transdução TAT
TNFα/CHX	Fator de necrose tumoral alfa com ciclohexamina
UW	Solução de preservação University of Wisconsin
VP22	Proteína de HSV-1 que funciona como PTD
S.I.	Short ischemia – Isquemia curta
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Resumo

O transplante de ilhotas pode ser uma opção para o tratamento do Diabetes do tipo I em função do aumento do controle metabólico e da qualidade de vida do paciente transplantado. As maiores limitações para a aplicação dessa técnica em larga escala são o alto número de ilhotas necessárias para a obtenção de um bom funcionamento do transplante e a deteriorização de ilhotas, causados por dano isquêmico por preservação a frio. Aqui propomos o uso de nova tecnologia denominada TAT- PTD como transportador de moléculas terapêuticas para o interior de ilhotas e pâncreas com a intenção de prevenir morte de ilhotas, aumentando, assim, o número e a qualidade das ilhotas para transplante de pacientes diabéticos.

Resultados da Parte I:
Construímos uma proteína de fusão composta por Heme oxigenase -1 (HO1) e o domínio de transdução protéica TAT- PTD (TAT) – um peptídeo viral com 11 aminoácidos provenientes do vírus da imunodeficiência humana (HIV) e com capacidade de penetrar em membrana plasmática. A transdução de TAT-PTD-HO1 em células produtoras de insulina protege contra a ação citotóxica de TNF-α. A transdução de TAT-HO1 para o interior de células produtoras de insulina não danifica a fisiologia de ilhotas como foi mostrado quando estas são transplantadas em camundongos imunodeficientes e com Diabetes quimicamente induzida. Finalmente, mostramos que transdução de ilhotas com a proteína de fusão melhora a viabilidade de ilhotas em cultura. Essa abordagem pode ter um impacto positivo no aumento da disponibilidade de ilhotas para transplante.

Resultados da Parte II:
A produção de proteínas de fusão ligadas a TAT-PTD pode ser obtida em larga escala e eficientemente com a expressão protéica em *E. coli*. No entanto, a contaminação com endotoxina representa um problema para a aplicação dessas proteínas *in vitro* e *in vivo*. Desenvolvemos várias proteínas de fusão ligadas a TAT que têm aplicação terapêuticas em ilhotas. Apresentamos aqui um novo método para a eficiente remoção de endotoxina de soluções protéicas usando tubos de polipropileno em combinação com tratamento em pH ácido, mantendo a atividade biológica da proteína com bom rendimento protéico.

Resultados da Parte III:
Para poder detectar possíveis moléculas que possam ser citoprotetoras e que possam ser associadas à tecnologia de TAT-PTD, estabelecemos um modelo de isquemia a frio em pâncreas de ratos. Isso nos permitiu estudar a modulação de vias ativadas por sinal de *stress* que levam ao impedimento da recuperação da qualidade e função de ilhotas. Observamos que JNK e p38 são proteínas kinase ativadas por *stress* (SAPK) que estão presentes por causa de dano causado por isquemia pancreática e isolamento de ilhotas. Um experimento preliminar usando TAT-D-JNKi, inibidor de JNK, tentou prevenir dano causado pela ativação de JNK.

Abstract

Islet transplantation can become a therapeutic option for the treatment of Type I Diabetes due to increased metabolic control and quality of life for transplanted patients. The major limitations for widespread application of this technique are, high number of islets needed to attain good function after transplantation and islet deterioration caused by pancreatic injury through cold ischemia storage. We here propose the use of a new technology using TAT-PTD as a transporter of therapeutic molecules into islets/pancreas with the attempt to prevent islet cell death and increase islet number/quality for transplantation of diabetic patients.

Results from Part I:
We have generated a fusion protein composed of HO-1 and TAT protein transduction domain (TAT/PTD), an 11-aa cell penetrating peptide from the human immunodeficiency virus TAT protein. Transduction of TAT/PTD–HO-1 to insulin producing cells protects against TNF-a-mediated cytotoxicity. TAT/PTD–HO-1 transduction to islets does not impair islet physiology, as assessed by reversion of chemically induced diabetes in immunodeficient mice. Finally, we report that transduction of HO- 1 fusion protein into islets improves islet viability in culture. This approach might have a positive impact on the availability of islets for transplantation.

Results from Part II: While production of TAT-PTD-bound protein can be efficiently attained by *E. coli* protein replication, contamination with endotoxin represents a major hindrance for application of these proteins in the context of *in vitro* and *in vivo* testing. We have developed several TAT-attached proteins that have therapeutic cytoprotection applications on islet. Here we present a novel method for efficient removal of endotoxin from proteins in polypropylene tubes in combination with acidic pH treatment that does not interfere with biological activity of the protein and has good protein recovery.

Results from part III: In order to identify possible molecular targets for islet cell cytoprotection by TAT-PTD technology, we have established a rat pancreas cold ischemia model and readout systems allowing for the study of the modulation of stress-activated cellular pathways leading to impairment of islet cell recovery and quality. We have observed that JNK and p38 stress activated protein kinases (SAPKs) are activated as a result of pancreas ischemia and islet isolation. A preliminary experiment was done using TAT-D-JNK inhibitor to try to prevent or reduce islet damage caused by activation of this SAPK pathway using this stringent model system.

1. Introdução

1.1 Transplante de ilhotas

Ilhotas são conjuntos de células encontradas em pâncreas (representam aproximadamente 1-2% do total de tecido) que são responsáveis pela síntese e secreção de hormônios, incluindo glucagon (células alfa), insulina (células beta), somatostatina (células delta) e polipeptídio pancreático (células pp) (1). Esses hormônios trabalham em conjunto para a manutenção da homeostase glicólica e da resposta apropriada à quantidade de açúcar presente no sangue. Portanto, transplante de ilhotas em pacientes diabéticos é uma opção mais fisiológica do que a reposição de insulina apenas. Esse transplante de ilhotas pode melhorar o controle metabólico e a qualidade de vida de pacientes com Diabetes do tipo I, demonstrando que transplante de ilhotas pode se tornar uma opção terapêutica para o tratamento de pacientes com controle instável da Diabetes (2-5).

O procedimento de isolamento de ilhotas consiste em digestão enzimática e mecânica de pâncreas cadavérico, permitindo a liberação de células endócrinas das células exócrinas do pâncreas, mantendo sua integridade estrutural **(Figura 1.1).** Isso é obtido por meio da câmara de dissociação (6), que se tornou o padrão ideal para o processamento automatizado de ilhotas pancreáticas humanas. Depois da digestão, as ilhotas são purificadas a partir do tecido digerido por centrifugação em gradiente de densidade. As ilhotas são infundidas no fígado de recipientes por meio da veia porta.

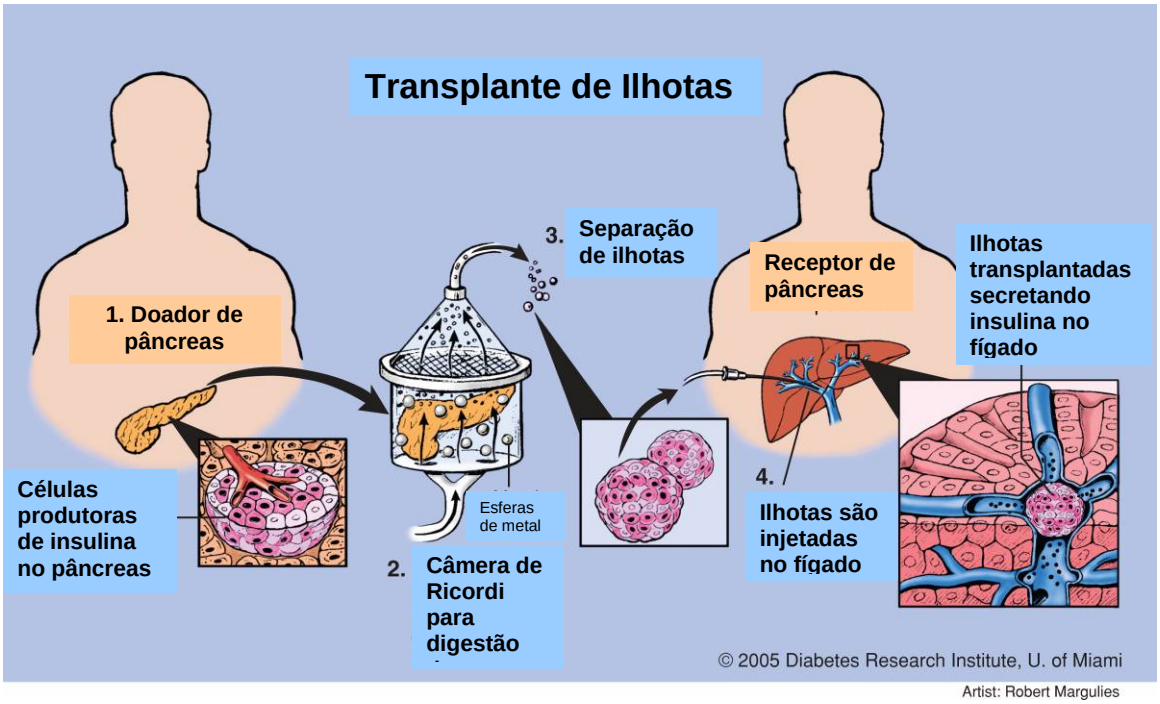


Figura 1.1 Transplante de ilhotas. Ilhotas representam aproximadamente 2% do total de tecido pancreático (1). O procedimento de isolamento visa a separar ilhotas do tecido acinar com integridade estrutural. Depois de injeção intraducto pancreático da enzima colagenase, a glândula é transferida para a câmara de digestão Ricordi (2) em que, por ação mecânica e enzimática, as ilhotas são liberadas do tecido acinar. A digestão pancreática é purificada usando gradiente de densidade (3) para enriquecimento da fração endócrina. Finalmente, ilhotas com diferentes graus de pureza são infundidas no fígado de recipientes diabéticos por canulação percutânea da veia porta (4), instalando-se nos sinusoides hepáticos.

As limitações para a aplicação do transplante de ilhotas são o alto número de ilhotas necessárias para o bom funcionamento do transplante e armazenamento pancreático para transporte (7). As ilhotas são normalmente isoladas de mais de um pâncreas e infundidas em pacientes, seqüencialmente ou em infusões misturadas. Diversas variáveis podem contribuir para a necessidade de mais de um doador por paciente. Os danos causados por *stress* oxidativo, seguido de morte cerebral(7), hipóxia durante a preservação do tecido, assim como as técnicas para retirada do órgão para uso em transplante, *stress* celular durante isolamento de ilhotas, cultura de ilhotas e o próprio transplante têm sido reconhecidos na literatura como meios que induzem morte celular (7-9). Em particular, hipóxia e *stress* oxidativo são os maiores contribuidores para a deteriorização, tanto em qualidade como em quantidade das ilhotas obtidas a

partir de órgão doado. O microambiente das ilhotas é altamente vascularizado e com alta tensão de oxigênio (1), quando as ilhotas são retiradas deste ambiente durante o processamento e cultura de ilhotas, a privação de oxigênio afeta significativamente o número, viabilidade, função e potência das mesmas.

Há uma necessidade urgente de desenvolvimento de métodos que permitam a preservação de células-beta de ilhotas, durante a fase crítica de retirada do órgão e do transplante, para melhorar a taxa de sucesso de experimentos clínicos. A importância de intervenção precoce tem sido reconhecida. A implementação novos de métodos de retirada de órgãos (10) e preservação de glândulas pancreáticas humanas para transplante que permitam a manutenção da atividade metabólica, assim como a disponibilidade de oxigênio (por exemplo o chamado *"two layer method")* (11), têm sido provados como sendo eficientes em aumentar o número de ilhotas obtidas a partir de um só órgão, permitindo, assim, uma melhor utilização dos órgãos doados em anos recentes (12,13).

1.2 Ativação de Proteínas Quinase Ativadas por Stress (SAPK) em isquemia pancreática.

Várias vias de transdução de sinal têm sido associadas com dano durante a preservação de pâncreas e processamento de ilhotas, afetando as células beta em sua função e viabilidade **(Figura 1.2).** Células beta de ilhotas têm deficiência em anti oxidantes intracelulares e são por isso particularmente vulneráveis a estímulos que envolvem *stress* oxidativo. Também se sabe que morte de ilhotas pode ocorrer via receptor de morte, mitocôndria e/ou MAP quinase (14-16). A via das MAPK consiste em três membros distintos: MAPK, MAPK quinase

(MAPKK) e MAPKK quinase (MAPKKK). MAPK é ativada por vários sinais extracelulares, tais como fatores de crescimento, citocinas, endotoxina e *stress* no ambiente (17,18). As proteínas ativadas por *stress* (SAPK) c-Jun NM2-terminal kinase (JNKs) e p38, pertencem à família de MAPK e estão envolvidas na geração de sinal de morte celular, principalmente via *stress* e hipóxia (19,20). Recentemente foi descrito na literatura que SAPK (p38, JNK) estão envolvidas em perda de ilhotas durante isquemia fria de pâncreas (11).

Em modelos de isquemia e reperfusão, ERK 1,2; AKT, IkB JNK e MAPp38 são estudados como sendo ativadores de MAP quinase (21). Sinal extracelular regulado por kinase (ERK) em modelo de isquemia e reperfusão em rato, *in vivo*, foi ativado por isquemia, mas não por reperfusão (22). Fosforilação de AKT tem sido associada à isquemia/reperfusão e pré-condicionamento isquêmico, em modelo de transplante de coração em rato (23,24). AKT está envolvida em mecanismos de sobrevivência celular que atenua a apoptose. Ela é uma serina/treonina quinase que mede diversas funções de fosforilação e inativação de quinases pro-apoptóticas (21). AKT modula as vias de p38MAPK e JNK por fosforilação e inibição de ativadores que antecedem essas quinases *(“upstream activators”)* como o regulador de sinal de apoptose quinase 1 (ASK1) (21) **(Figura 1.3).** IkB é um inibidor de NFkB que é encontrado no citoplasma quando associado a IkB. NFkB é ativado quando IkB é fosforilado e , subseqüentemente, degradado por proteosoma, permitindo assim a liberação de dímeros de NFkB que, ao entrar no núcleo, iniciam a transcrição de genes (25). Em modelo de rato para transplante de coração e em modelo em caninos, a isquemia não ativou p55JNK (26). O mesmo foi encontrado em modelo com rato, relacionado à ativação de p38MAPK (21).

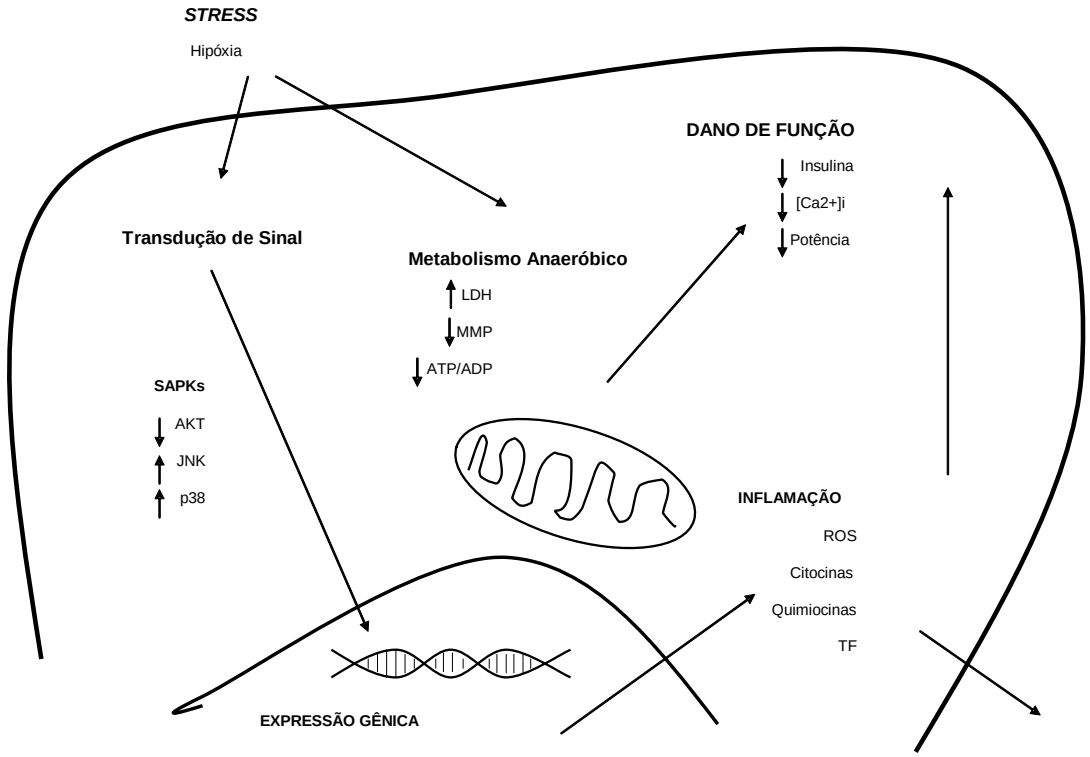


Figura 1.2 Esquema simplificado representando dano celular causado por *stress* de células de ilhotas. O destino de células de ilhotas sob condições de *stress* depende de múltiplos fatores. Sinal de *stress* (i.e., hipóxia, inflamação, sinal mediado por receptor de stress dentre outros) pode ativar a transdução de sinal (i.e., MAPK) com amplificação de inflamação local e/ou stress oxidativo. Hipóxia pode ainda ativar metabolismo anaeróbico levando ao bloqueio de viabilidade e função celular.

O desenvolvimento de estratégias de intervenção molecular que modulam os níveis de expressão de determinados SAPK durante a retirada do pâncreas de doadores, assim como durante preservação a frio, pode ser importante na melhora da recuperação de ilhotas e, também, na qualidade das mesmas. Temos explorado várias abordagens que poderiam promover a citoproteção de ilhotas nos últimos anos, incluindo fármacos e terapias moleculares (9,27-31). A tecnologia de transdução protéica, assim como seus domínios denominados domínios de transdução protéica (PTD), é uma

tecnologia emergente que tem mostrado grande potencial em relação à citoproteção de tecidos e células (32-34).

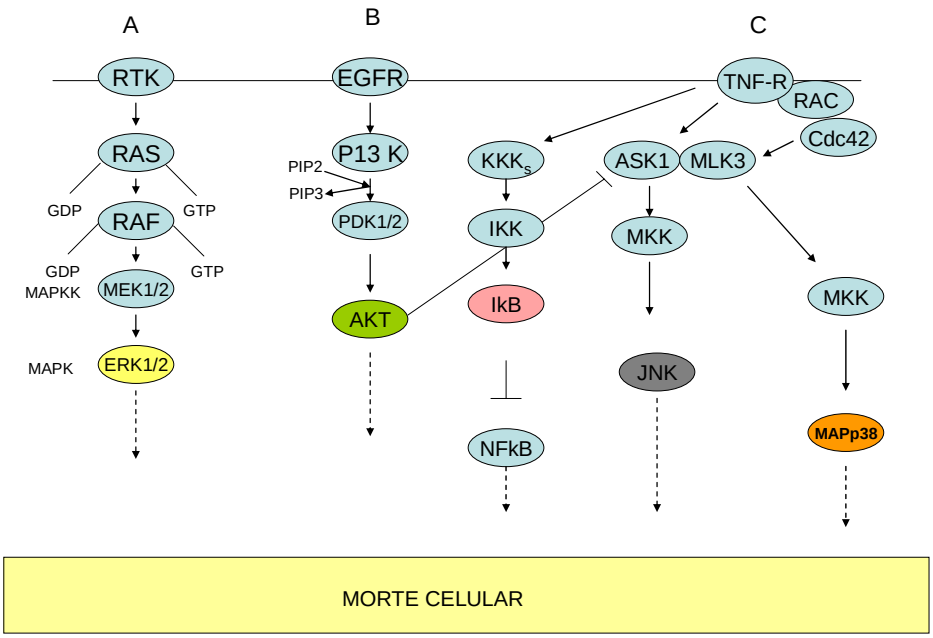


Figura 1.3 Ativação de vias de proteína quinase por *stress*. (A) A associação de Ras/Raf com o receptor de tirosina quinase (RTK) e com MEK1, mediador da ativação de ERK, permite a fosforilação do substrato. (B) A associação de PI-3 quinase (PI-3K) com o receptor de fator de crescimento epidermal (EGFR) induz à produção de 3-fosfatidil-inositol-trifosfato (PIP3), à ativação de fosfoinositol quinase dependente (PKD 1,2),à fosforilação de AKT Thr308/Ser473 e substratos. (C) O receptor de TNF (TNF-R) ativa a quinase sinal de regulação da ativação de apoptose (ASK1) ou *mixed lineage kinase* (MLK), iniciando a fosforilação de MKK4/7 e MKK3/6 com ativação posterior de JNK e p38MAPK, respectivamente (Adaptação do paper Armstrong SC/ Cardiovascular research 61 (2004) 427-436).

1.3 Usando PTD para citoproteção de ilhotas

A tecnologia de transdução protéica é uma nova tecnologia pela qual proteínas e peptídeos podem ser diretamente transferidos para dentro de células quando covalentemente ligados a pequenos peptídeos conhecidos como domínios de transdução protéica (PTDs). PTDs oferecem uma grande vantagem na internalização de proteínas em diferentes tipos de células de mamífero. A

capacidade de atravessar a membrana celular da maioria dos tipos celulares, independentemente de receptor, pode ser altamente interessante no tratamento terapêutico de células e tecidos. Uma vez que PTD seja fundido a proteínas, elas podem ser transportadas para o interior de células, abrindo caminho para a aplicação de uma nova abordagem terapêutica.

Existem diferentes tipos de PTDs que foram identificados como proteínas que ocorrem naturalmente. Dentre elas, as mais comumente estudadas foram encontradas no domínio de transcrição (*homeodomain transcription*) de Drosófila (35), no vírus simplex da herpes tipo I (HSV-1) (36), na proteína VP22 e na proteína transativatora TAT do vírus de imunodeficiência humana (HIV)(37,38).

Dentre todos os PTDs, TAT é o mais estudado, portanto, escolhemos trabalhar com esse peptídeo, em particular como transportador de proteínas que previnem a morte celular de ilhotas iniciada durante o período de isquemia, aumentada durante o isolamento de ilhotas e agravada uma vez que as ilhotas sejam transplantadas em organismos vivos**.**

O mecanismo pelo qual a proteína de fusão é internalizada em células é descrito como sendo macropinocitose de lipídio dependente (*lipid raft-dependent macropinocytosis*)(39).A ligação inicial de PTD com a superfície da célula envolve interações iônicas (com glicofosfatidilinositol ancorado a proteoglicanas e glicoproteínas em “rafts” lipídicos), seguidas de múltiplos passos que são receptores independentes, estimulando a rápida internalização via macropinocitose. Há queda de pH e desestabilização da integridade da vesícula

do macropinosomo bi-lipídico, com liberação da carga fusionada a TAT para o interior celular.

Produzimos várias proteínas citoprotetoras como TAT-HO1(31), TAT-Bcl-XL/TAT-BH4 (28), TAT-Ngb (29) e TAT-NgN3 (40) usadas para reverter a morte celular e estimular a diferenciação celular, aumentando a disponibilidade de ilhotas para transplante. Nossos resultados preliminares sugerem que podemos internalizar eficientemente proteínas grandes como beta-galactosidase (27), assim como agentes citoprotetores (i.e.; heme-oxigenase 1, Bcl-XL e neuroglobina) (28,29,31) em células de ilhotas, em ilhotas inteiras ou em pâncreas, via injeção intraducto pancreático. A mesma abordagem pode se provar eficiente quando usada em via de transdução de sinal que leva à perda de ilhotas durante a preservação de ilhotas pancreáticas.

Neste trabalho, nos concentraremos em descrever a transdução protéica com relação a TAT-HO1 já que esse é o artigo em consideração para o doutorado. Este trabalho descreve os efeitos da transdução da proteína recombinante TAT/PTD-heme oxygenase-1 (TAT-HO1) em viabilidade de ilhotas pancreáticas. Heme-oxigenase-1 (HO-1) foi identificada como uma proteína ubiquita induzida em vários tipos celulares em resposta a condições de *stress* (31). A indução da expressão de HO-1 é considerada uma resposta adaptativa do mecanismo de defesa celular. HO-1 é a enzima fator limitante da degradação de Heme nos produtos monóxido de carbono (CO), ferro e biliverdina. CO tem papel citoprotetor em diferentes sistemas, incluindo células (41-44) beta do pâncreas (45). O ferro induz à síntese de ferritina que também já foi descrito como tendo papel citoprotetor em células endoteliais (46). A

biliverdina é, subseqüentemente, reduzida a bilirrubina, um anti-oxidante potente (47). A superexpressão de HO-1, por terapia genética ou por indução química, foi usada para reduzir os efeitos deletérios de apoptose e *stress* oxidativo, induzidos por citocinas em vários tipos celulares e em modelos animais (48-52).

O objetivo do nosso estudo era gerar a proteína de fusão TAT-PTD-HO-1 que pudesse transduzir células produtoras de insulina, conferindo citoproteção a elas. Produzimos e purificamos HO-1 recombinante fundida em fase com o TAT-PTD. Transduzimos TAT-PTD-HO-1 em insulinoma de células beta (β-TC3-Deutsche Sammlung von Mikroorganismen und Zelkulturem GmbH-Braunschweig, Alemanha) o que conferiu proteção contra a ação da citotoxina TNF-α. A presença da proteína de fusão em cultura de ilhotas de rato prolongou a viabilidade da produção/secreção de insulina em cultura e não se mostrou tóxica quando transplantada *in vivo* em camundongos **(Parte I)**.

As demais proteínas escolhidas para aplicação da tecnologia de TAT-PTD também tiveram sua região codante do DNA clonada no vetor de expressão pTAT (generosamente disponibilizado pelo Dr. Steven Dowdy- USDC), da mesma forma como descrita para TAT-HO1 (31). Vetores foram transfectados em BL21 (*E.coli)* e foram expressos para a produção em larga escala de proteínas. Um problema freqüente entre as proteínas recombinantes produzidas por bactérias é a presença de altas concentrações de endotoxina o que se tornou um impedimento para a aplicação das proteínas produzidas *in vivo* e um problema em potencial para a interpretação de resultados *in vitro*.

1.4 Remoção de endotoxina de proteínas recombinantes

As endotoxinas são lipopolisacarídeos (LPS) responsáveis pela organização e estabilidade de bactérias (53). Elas são associadas à membrana externa de bactérias gram-negativas e se tornam contaminantes de produtos produzidos por ela na hora da lise celular. Reações adversas como aumento de temperatura corpórea, ativação da cascata de coagulação, modificação hemodinâmica e choque séptico podem ocorrer *in vivo* por causa da exposição a produtos contaminados por endotoxina (54). Além disso, ensaios *in vitro* podem ter a interpretação dos resultados significativamente alterada em função da presença de endotoxina (55). Portanto, a remoção de endotoxina de proteínas recombinantes geradas em *E. coli,* de uma forma eficiente e econômica, é fundamental para a viabilização do uso dessas proteínas.

As endotoxinas são consideradas resistentes à temperatura e pH, o que faz da sua eliminação um dos passos mais difíceis no processo de purificação de proteínas (56). Vários métodos são usados para a eliminação de endotoxina. A ultrafiltração remove endotoxina de água, no entanto, as forças físicas necessárias nesse processo podem danificar proteínas (57). A cromatografia de troca iônica utiliza a carga negativa da endotoxina para ligação na resina. No entanto, esse método é aplicado em proteínas básicas que não se ligam à coluna. A competição entre troca iônica e carga positiva da proteína pode levar a proteína a arrastar endotoxina através da coluna (58). A adsorção por afinidade como no caso da histidina, histamina, polimixina B e poli-L-lisina facilitam a adsorção de endotoxina na matriz, por interações eletrostáticas ou interações hidrofóbicas. Ainda que as interações entre adsorventes e endotoxina sejam

seletivas, múltiplas rodadas podem diminuir o rendimento do produto a ser recuperado (59). Triton X-114 é descrito como um composto eficiente na remoção de endotoxina de proteínas recombinantes (60,61). Pode ser usado em separação de fase ou em lavagens de coluna de afinidade (62). No entanto, a contaminação por detergente deve ser removida por repetidas adsorções ou gel filtração. Cada processo tem em média uma perda da ordem de 10-20%. Além disso, em escala industrial, detergentes são caros e podem, potencialmente, afetar a atividade biológica da proteína purificada (63). Solventes inflamáveis como etanol e isopropanol são considerados solventes eficientes na remoção de endotoxina (64), mas, quando usados em escala industrial, o requerimento por procedimentos de segurança encarece significativamente o custo de produção. Os alcanediols são vistos como produtos alternativos, mais seguros e mais baratos, que podem ser usados para a remoção de LPS, associados a proteínas imobilizadas em colunas de cromatografia (63). Uma desvantagem desse processo é o aumento da viscosidade dependendo do alcanediol usado.

A composição química do recipiente onde a amostra é estocada também exerce um papel importante na dosagem do conteúdo de endotoxina. Polipropileno foi descrito como sendo um adsorvente de endotoxina, enquanto poliestireno adsorvia endotoxina com menor intensidade (65). Portanto, era recomendado evitar estocagem de amostras em tubos de polipropileno, antes da dosagem analítica de endotoxina (63).

Tendo como base essa observação, testamos a hipótese de que adsorção de endotoxina em tubos de polipropileno poderia ser usada como um método

para a remoção de endotoxina de proteínas recombinantes produzidas em *E.coli*..

Descrevemos aqui as condições em que o polipropileno associado a tratamento em pH ácido elimina a endotoxina de proteínas recombinates produzidas em bactérias. Esse é um método eficiente, barato, rápido e que poderia ser incorporado em processos de purificação de proteína em laboratórios, desde que a qualidade do tubo de polipropileno usado fosse previamente testada, garantindo não haver a presença de inibidores de LAL (Lymulus Amebocyte Lysate) **(Parte II).**

Concentramos, também, os esforços no desenvolvimento de um modelo em rato de isquemia pancreática que foi feita nos moldes técnicos descritos para retirada do órgão para transplante clínico. Isso nos permitiu avaliar e controlar todas as variáveis que estão envolvidas no processo de retirada de pâncreas do doador, como estocagem a frio, isolamento de ilhotas para transplante, cultura de ilhotas, antes do transplante além do transplante. Isso nos permitiu intervir usando tratamentos terapêuticos com proteínas ligadas ao transportador TAT-PTD **(Parte III).**

Podemos dizer que, durante o curso deste doutorado, desenvolvemos um método eficiente para a retirada de endotoxina de proteínas recombinantes e que submetemos um manuscrito para publicação no periódico *Journa of Biotechnology*, que está sob revisão no momento. Estamos analisando processos moleculares para identificar danos causados por isquemia a frio em pâncreas de ratos, caracterizando a conseqüência disso na qualidade das ilhotas disponíveis para transplante (esse manuscrito será submetido em breve). Esses dois

manuscritos nos permitirão atingir nosso objetivo maior que é usar proteínas terapêuticas ligadas ao transportador TAT-PTD para tentar prevenir os danos causados por isquemia a frio, melhorando, assim, o número e a qualidade de ilhotas provenientes de um só pâncreas.

Como as técnicas para eliminação de endotoxina foram desenvolvidas em paralelo com as técnicas para estabelecimento do modelo em rato de isquemia pancreática, não fomos capazes de usar as proteínas ligadas a TAT, descritas na secção de produção científica, no modelo de isquemia em pâncreas de rato durante o período de desenvolvimento da tese. Esse trabalho será feito em futuro próximo.

No estudo aqui relatado, usamos o modelo experimental de isquemia de pâncreas de rato para determinar quais as vias de transdução de sinal sofrem alterações em conseqüência desse processo. Para provar que proteínas ligadas ao transportador TAT podem ser usadas dentro desse contexto, mandamos sintetizar quimicamente um inibidor de JNK fundido a TAT para uso na prevenção de via ativada no processo isquêmico. Esse inibidor, como era sintetizado quimicamente, não tinha contaminação por endotoxina e, portanto, não precisava esperar pela finalização da caracterização do método de eliminação de endotoxina para poder ser usado no modelo descrito.

2. Hipótese e objetivos

Hipótese I

Pode o tratamento de ilhotas em cultura com TAT-HO1 proteger contra a ação de TNFα/CHX e prolongar a viabilidade de células para transplante?

Objetivo 1: Clonar, expressar e purificar a proteína TAT-HO1 recombinante em grande quantidade, mantendo sua atividade biológica.

Objetivo 2: Usar TAT-HO1 para proteger células de insulinoma contra a ação de TNFα/CHX em cultura.

Objetivo 3: Cultivar células de ilhotas na presença de TAT-HO1 antes do transplante em camundongos diabéticos, para verificar a eficiência da proteína na preservação das ilhotas em cultura. Transplantar essas mesmas ilhotas em camundongos diabéticos para verificar sua capacidade em reversão da diabetes assim como possível toxicidade da proteína ás ilhotas.

Hipótese II:

Será o tratamento em pH ácido, em recipiente de polipropileno de proteína contaminada com endotoxina, eficiente na remoção do contaminante?

Objetivo 4: Desenvolver um método eficiente na remoção de endotoxina (de *E. coli*) de soluções protéicas. Isso nos permitirá usar as proteínas de fusão ligadas a TAT-PTD, no modelo desenvolvido de isquemia pancreática a frio em rato.

Hipótese III:

Seremos capazes de testar efeitos terapêuticos de proteínas em etapas críticas, como antes do isolamento de ilhotas e cultura, usando o modelo de isquemia pancreática em rato, para poder tentar aumentar a viabilidade de ilhotas para transplante?

Objetivo 5: Determinar os diferentes mecanismos pelo qual a morte celular de ilhotas ocorre durante a isquemia pancreática por preservação a frio.

Objetivo 6: Estabelecer instrumentos que nos permitam analisar a qualidade, o número e a potência de ilhotas isoladas.

Objetivo 7: Intervir com proteínas terapêuticas fundidas a TAT, em vias específicas que se mostrarem alteradas como conseqüência de isquemia a frio no modelo em rato.

3. Materiais e Métodos

3.3 Materiais e Métodos Parte III

Ribeiro MM et al. Effects of pancreas cold ischemia on the expression of stress-activated protein kinases in islets: impact for islet transplantation outcome. (Manuscrito em atual preparação para submissão em revista científica)

3.3.1 Protocolo para isquemia fria em pâncreas de rato.

Pancreático-duodenoctomia foi feita sob anestesia geral em ratos doadores. Uma combinação de incisão bilateral sub-costal foi executada para melhor exposição de órgãos. O ducto biliar foi canulado com um tubo de polietileno que foi assegurado por sutura. O duodeno foi dissecado distalmente em direção ao lúmen depois de fechá-lo. O estomago foi imobilizado para expor o esôfago em posição distal que foi suturado e cortado (o estômago foi retirado em bloco com o duodeno, pâncreas e baço). A aorta abdominal foi identificada e canulada com um angiocateter 24G conectado a uma seringa, contendo solução de preservação University of Wisconsin (UW). A solução UW foi injetada após a dissecção da veia porta, com veia cava inferior e aorta fechada por meio de presilias, acima do eixo cilíaco. Gelo de solução salina foi adicionado ao abdômen para redução da temperatura pancreática durante a perfusão e retirada do órgão. A glândula foi rapidamente dissecada e estocada em um recipiente estéril, resfriado em gêlo e com UW gelado para a preservação a frio (18 horas- isquemia longa- L.I.), antes de acessar dano causado por isquemia ou início de isolamento de ilhotas. O pâncreas controle teve uma média de isquemia a frio de aproximadamente 3 horas (isquemia curta – S.I.).

3.3.2 Injeção de inibidor de JNK (TAT-D-JNKi) em isquemia de pâncreas.

Em experimentos preliminares, injetamos em pâncreas que sofreu L.I. o inibidor de JNK, que consiste em uma seqüência de 320- aminoácidos do domínio da proteína Islet/Brain-1 fundida ao transportador TAT-PTD, comparamos com um grupo controle que não recebeu a mesma proteína e que também sofreu L.I.. Isso foi feito para poder observar os efeitos seletivos do inibidor no pâncreas que sofreu preservação a frio em nosso modelo em rato. Para alcançar esse objetivo, TAT-JNKi (Alexis Biochemicals; San Diego, CA) foi injetado em (114 µM) intraducto pancreático junto com 0,5ml de solução UW, imediatamente antes da captação do órgão (procurement). **Pâncreas controle** foi injetado com o mesmo volume de UW sem inibidor.

3.3.3 Isolamento de ilhotas

O pâncreas foi obtido de ratos doadores e suas ilhotas isoladas usando Liberase® (0,17mg/ml; Roche), preparada com solução salina balanceada e tamponada Hank´s (HBSS; Mediatech), como previamente descrito (66). Em seguida, o animal sofreu incisão de laparatomia sob anestesia geral. O ducto biliar foi localizado e fechado com presilhas na entrada do duodeno. Solução com Liberase® foi, então, injetada através do ducto pancreático, usando um tubo de polietileno conectado a uma seringa. O pâncreas foi removido e incubado em solução de Liberase® a 37ºC por 18-20 minutos. Depois da incubação a ação enzimática foi cessada por diluição com HBSS resfriado, contendo 10% de soro bovino fetal inativado por calor (HI-FCS HyClone) e DNAse (100µg/ml; Roche). A punção mecânica do tecido foi feita passando o mesmo por uma malha metálica usando uma agulha 14Ga. A purificação de

ilhotas foi feita por centrifugação (950g, 15 min a 4ºC) em gradiente de Euroficoll (Mediatech) densidades 1,111, 1,096,1,069 e 1,037g/ml. As ilhotas foram cultivadas em meio CMRL-1066 (Gibco), suplementado com 10% de FCS, 25mM/L de tampão HEPES, 2mM/L L-glutamina, 100U/ml de penicilina e 100 µg/ml de estreptomicina (todos componentes Mediatech), a 37ºC na presença de 5% de CO_2.

3.3.4 Contagem de ilhotas

As ilhotas foram coradas com corante que se liga ao zinco da insulina (DTZ) e classificadas por tamanho (67,68). Um algoritmo foi usado para converter as contagens em tamanhos considerados “ideais” de ilhotas com diâmetro de 150 µm (Equivalente de ilhotas; IEQ) que, quando multiplicado pelo fator diluição, nos permite estimar o número total de IEQ obtido para cada condição (67). O número de ilhotas foi estimado após isolamento e após cultura (*overnight)*. A taxa de perda de ilhotas durante a cultura *(overnight*) é considerada um marcador para a qualidade das ilhotas, sendo que as preparações de ilhotas com maior perda em número são consideradas de baixa qualidade.

3.3.5 **Viabilidade de ilhotas**

As ilhotas foram dissociadas (ficando em suspensão) e analisadas usando "*fluorescence activated cell sorting*" (FACS), como previamente descrito (69). Em seguida, 1000 a 2000 IEQ foram incubadas por 10 min a 37ºC com 1 ml de solução Accutase (*Innovative Cell Technologies, Inc, San Diego, CA),* seguido de leve pipetagem. Células em suspensão foram filtradas através de um filtro de nylon de 500 µm. Análise em FACS foi feita em células dissociadas depois de corar com o fluoróforo 7AAD (69). Para acessar a viabilidade das células em suspensão elas foram incubadas com 7-aminoactinomicina D (7-AAD; Molecular Probes), que se liga a DNA, quando a membrana celular está alterada, como resultado de morte celular (REF 99). Células em suspensão são analisadas (mínimo eventos de 3,0 X 10^4) usando citômetro FACScan (Becton Dickinson, Mountain View, CA) com CellQuest software.

3.3.6 Transplante de ilhotas

Transplante de massa marginal à ideal em recipientes diabéticos (*athymic nu/nu; camundongos nude*) resultou em maior tempo na reversão para atingir glicemia normal e com alta proporção de enxerto primário não funcional -"*primary non function*" (PNF), que se correlaciona inversamente com o número e a qualidade das ilhotas implantadas e com o nível de inflamação gerada no sítio de implante (70). Tratamento que favorece *engraftment* (por meio de aumento de viabilidade e função e/ou prevenção reduzindo a ativação do microambiente que recebeu o implante) resulta em taxas menores de PNF e em menor tempo de reversão do diabetes, quando comparado a controles não tratados. Na ausência de elementos que causem a autoimunidade, esse modelo *in vivo* nos permite detectar

diferenças em qualidade de ilhotas transplantadas quando uma massa marginal à ideal é implantada em um recipiente, medindo assim o tempo de reversão do diabetes e a proporção de animais que atingiram glicemia normal após o transplante. Transplante de massa ideal de ilhotas (300 IEQ/recipiente, ilhota de rato transplantadas em camundongo) geralmente resultam na reversão do diabetes em 2-3 dias, como observado na **Figura 3.2**. Transplante de massa marginal à ideal (100 IEQ/*recipiete-marginal mass*) resulta em reversão atrasada do diabetes, dependendo da qualidade das ilhotas transplantadas (i.e., viabilidade e função). Animais foram feitos diabéticos usando estreptozotocina (200 mg/Kg; Sigma) dissolvida em tampão de citrato imediatamente antes do uso. O diabetes foi caracterizada como glicose sanguínea de 350 mg/dl por três ou mais dias, usando um glucômetro portátil (One Touch Ultra; Lifescan) em amostras de sangue obtidas da cauda. Sob anestesia geral (isoflurane 5% em oxigênio), o rim esquerdo foi exposto por meio de incisão cirúrgica na região lateral. A cápsula do rim foi quebrada na região caudal usando pinça de ponta fina (*jeweler fórceps*) e um cateter de polietileno para avançar até a região subcapsular polar cranial *(cranial pole*), depositando as ilhotas com a ajuda de uma seringa de precisão (Hamilton; Reno, NV). O cateter foi retirado e a região em que a cápsula foi quebrada e fechou-se por cauterização. Músculo e pele foram imediatamente suturados. Nefrectomia do rim que continha o enxerto foi feita para confirmar que o efeito de glicemia normal atingido era devido ao enxerto e não à ação residual de ilhotas que não haviam sido destruídas pela droga estreptozotocina.

3.3.7 Análise histopatológica

Pâncreas de rato foi submetido a S.I. ou L.I. em solução resfriada de UW. O tecido foi fixado em formalina 10% embebido em parafina e slides com espessura de 4µm foram cortados a uma distância de 100 µm cada um. A partir de cada pâncreas obtivemos um mínimo de sete slides por glândula. Os slides eram corados com Hematoxilina e Eosina (H&E) para subseqüente análise de tecido por um patologista certificado (análise a cega). Pelo menos 25 secções foram analisadas por glândula (secções foram feitas por toda a glândula) em um total de três pâncreas por grupo. Um sistema de classificação/nota foi utilizado para detectar dano pancreático (0-5; 0 = tecido normal sem alterações patológicas; 1 = alterações mínimas; 2 = alterações leves; 3 = alterações moderadas; 4 = alterações agudas; 5 = alterações severas/crônicas). Os parâmetros considerados para a avaliação final de cada pâncreas levaram em conta o número de células apoteóticas, o número e figuras mitóticas em células acinar epiteliais, o grau de necrose, o grau de apoptose acinar e o grau de apoptose de ilhotas.

3.3.8 Análise de citocinas e quimocinas

O nível de citocinas e quimocinas em sobrenadante de cultura e em ilhotas isoladas foi analisado usando ELISA, por meio da tecnologia de Bio-Plex (Biorad) que se baseia na medida simultânea de proteínas múltiplas em pequenas quantidades de amostra. Sobrenadante de cultura e *pellets* são usados separadamente para a medida de citocinas e quimocinas (proteínas). Cada proteína se liga a um anticorpo específico, imobilizado na superfície de microcápsulas fluorescentes. Cada anticorpo é ligado a um tipo específico de

microcápsulas com características próprias, permitindo, assim, que se identifique e quantifique vários elementos simultaneamente. Kits com alvos múltiplos estão disponíveis e usamos painel com citocina e quimoquinas da Linco (LINCOplex kit 24-plex pré-mesclado: Beads Eotaxin – GMCSF, GCSF, IL-1α, MCP-1, Leptin, MIP-1α, IL-4, IL-1β, IL-2, IL-6, IL-9, IL-13; IL-10, IL-12, IL-5, IFNγ, IL-17, IL-18, IP-10, GRO/KC, RANTES, TNFα, VEGF), de acordo com as instruções do fabricante.

3.3.9 Análise de proteínas quinase ativadas por *stress* (SAPK)

A atividade de algumas SAPK foram medidas em lisados de ilhotas recém-isoladas, usando a plataforma Bio-Plex (71). Logo em seguida, amostras foram lisadas com kit (Bio-Rad) e processadas de acordo com recomendações do fabricante. Ilhotas foram lavadas com PBS, centrifugadas a 1000 rpm por 5 min e ressuspendidas em 250 µl de tampão de lisado. O extrato celular passou por dois ciclos de congelamento seguido de descongelamento para garantir completa lise celular. As amostras foram então sonicadas em gelo e centrifugadas a 4500 rpm por 15-20 min. O sobrenadante coletado da concentração protéica do lisado foi determinado usando o kit de determinação protéica BioRad DC. As amostras foram diluídas para a concentração final de 500 µg/ml e um total de 25 µg de proteínas foi utilizado em BioPlex para a detecção de proteína fosforiladas, assim como o total protéico das seguintes kinases: JNK, ERK1,2, p38, AKT, Ik-B. Os resultados foram expressos como razão de proteína fosforilada sob total de proteína em cada amostra.

3.3.10 Análise estatística

Análise dos dados foi feita usando Microsoft Excel e Statistica para Windows. As comparações entre os dois grupos foram feitas usando teste estudante t dispariado. Significância estatística foi considerado a partir de $p < 0,05$.

3.3.11 Análise proteômica de pâncreas de rato submetido a L.I. *versus* S.I.

Pâncreas foi retirado e estocado a -80ºC, depois de incubação pelo tempo determinado. A análise de expressão protéica foi feita usando géis 2-DIGE com espectrômetro de massa, um serviço prestado por Applied Biomics (Hayward, CA). A proteína total foi extraída do pâncreas e marcada com Cy3 e Cy5. O focus isoelétrico (*Isoelectric focusing – IEF*) foi feito na primeira dimensão entre os pHs 3 e 10. A segunda dimensão foi feita com eletroforese em gel de gradiente de poliacrilamida entre 8-14%. Proteínas diferencialmente expressas foram cortadas do gel e submetidas à digestão com tripsina, antes de análise espectral de massa.

4. Resultados

4.3 Resultados Parte III

4.3.1 Padrão histopatológico de tecido de pâncreas após isquemia a frio.

A análise histopatológica foi feita em secções de pâncreas de rato que foram submetidas a L.I. e S.I. A análise mostrou que os efeitos da isquemia fria são significativamente mais acentuados em pâncreas submetido a L.I. do que em pâncreas submetido a S.I. e que esse fenômeno contribui para as mudanças patológicas observadas como um todo **(Tabela 3.1).**

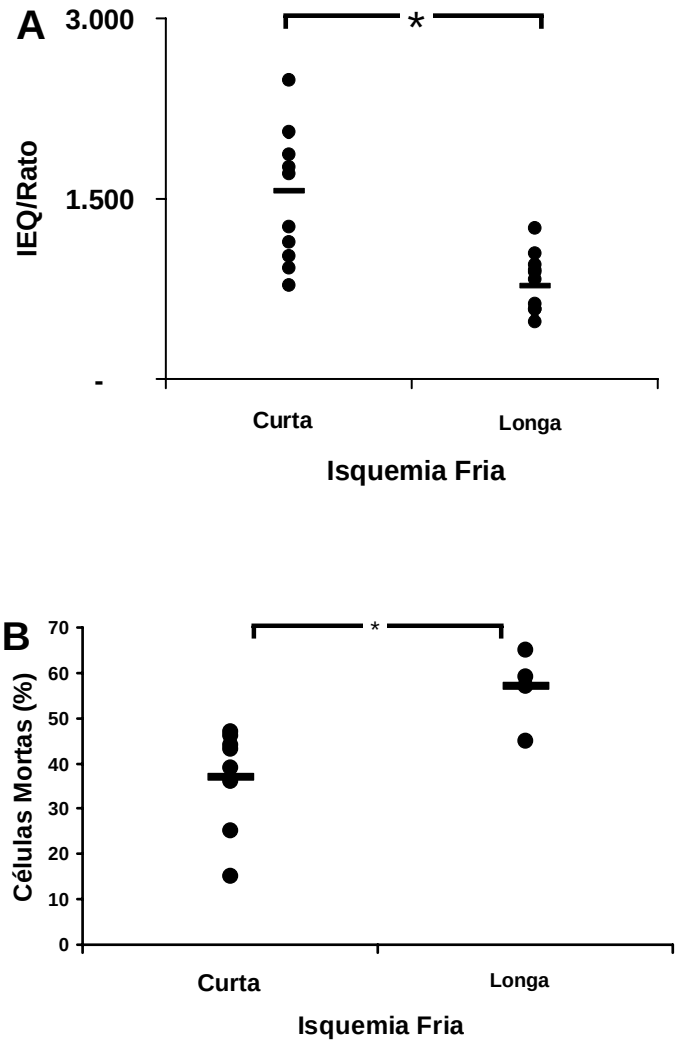
Table 3.1. Análise histopatológica de dano a pâncreas causado por isquemia a fria.

	Isquemia Curta	Isquemia Longa	p
Ilhota	0.7 ± 0.9	0.9 ± 0.9	NS
Apoptose de ilhota	0.6 ± 0.9	0.8 ± 0.9	NS
Tecido Acinar	1.6 ± 0.3	2.4 ± 0.9	0.003
Apoptose acinar	0.6 ± 0.9	0.8 ± 0.9	NS
Tecido Ducto (ductal tissue)	0.6 ± 0.9	0.7 ± 0.9	NS
Necrose	1.4 ± 1.3	1.8 ± 1.1	NS
Avaliação patológica total	1.7 ± 1.3	2.4 ± 0.9	0.004

4.3.2 Efeitos de isquemia a frio em viabilidade e potência de ilhotas

Pâncreas submetidos a S.I. e L.I. e foram processados para obter ilhotas. O rendimento de ilhotas de pâncreas de rato que sofreu L.I. (868.3 ± 235,8 IEQ/rato; N=14) foi menor se comparado a ilhotas obtidas a partir de pâncreas que sofreu S.I. (1560,8 ± 528,4 IEQ/rato, N=12; p = 0,00004) **(Figura 3.1A).** Quando analisamos ilhotas recém-isoladas após dissociação e após corar com 7AAD para citometria de fluxo (*flow cytometry*), células provenientes de pâncreas que haviam sido expostas a L.I. apresentaram maior proporção de células mortas (56,6 ± 8,2%; N=8) do que as provenientes de pâncreas de S.I.

(36,8 ± 11,3; N=4; p = 0,011) **(Figura 3.1B).** A recuperação das ilhotas depois da cultura durante a noite (overnight culture) é usada como um marcador de qualidade de ilhotas. No presente estudo, a recuperação das ilhotas provenientes do grupo que sofreu L.I. foi reduzida (55% ± 20; N=12) se comparada ao grupo que sofreu S.I. (82% ± 13; N = 7; p = 0,005) **(Figura 3.1 C).**



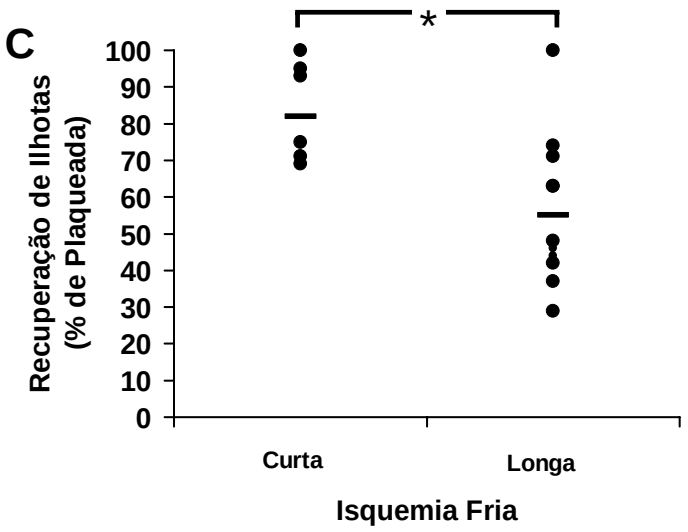


Figura 3.1 Análise do rendimento e viabilidade das ilhotas (A). Rendimento de ilhotas/rato obtidas após isolamento. **(B)** Viabilidade de células de ilhotas foi analisada por meio de citometria de fluxo com células coradas com o marcador 7AAD (indica morte celular) em ilhotas dissociadas. **(C)** Recuperação de ilhotas após cultura durante a noite foi estimada em % de IEQ separadas para cultura no dia anterior.

Para poder acessar a potência das ilhotas em restaurar euglicemia de recipientes diabéticos, transplantamos massa marginal à ideal de ilhotas em camundongos nude com diabetes química. Nossos dados sugerem que os efeitos de isquemia fria prolongada do pâncreas podem resultar em pior qualidade e pior função de ilhotas, quando comparadas com as do grupo controle (S.I.), por meio de transplante no modelo "marginal mass". **(Figura 3.2)**. Todos os recipientes de massa margina à ideal de ilhotas obtidas de S.I. reverteram diabetes, enquanto apenas 80% (4/5) reverteram no grupo L.I.. O tempo médio de reversão da diabetes foi de cinco dias no grupo S.I. e de 18 dias no L.I. **(Figura 3.2).**

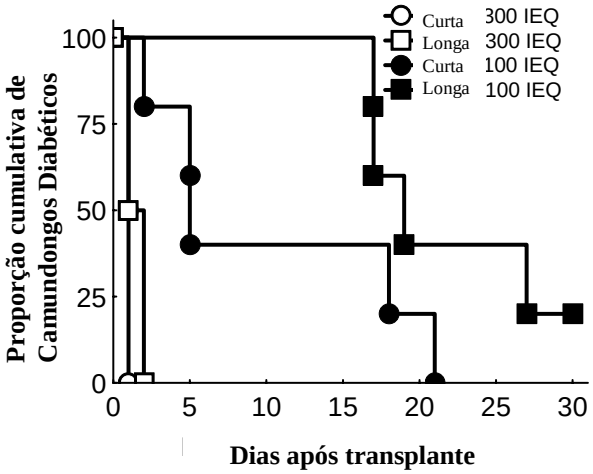


Figura 3.2. Avaliação *in vivo* da potência de ilhotas. Massa maginal à ideal (100 IEQ) e massa ótima (300IEQ) foram transplantadas debaixo da cápsula do rim de camundongos nude diabéticos. Massa ótima reverteu diabetes em recipientes de ilhotas provenientes tanto do grupo S.I. quanto do grupo L.I..Quando comparamos as mesmas ilhotas em massa marginal à ideal, as que vieram do grupo S.I. reverteram mais rapidamente do que as ilhotas que vieram do grupo L.I..

4.3.3 Modulação de proteínas quinase ativadas por *stress* em pâncreas que sofreu isquemia fria.

Para que se possa estudar os possíveis mecanismos de morte celular de ilhotas obtidas de pâncreas expostos a L.I. e S.I., analisamos SAPK em ilhotas recém-isoladas. Encontramos fosforilação aumentada de JNK e p38 em ilhotas recém-isoladas de glândulas L.I. em comparação com S.I. **(Figura 3.3).** A análise de quinases adicionais (como as ERK1,2, AKT e Ik-B) não mostram diferença em fosforilação entre os dois grupos. A análise de níveis de citocinas e quimocinas em sobrenadante de ilhotas lisadas não demonstrou significativas diferenças entre os dois grupos analisados (dano não mostrado).

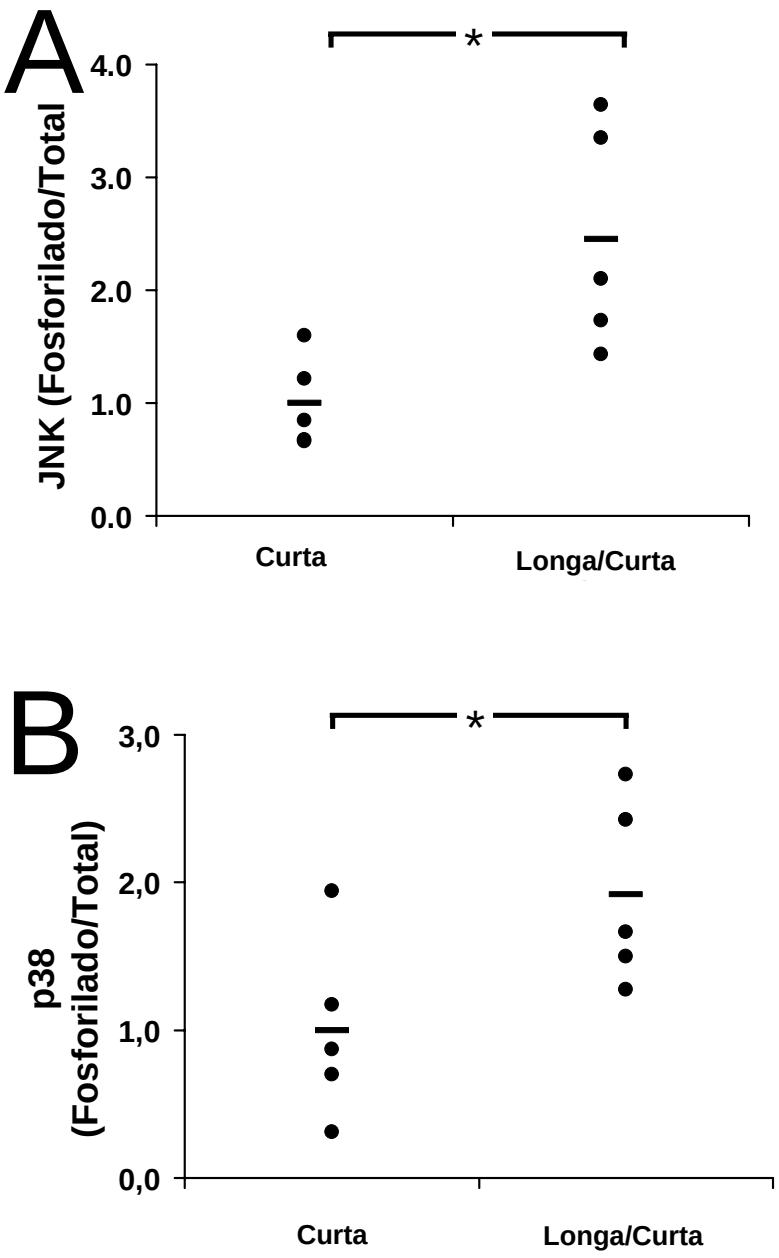


Figura 3.3. Ativação da fosforilação de JNK e p38 em ilhotas de rato provenientes de pâncreas exposto a S.I. e L.I.. **(A)**Ativação de JNK foi significativamente mais elevada em ilhotas de L.I. (2,45 ± 0,99) do que em ilhotas de S.I. (1,00 ± 0,40; p = 0,016). **(B)** A ativação de p38 também é mais pronunciada em L.I. (1,92 ± 0,63) do que em S.I. (1,0 ± 0,61; p = 0,047).

4.3.4 Efeitos de inibidor de JNK durante isquemia a frio de pâncreas.

Diante da ativação da fosforilação de JNK observada, decidimos executar experimentos preliminares com inibidor de JNK (JNKi), durante isquemia a frio. A TAT-JNKi diluída em solução UW foi injetada no ducto pancreático após lavagem do mesmo para retirada de sangue. Os resultados preliminares foram encorajadores. No grupo de L.I. que recebeu o inibidor TAT-JNKi obtivemos 1200 IEQ/rato de ilhotas em comparação com 600 IEQ/rato de um segundo grupo de L.I. que não recebeu o inibidor. Experimentos adicionais estão sendo planejados para confirmação desse dado.

Também analisamos a potência das ilhotas provenientes do grupo tratado com TAT-JNKi em comparação com o grupo não tratado, usando massa marginal à ideal (*marginal mass transplantation*) de ilhotas em camundongos diabéticos. Depois de cultivar ilhotas durante a noite, elas foram contadas e transplantadas debaixo da cápsula do rim de nude diabéticos. O tempo de reversão de diabetes foi comparável nos dois grupos, sugerindo que TAT-JNKi foi suficiente para aumentar o número de ilhotas disponíveis, no entanto, não afetou a função, o que se reflete em igual capacidade de reversão de diabetes *in vivo* **(Figura 3.4).**

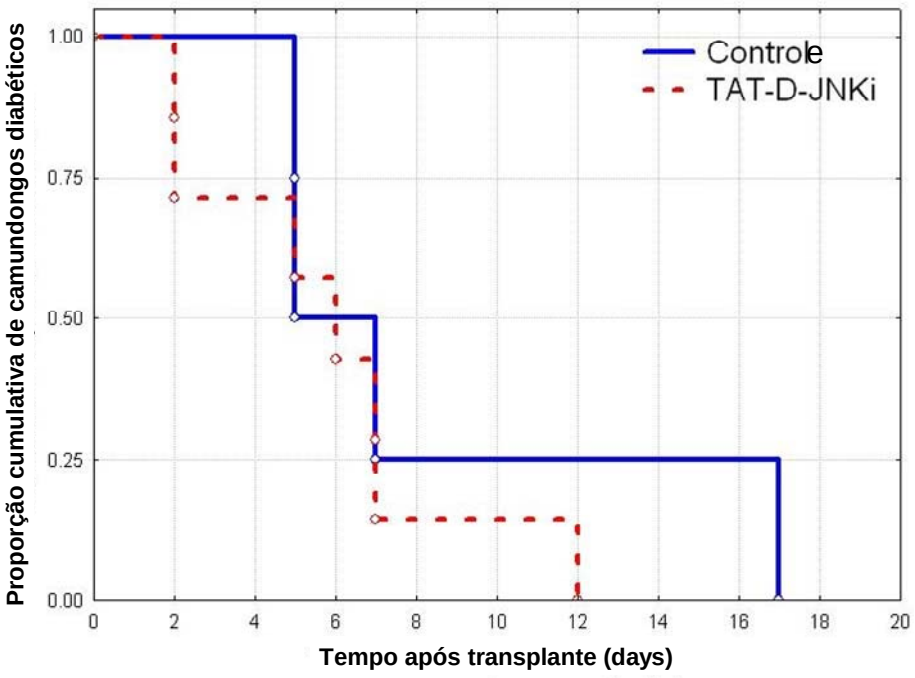


Figura 3.4 Transplante de massa de illhotas marginal á ideal (100IEQ) debaixo do rim de camundongos nude diabéticos. Animais receberam ilhotas isoladas de pâncreas que sofreram isquemia longa com (n=6) ou sem (controle; n=7) o inibidor de JNK, TAT-D-JNKi na hora de captação (procurment).

4.3.5 Análise de proteômics em pâncreas de rato submetido a S.I. versus L.I.

A proteína foi extraída de pâncreas e tratada com Cy3 ou Cy5. Diferente de géis 2D convencionais em que duas amostras são corridas em géis separados, esse método separa as duas amostras com corantes fluorescentes diferentes em um mesmo gel, eliminando assim variações entre géis e permitindo uma comparação de diferentes níveis de expressão relativa. Depois da separação de proteínas por focus isoelétricos (isoelectric focusing) e SDS-PAGE, encontramos algumas proteínas que estavam superexpressas ou subexpressas, como mostrado em cor vermelha ou verde, no gel da **Figura 3.5.**

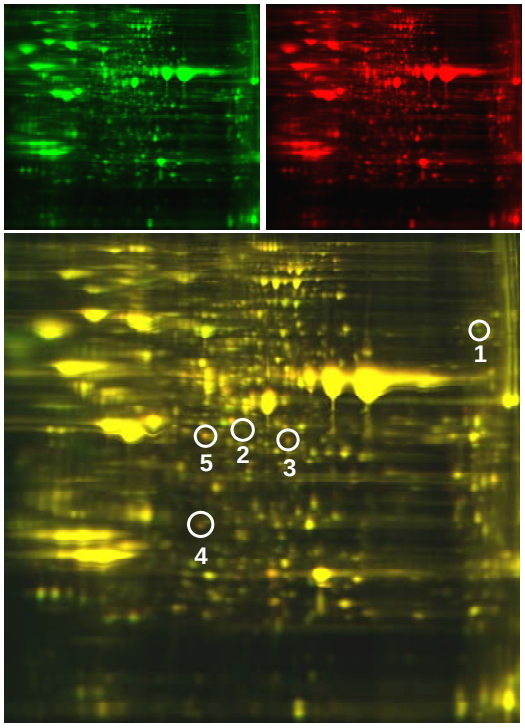


Figura 3.5 2-D gel de proteínas de pâncreas submetidos a S.I. e L.I. Identificação de expressão diferencial de proteínas de pâncreas de S.I. (Cy3 – verde) e L.I. (Cy5 – vermelho). Gel amarelo representa a imagem sobreposta dos géis verde e vermelho. Perfil de proteínas potencialmente interessantes é aquele que depois da imagem sobreposta ainda se apresenta em cor verde ou vermelha. 2D-gel de S.I. (Cy3) e L.I. (Cy5) mostrando 5 pontos escolhidos para análise em espectrometria de massa.

Table 3.2. Tabela mostra expressão diferencial entre S.I. e L.I. pâncreas.

ID	Name	Symbol	Accession No.	Function	Fold Change
1	Eukaryotic Translation Elongation Factor-1 Alpha-2	EEF1 EEF1A1 EF1A2	gi|15805031	Translation regulation	-1.5
2	Heterogeneous Nuclear Ribonuclearprotein H1	HNRPH1 hnRNPH	gi|18104446	Ribonuclease	-1.97
3	Poly(rC) Binding Protein 3	PCBP3	gi|50927011	Kinase	1.5
4	Carboxipeptidase B1	CPB1 PASP PCPB PCB	gi|6978697	Peptidase	1.54
5	Similar to Serine or Cysteine Proteinase Inhibito, clade		gi|34875374		1.85

Estamos particularmente interessados em proteínas que apresentam expressão diferencial da ordem de 1,5 vezes ou mais. Como mostra a **Tabela 3.2,** encontramos cinco proteínas que atendem a esse quesito e, portanto, são possíveis candidatas a identificação e caracterização.

5. Discussão

5.1 Discussão Parte I (*Heme oxigenase -1 fused to TAT peptide transduces and protects pancreatic β-cell.* – artigo em consideração como requisito para PhD).

Depois de clonar a região codante do DNA da HO1 no vetor de expressão pTAT e subclonar esse vetor em Bl21 para expressão protéica, percebemos que a cultura tinha uma coloração verde **(Figura 1B – Anexo 1).** Isso era consistente com a acumulação de biliverdina em bactéria, em razão da falta de biliverdina redutase em bactérias. A coloração foi um bom indicador do nível de proteína produzido.

Conseguimos purificar TAT-HO1 e encontramos as duas formas dessa proteína descrita na literatura: a forma ligada à membrana (39Kda) e a forma citoplasmática (37Kda) **(Figura 1C-Anexo 1).** Ambas as formas são reconhecidas por anticorpo contra histidina em *Western bloting* - seis histidinas em tândem foram adicionadas a proteína antes do transportador TAT, durante clonagem para facilitar a purificação protéica em coluna de afinidade em Ni-agarose".

A atividade específica da proteína foi medida em 24 X $10^3\eta$ mol de bilirrubina/mg de proteína/h o que é mais alta que atividades reportadas em outras fontes (72,73).

Quando adicionamos TAT-HO1 a células β-TC3 (células de insulinoma de camundongo) percebemos que a transdução acontecia em toda célula analisada por microscopia confocal **(Figura 2- Anexo 1).** As células eram simultaneamente coradas com PI (propidium iodine) para excluir células que

tivessem dano em membrana. TAT-HO1 não só foi muito eficiente em penetrar células de insulinoma, mas o fez através de toda célula sem citolocalização preferencial. TAT é um transativador viral e, como tal, poderia ter uma citolocalização nuclear preferencial, no entanto isso parece não ocorrer.

Uma vez analisada a eficiência de transdução da HO1 em células, testamos a atividade biológica da enzima em citoproteger contra a ação de TNFα/CHX. TAT-HO1 foi adicionada à cultura de β-TC3 duas horas antes do tratamento com TNFα/CHX e foi capaz de proteger as células de morte celular (60-80% de células vivas) em comparação com células tratadas apenas com TNFα/CHX (30% de células vivas). Isso indica que TAT-HO1 é biologicamente ativa e que TAT é um eficiente transportador de proteína. Insulinoma, tratado apenas com HO1 sem a fusão TAT, não foi capaz de proteger células contra a ação de TNFα/CHX **(Figura 4-Anexo 1).**

Uma vez que proteínas bioativas foram previamente demonstradas como sendo tóxicas a células, era importante mostrar que TAT-HO1 não afetaria a fisiologia de liberação de insulina. Para isso ilhotas recém-isoladas foram incubadas com TAT-HO1 e transplantadas debaixo do rim de camundongos diabéticos e imunodeficientes após oito dias de cultura. Tanto os camundongos que receberam ilhotas tratadas como os que receberam ilhotas não tratadas com TAT-HO1 reverteram o diabetes. Após nefrectomia e retirada do enxerto em alguns animais, o diabetes retornou prontamente, confirmando que o enxerto é que sustentava euglicemia **(Figura 3- Anexo 1).**

Investigamos, também, qual era o efeito da transdução da proteína TAT-HO1 em preservação de ilhota em cultura. Percebemos que TAT-HO1 preserva

ilhotas da deteriorização causada por cultura. Após 16 dias de cultura, ilhotas tratadas com TAT-HO1 apresentavam menos sinais de hipóxia (necrose) e formação de grumos se comparadas a ilhotas sem o mesmo tratamento. Consistente com uma morfologia melhor, as ilhotas tratadas com TAT-HO1 tinham bom índice de estimulação quando comparadas a ilhotas não tratadas, que, por sua vez, não eram responsivas a variação na presença de glicose **(Figura 5- Anexo-1).** No presente momento, não sabemos se, além da preservação de células beta em cultura, TAT-HO1 também seja capaz de melhorar a performance de células beta. Para responder a essa questão, transplante usando uma massa marginal à ideal para reversão da diabetes terá que ser usada.

5.2 Discussão da Parte II (Remoção de endotoxina de proteínas recombinantes após tratamento em pH ácido dentro de tubos de polipropileno - Manuscrito submetido para *Protein Expression and Purification*).

Eliminação de endotoxina de soluções protéicas é um dos passos mais desafiadores de purificação protéica, uma vez que a eficiência do protocolo varia com as propriedades físico-químicas de cada proteína. A maior parte dos procedimentos são caros, longos e com baixo rendimento protéico. Propomos aqui o uso de um método fácil, rápido e barato que pode ser executado na maior parte dos laboratórios do mundo. A metodologia vem da simples observação de que, depois da purificação protéica e preservação a 4°C em cones de 50 ml (Corning ou Falcon), o conteúdo de endotoxina diminui com o tempo. Depois de executar experimentos com FITC-LPS nos mesmos cones, observamos uma diminuição similar em fluorescência ao longo do tempo, levando-nos a acreditar que o plástico de polipropileno poderia estar adsorvendo endotoxina como

previamente descrito na literatura (65) (Dados não mostrados). Notamos, também, que depois que diminuíamos o pH para pH abaixo do fisiológico, a eficiência da eliminação de endotoxina de soluções protéicas era ainda maior, chegando a 99,9% em alguns casos. O rendimento protéico na maioria dos casos variava com o tratamento em diferentes pHs. As proteínas aqui testadas tiveram rendimento protéico que variava entre 60 e 97%, indicando que é necessário tentar diferentes pHs até que se encontre o pH mais adequado para a eliminação da endotoxina e a preservação do rendimento protéico.

Uma vez aplicado o método de eliminação de endotoxina nas proteínas aqui testadas, medíamos os níveis de endotoxina usando o método LAL. Esse método mostrou que níveis significativos de eliminação de endotoxina haviam sido atingidos. A extração de inibidores provenientes de tubos de polipropileno **(Figura 3 Anexo 2),** assim como presença de β-glucana, são conhecidos como inibidores do ensaio de LAL. Ambos os inibidores não foram detectados nos experimentos.

Testamos a atividade biológica e a qualidade da TAT-HO1 após a eliminação de endotoxina e percebemos um aumento da atividade biológica da proteína quando desprovida da endotoxina contaminante. A atividade biológica foi testada usando insulinoma β-TC3 tratado com TNFα, ciclohexamina e TAT-HO1 (31), na presença ou ausência de endotoxina **(Tabela 1.3- Anexo 2).** Isso mostra não só que a proteína TAT-HO1 permanecia biologicamente ativa, mas que também a endotoxina parecia estar contribuindo para a toxicidade celular (55). A qualidade da proteína TAT-HO1 foi analisada após eliminação de endotoxina, usando o sistema Agilent 2100 Bioanalyzer, e não se mostrou

diferente da mesma proteína com a presença de endotoxina. Isso indica que o tratamento em pH ácido não alterou o padrão protéico apresentado.

Como o nosso objetivo era atingir níveis de eliminação de endotoxina que fossem compatíveis com estudos *in vivo*, decidimos testar *in vivo* a pirogenicidade em coelhos das proteínas tratadas para eliminação de endotoxina. Nesse contexto, as proteínas seriam expostas ao sistema imunológico de um animal que é muito mais complexo que o ensaio *in vitro* de LAL.

O mesmo preparado protéico foi dividido entre dois grupos de coelhos: um grupo que recebeu a proteína com 20 EU/ml e o segundo que recebeu a proteína com 0,3EU/ml, após ser submetido ao protocolo de eliminação de endotoxina. O procedimento de eliminação de endotoxina mostrou-se eficiente *in vivo*, confirmando os testes *in vitro* feitos com ensaio LAL. Já a proteína que não foi submetida à retirada de endotoxina mostrou-se altamente pirogênica **(Figura 4 Anexo 2)**

Não realizamos nenhum experimento mecanístico para desvendar como a eliminação de endotoxina acontece. Acreditamos que a adsorção pelo plástico polipropileno, assim como a mudança em pH fisiológico da proteína, exerça algum papel no processo de descontaminação. No entanto, experimentos futuros precisam ser feitos para elucidar essa questão.

Existe a possibilidade ainda de que não eliminamos a endotoxina da solução protéica, mas apenas induzimos uma mudança conformacional na estrutura da molécula, fazendo com que a porção correspondente ao lipídio A fique indisponível para detecção por meio do ensaio LAL. Ainda assim, é

improvável que essa mudança conformacional seja micela, uma vez que essa formação acontece em pHs básicos e não ácidos como foi o caso aqui descrito.

Em função da nossa preocupação com a possibilidade dessa mudança conformacional ser temporária e da possibilidade de haver nova organização da molécula de endotoxina uma vez dentro de um ambiente *in vivo*, decidimos executar experimentos *in vivo* em coelhos com proteínas que haviam sido submetidas à eliminação de endotoxina aqui descrita. Nossos resultados mostraram que a possível mudança conformacional era provavelmente permanente, uma vez que não havia pirogenicidade na proteína submetida à eliminação de endotoxina nem *in vitro* nem *in vivo*.

O método aqui descrito poderia ser usado em larga escala ou escala industrial se pequenas adaptações fossem incorporadas ao sistema. Uma idéia seria fazer microesferas do mesmo polipropileno usado nos tubos Corning e Falcon. Essas esferas poderiam ser usadas tanto em soluções contaminadas com endotoxina sob agitação como em colunas em que se poderia passar a solução para aumentar a exposição de superfície. Isso provavelmente aumentaria a eficiência e diminuiria o tempo de eliminação de endotoxina de soluções protéicas.

Concluindo, fomos capazes de desenvolver um método simples, eficiente, rápido e barato para eliminar endotoxina de proteínas recombinantes que nos permitiu proceder com a aplicação *in vivo* de diversas proteínas produzidas no nosso laboratório. É importante notar que o ajuste em pH deve ser feito para cada proteína a ser testada com esse método de eliminação de

endotoxina, com o objetivo de atingir máxima eliminação de endotoxina, juntamente com um nível de rendimento protéico aceitável.

5.3 Discussão da Parte III (Efeitos de isquemia fria de pâncreas na ativação da expressão de proteínas quinase ativadas por *stress* em ilhotas: impacto em transplante de ilhotas).

Constantes progressos têm sido reportados na área de transplante de ilhotas (74). O número de pâncreas processados para obtenção do número adequado de ilhotas necessárias para transplante ainda é desproporcional ao número de potenciais recipientes, tendo em vista que há em torno de 50% de sobrevivência de ilhotas por procedimento (2,10,75-83). Ainda que se atinja a independência de insulina após o transplante de ilhotas provenientes de um único órgão doado, são normalmente necessários pelo menos dois pâncreas por recipiente para se atingir a independência da insulina (76,84). Isso se deve em parte às limitações em isolamento de ilhotas e à qualidade subótima de órgãos (82). Morte cerebral é associada com desbalanceamento hemodinâmico, que pode levar à hipóxia nos distritos esplênicos, incluindo pâncreas, resultando em perda dramática de função e número de ilhotas (7). A isquemia fria prolongada pode ser danosa à preservação de pâncreas e à recuperação de ilhotas (10,80,85-87). O uso do *"two-layer method"* para preservação de pâncreas tem sido reconhecido pelos efeitos positivos que exerce no rendimento de ilhotas após isquemia, usando doadores considerados marginais e doadores de morte por motivos cardíacos (82,88-95).

Obter números adequados de ilhotas funcionais é um pré-requisito fundamental para o sucesso de transplante de ilhotas. Superar as limitações atuais na obtenção de maior número e disponibilidade de células beta é uma das

prioridades da área de reposição de ilhotas(12,96). Enquanto alternativas para a reposição de células produtoras de insulina (incluindo células ramo ou xenogênicas) estão sendo buscadas (97-99), há urgência na otimização da disponibilidade de ilhotas cadavéricas humanas. A implementação de intervenção molecular pode representar uma alternativa viável para conferir citoproteção a ilhotas, melhorando a recuperação e a qualidade das mesmas.

O modelo de isquemia em rato foi estabelecida com a intenção de avaliar os efeitos de isquemia fria de pâncreas em ilhotas para transplante e, ao mesmo tempo, poder desenvolver a intervenção molecular específica para tentar prevenir esses efeitos. Nossos dados indicam que isquemia por longos períodos de tempo resulta em redução significativa do rendimento de ilhotas, da recuperação durante incubação (*overnight incubation*) e do potencial de reversão de diabetes **(Figuras 3.1 e 3.2).**

O efeito de isquemia no pâncreas também foi avaliado usando análise histopatológica, que mostrou que o tecido acinar foi o maior contribuinte para a avaliação de dano pancreático como um todo **(Tabela 3.1; p=0,004).** Nossos dados estão em concordância com trabalhos previamente reportados de que a apoptose de célula acinar é conseqüência de dano causado por isquemia e reperfusão (100,101), assim como por pancreatite. É possível que o dano isquêmico acinar seja exacerbado durante o processo de digestão pancreática, quando a temperatura aumenta para 37°C para a ativação da enzima, expondo o pâncreas a *stress* químico e mecânico. O pâncreas exposto à isquemia longa tem menor rendimento em número de ilhotas se comparado a pâncreas proveniente de isquemia curta, indicando o impacto negativo de isquemia no rendimento

final de ilhotas. Nos grupos de pâncreas submetidos à longa isquemia fria, a perda de ilhotas durante cultura (*overnight culture*) foi maior do que em grupos controle (curta isquemia), sugerindo que isquemia de pâncreas afeta dramaticamente ilhotas no que diz respeito a número e viabilidade. Os efeitos deletérios de isquemia de pâncreas também são notados em ensaios mais extringentes, como o transplante *in vivo* de ilhotas no modelo de massa marginal à ideal -"*marginal mass"* em camundongos. A reversão da diabetes ocorre de forma retardada comparada ao transplante feito com ilhotas provenientes de isquemia curta.

Para poder identificar mecanismos moleculares fundamentais associados à deteriorização de ilhotas, decidimos avaliar SAPK em ilhotas recém-isoladas. A contribuição de p38 MAPK para o destino das ilhotas, durante e depois do isolamento, ainda não é compreendido em sua totalidade. A ativação de p38 MAPK durante o isolamento foi recentemente descrito em um modelo em rato (11). Relatos prévios sugerem que a ativação de p38 pode ser menor logo depois do isolamento de ilhotas, aumentando com o passar do tempo (14). Não há consenso se o aumento da ativação de p38 resulta em sobrevivência celular ou apoptose (102). Relatos recentes usaram inibidores de p38 MAPK durante criopreservação de ilhotas, resultando em melhor recuperação no que diz respeito à qualidade de ilhotas (103). No presente estudo, encontramos p38 e JNK significativamente aumentados em ilhotas provenientes de pâncreas submetido à isquemia longa, quando comparado a glândulas expostas à curta isquemia **(Figura 3.3).** Nossa observação difere da de Matsuda (11) que descreve níveis comparáveis de p38 ativado em ilhotas recém-isoladas, quando comparadas a glândulas que sofreram longa e curta isquemia.

Nosso estudo também mostrou aumento da ativação de JNK em ilhotas recém-isoladas de pâncreas submetido à longa isquemia, quando comparada à curta isquemia. Essa observação está em concordância com relatos prévios da literatura. A prevenção da ativação de JNK pelo uso de inibidores de JNK tem levado à sobrevivência de maiores números de células (32,71,102,104,105). Inspirados pelas observações descritas, decidimos fazer experimentos usando TAT-PTD como transportador de inibidor de JNK em pâncreas de rato antes da preservação a frio. Administramos TAT-JNKi no ducto pancreático antes de submeter o pâncreas à isquemia longa a frio. Descobrimos que o rendimento de ilhotas era o dobro quando comparado a um grupo de controle também submetido à longa isquemia, mas sem tratamento com inibidor. Transplantamos essas ilhotas *in vivo* no modelo de transplante "*marginal mass*" em camundongos. Os valores de glicemia foram monitorados para detecção da reversão da diabetes e obtivemos valores de reversão similares nos dois grupos testados. Esses resultados, ainda que preliminares, sugerem que o tratamento com TAT-JNKi não afetou a potência das ilhotas. O aumento do número de ilhotas recuperadas a partir do pâncreas submetido à longa isquemia e tratado com TAT-JNKi pode contribuir para a redução do número de órgãos necessários por receptor para atingir a independência de insulina.

Inicialmente selecionamos o enantiômero D da molécula inibidora de JNK pelo seu potencial de meia-vida prolongada em comparação com o enantiômero L. Vale ressaltar que nosso grupo recentemente encontrou que TAT-D-JNKi é tóxico para ilhotas de suíno *in vitro* e, portanto, não deve ser a molécula mais eficiente na citoproteção de ilhotas (100). O resultado parcialmente positivo observado no modelo de preservação de pâncreas a frio

pode ser atribuído, ainda que em parte, às diferentes condições experimentais *ex-vivo,* em que a ativação de JNK pode se dar em diferentes vias de transdução de sinal se comparadas ao modelo *in vitro*. Atualmente estamos no processo de preparação para experimentos adicionais com TAT-L-JNKi, para poder avaliar as possíveis diferenças em citoproteção de ilhotas das duas isoformas.

A intervenção molecular, usando TAT-PTD para atingir seletivamente vias moleculares que estejam envolvidas com morte de ilhotas, pode auxiliar na melhora da qualidade e do número de ilhotas recuperadas a partir de um só órgão o que melhoraria o índice de sucessos em transplante de ilhotas. Coletivamente, nossos estudos nos permitiram estabelecer um modelo de isquemia a frio em pâncreas de rato para poder avaliar o mecanismo molecular associado com dano em órgão que leva a menores números e a pior qualidade das ilhotas isoladas. Esse modelo nos permitiu identificar contribuintes, tais como p38 e JNK das SAPK, como transdutores de sinais que levam a dano isquêmico nesse modelo. Os resultados preliminares obtidos com TAT-JNKi são animadores e nos incentivam a seguir com futuros experimentos tentando encontrar moléculas ainda mais adequadas para a citoproteção de ilhotas. Atualmente estamos analisando resultados provenientes de microarranjo (*microarray)* e proteômics em pâncreas submetidos à isquemia longa e curta. Identificamos alguns candidatos que poderiam servir de biomarcadores para danos causados por isquemia fria. A análise de microarranjo de pâncreas nas mesmas condições também estão sendo feitas e nos permitirão identificar genes que são expressos como conseqüência de isquemia. Isso por sua vez nos permitirá identificar genes que poderão ser usados como alvos terapêuticos para intervenção no modelo de isquemia em rato.

6. Objetivos atingidos e perspectivas

Objetivo1 e 2:

A proteína TAT-HO1 foi purificada em grande quantidade. A atividade biológica foi testada por meio da atividade enzimática e da proteção de insulinoma de células beta à ação da citocina TNFα associada à ciclohexamina (CHX). A atividade enzimática mostrou-se maior do que o descrito na literatura. A proteção de insulinoma de células beta ficou em torno de 60 a 80% (sobrevivência), quando submetido ao tratamento de TAT-HO1 + TNFα/CHX, se comparada às mesmas células tratadas com TNF/CHX apenas (30% de sobrevivência).

Objetivo 3:

Ilhotas de rato foram cultivadas por oito dias na presença de TAT-HO1 e transplantadas embaixo da cápsula do rim de camundongos diabéticos e imunodeficientes. A diabetes foi revertida nesses animais e em animais que receberam ilhotas cultivadas pelo mesmo período de tempo, sem TAT-HO1. Esse experimento mostra que essa proteína não é tóxica para a ação de liberação de insulina nas ilhotas pancreáticas.

Objetivo 4:

Desenvolvemos um método eficiente, simples e barato para remover endotoxina contaminante de soluções protéicas que pode ser aplicado em qualquer laboratório que tenha a seu dispor cones de polipropileno (Corning ou Falcon), desde que devidamente testados para a presença de inibidor do ensaio de detecção de endotoxina LAL. O rendimento protéico e a atividade biológica

foram altas após eliminação de endotoxina contaminante. O método de eliminação de endotoxina baseou-se no tratamento em pH ácido da solução protéica em tubos de polipropileno. O mecanismo mais provável envolve mudanças conformacionais na proteína ou na endotoxina, como conseqüência da mudança de pH além de adsorção na superfície de polipropileno. Experimentos adicionais terão que ser feitos para elucidar os detalhes do mecanismo de eliminação de endotoxina.

Objetivo 5:

Depois de analisar diferentes SAPK, fomos capazes de detectar o aumento da fosforilação de JNK e p38 como conseqüência de isquemia pancreática. Testamos a redução de morte de ilhotas provenientes de pâncreas que sofreram isquemia longa tratadas com a molécula quimicamente sintetizada TAT-D-JNKi e fomos capazes de dobrar o número de ilhotas obtidas a partir desses pâncreas se comparados a pâncreas que também sofreram isquemia longa sem o tratamento com a mesma molécula. Experimentos adicionais precisam ser feitos para confirmar esses dados. Atualmente estamos preparando experimentos *in vivo* com a molécula TAT-L-JNKi.

Objetivo 6:

O número de ilhotas obtidas foi analisada usando DTZ e convertendo esse número ao equivalente em ilhotas (IEQ). A qualidade de ilhotas foi determinada usando as técnicas de "*marginal mass*" em que ilhotas são transplantadas em camundongos diabéticos e imunodeficientes para a reversão da diabetes. As

duas técnicas acima descritas são rotineiramente usadas na área de transplante de ilhotas e encontram-se descritas na literatura. Tentamos aplicar novos métodos usados em ilhotas humanas (REF 99) para analisar a composição das células beta e sua viabilidade em ilhotas de rato e esse método mostrou-se ineficiente nesse modelo, ou seja, não foi capaz de predizer a qualidade das ilhotas de rato.

Análise em Bioplex de ilhotas recém-isoladas pode predizer a diferença na ativação da fosforilação de JNK e p38.

Avaliação histopatológica mostrou-se eficiente em predizer danos causados por isquemia a frio por meio de alterações significativas em tecido acinar.

Estamos analisando no momento dados provenientes de “proteômics” e “microarray” (microarranjo) com o objetivo de verificar se há como determinar um biomarcador protéico de dano causado por isquemia a frio, assim como genes que são diferencialmente expressos e que possam servir como alvos terapêuticos para a prevenção de morte de ilhotas.

Objetivo 7:

Esse objetivo foi alcançado apenas parcialmente com o uso de TAT-D-JNKi em pâncreas que sofreria longa isquemia a frio. As demais proteínas fundidas a TAT, reportadas na secção de produção científica, ainda não foram testadas no modelo de isquemia em pâncreas de rato. Somente agora, após desenvolver um método barato para a eliminação da endotoxina de soluções protéicas, poderemos usar as proteínas de fusão no ducto pancreático de pâncreas que sofrerão isquemia a frio. Esperamos com isso poder prevenir a morte celular de

ilhotas, aumentando assim o número de ilhotas obtidas a partir de pâncreas que tenha sofrido isquemia.

7. Produção científica durante o período de desenvolvimento do PhD (2003-2007).

Publicações em revistas científicas durante o curso do PhD:

1. **Ribeiro MM**, Klein D, Pileggi A, Damaris Molano R, Fraker C, Ricordi C, Inverardi L, Pastori RL. Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic beta-cells. Biochem Biophys Res Commun 2003;305(4):876-81. PMID: 12767912

2. Pastori RL, Klein D, **Ribeiro MM**, Ricordi C. Delivery of proteins and peptides into live cells by means of protein transduction domains: potential application to organ and cell transplantation. Transplantation 2004;77(11):1627-31. PMID: 15201657

3. Klein D, **Ribeiro MM,** Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, Molano RD, Inverardi L, Ricordi C, Pastori RL. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 2004;323(2):473-8. PMID: 15369775.

4. Pastori RL, **Ribeiro MM,** Klein D, Ricordi C. Protein Transduction Domain as a Novel Tool for Delivery of Proteins, Peptides and Nucleic Acids. In*: "Biomaterials for Delivery and Targeting of Proteins* and *Nucleic Acids"* RI Mahoto Editor. CRC Press 2005. ISBN 0-8493-2334-7

5. Mendoza V, Klein D, Ichii H, **Ribeiro MM**, Ricordi C, Hankeln T, Burmester T, Pastori RL. Protection of islets in culture by delivery of oxygen binding neuroglobin via protein transduction**.** Transplant Proc 2005;37(1):237-40. PMID: 15808606

6. Domínguez-Bendala J, Klein D, **Ribeiro M**, Ricordi C, Inverardi L, Pastori R, Edlund H. TAT-Mediated Neurogenin 3 Protein Transduction Stimulates Pancreatic Endocrine Differentiation In Vitro. Diabetes 2005;54(3):720-6. PMID: 15734848

Manuscrito atualmente em processo de revisão editorial:

7. **Ribeiro MM**, Ricordi C, Pastori RL. Removal of endotoxin from recombinant proteins after acidic pH treatment in polypropylene tubes. *Protein Expression and Purification.*

Manuscrito em preparação para ser enviado para revisão editorial até final de agosto de 2007

8. **Ribeiro MM, *et al*.** Effects of pancreas cold isquemia on the expression of stress-activated protein kinases in islets: impact for islet transplantation outcome.

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ANEXO 1

8.1 Ribeiro MM, Klein D, Pileggi A, Damaris Molano R, Fraker C, Ricordi C, Inverardi L, Pastori RL. Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic beta-cells. Biochem Biophys Res Commun 2003;305(4):876-81

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Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic β-cells

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Abstract

Transplantation of islets is becoming an established method for treating type 1 diabetes. However, viability of islets is greatly affected by necrosis/apoptosis induced by oxidative stress and other insults during isolation and subsequent in vitro culture. Expression of cytoprotective proteins, such as heme oxygenase-1 (HO-1), reduces the deleterious effects of oxidative stress in transplantable islets. We have generated a fusion protein composed of HO-1 and TAT protein transduction domain (TAT/PTD), an 11-aa cell penetrating peptide from the human immunodeficiency virus TAT protein. Transduction of TAT/PTD–HO-1 to insulin-producing cells protects against TNF-α-mediated cytotoxicity. TAT/PTD–HO-1 transduction to islets does not impair islet physiology, as assessed by reversion of chemically induced diabetes in immunodeficient mice. Finally, we report that transduction of HO-1 fusion protein into islets improves islet viability in culture. This approach might have a positive impact on the availability of islets for transplantation.

Keywords: Protein transduction domain; TAT; Heme oxygenase-1; Islets; Pancreatic β-cells

Transplantation of pancreatic islets has become a promising approach for treating diabetes. The introduction of a novel glucocorticoid-free immunosuppressive regiment [1] has shown that long-term insulin independence can be achieved following islet transplantation. Despite this success, more than one pancreas preparation per recipient is generally required to observe insulin independence. This limitation is in part due to the quality/viability of pancreatic islets. Once isolated, non-vascularized pancreatic islets are easily damaged in in vitro culture by hypoxia and diffusion-limited availability of nutrients, resulting in decreased number and function. Therefore, improvement of islet culture conditions is an important goal in islet transplantation. Such improvement could be provided by direct delivery of protective anti-oxidative stress/anti-apoptotic proteins into islets during culture. This approach has the distinct feature of expressing the desired protein transiently. Temporary expression is an important advantage when the expression of a specific protein is required only for a short period of time. Lipid carriers and protein transduction represent two possible alternatives for protein delivery. Liposome carriers have recently been designed to transport active proteins across cell membranes [2]. Protein transduction is a novel technology by which proteins/peptides can be directly transferred into cells when covalently linked to small peptide domains, known as protein transduction domains (PTDs) [3]. The most common PTDs are part of naturally occurring proteins, among them being homeodomain transcription factors [4], the herpes simplex virus type I protein VP22 [5], and the human immunodeficiency virus *trans*-activator TAT protein [6–8]. Other peptides, derived from screening peptide-libraries, are capable of transducing different types of cells including pancreatic islets [9]. In particular, PTD-5 was used for the delivery of a peptide inhibiting nuclear factor κ B activation in pancreatic islets [10]. TAT/PTD is one of the most widely studied PTDs. We have previously used TAT/PTD

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fusion protein transduction to antagonize apoptosis/cell death in pancreatic β-cells [11]. The mechanism of internalization of TAT/PTDs is currently unknown, although endocytosis has been reported as having a role in protein transduction [12,13].

This work describes the effects of protein transduction of a recombinant TAT/PTD–heme oxygenase-1 on pancreatic islet viability. Heme oxygenase-1 (HO-1) has been identified as a ubiquitous protein induced in many cell types in response to stress conditions [14]. The induction of HO-1 expression is considered a primary adaptive response of the cellular defense mechanism. HO-1 is the rate-limiting enzyme of heme degradation into its by-products carbon monoxide (CO), iron, and biliverdin. CO has a cytoprotective role in different systems [15–18] including pancreatic β-cells [19]. Iron induces the synthesis of ferritin reported as having a cytoprotective role in endothelial cells [20]. Biliverdin is subsequently reduced into bilirubin, a powerful anti-oxidant [21]. Overexpression of HO-1 by gene therapy and chemical induction has been used to reduce the deleterious effects of cytokine-induced apoptosis and oxidative stress in various cell types and animal models [22–26]. The aim of this study was to generate a TAT/PTD–HO-1 fusion protein that could transduce insulin-producing cells, conferring cytoprotection. We produced and purified recombinant HO-1 protein fused in-frame with TAT/PTD. Transduction of TAT/PTD–HO-1 into pancreatic βTC-3 cells conferred protection against TNF-α cytotoxicity. Furthermore, transduction with TAT/PTD–HO-1 was not toxic to islets and remarkably enhanced the insulin secretion capability of cultured islets.

Materials and methods

Cloning and related techniques

The recombinant TAT/PTD anti-apoptotic protein construct was generated by inserting the DNA coding region of murine heme oxygenase-1 (HO-1) (produced by Robert Oliver University of Miami) in the *Eco*RI site of the pTAT expression vector (kind gift from Dr. Steven Dowdy from USDC).

Protein generation and purification

The isolation and purification of TAT/PTD–HO-1 and HO-1 (no TAT) were done as previously described [11] with some modifications in the washing steps: briefly, the Ni–NTA column, with bacterial extract, was sequentially washed with 20 mM imidazole and 30 mM imidazole, both in cold PBS. Elution was performed with 100 mM imidazole. Proteins were preserved at −80 °C with 10% glycerol and quantified with Bio Rad protein assay. Purity was assayed by Tris–HCl SDS–PAGE 12.5% gel. Western blot was done with primary mouse Penta His antibody (Quiagen) (1:1000).

Isolation of rat islet of Langerhans

Islets were isolated from Lewis rats (Charles River Labs) by dissociation of the pancreatic tissue using Liberase RI purified enzyme blend (Roche Molecular Biochemical, Indianapolis, IN) at a concentration of 0.16 mg/ml following procedure described previously [11].

Cell line and rat pancreatic islet culture

βTC-3 cells, DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were cultured in DMEM (high glucose, 4.5 g/L), with 10% fetal bovine serum, and 1% sodium pyruvate (100 mM) at 37 °C and 7.5% CO_2. Rat islet cells were cultured in CMRL 1600 supplemented with 10% FBS under the same conditions. When culturing islets for static glucose challenge, an IEq (islet equivalent) = 50 was distributed into 70 ml non-treated culture flasks, one day after islet isolation. Media with TAT/PTD protein were changed on average every 3–4 days. The content of one 70 ml flask was used for each static incubation column. Rat islets were incubated with TAT/PTD–HO-1 the following morning after isolation and kept in culture for 16 days.

Transduction of βTC-3 with labeled TAT–HO-1

TAT–HO-1 was labeled using FluoReporter FITC Protein Labeling Kit (Molecular Probes). TAT–HO-1-FITC (6.7 μM) was added to a tube containing floating βTC-3 in 500 μl of DMEM. Tube with cells was incubated for 3 h at 37 °C and washed extensively with DMEM. Pellet was resuspended in 500 μl DMEM and plated in a glass-bottomed number 0 uncoated γ-irradiated petri dish for confocal microscopy. Forty microliters of a 0.5 mg/ml solution of propidium iodide (PI) was added to cells before analysis to rule out internalization of the TAT-fused protein due to damaged cell membrane. The samples were examined under a ZEISS confocal laser scanning microscope (LSM-510) equipped with image analysis software.

βTC-3 cell viability after induced TNF-α cytotoxicity

βTC-3 (10^6) cells were transduced for 2 h with TAT/PTD–HO-1 (200 nM) before adding 4000 U of mouse (TNF-α) and 10 μg/ml cycloheximide (CHX). After incubation for 16 h cells were assayed with Live/Dead viability Cytotoxicity Kit (Molecular Probes). Pictures were captured by a charge-coupled device camera (Lei-750; Leica) using Video Vixen software and analyzed by Photoshop version 5.5.

Static glucose challenge of islets of Langerhans

Rat islets were subjected to low (40 mg/dl), high (400 mg/dl), and low (40 mg/dl) static glucose challenge as described previously [11].

Reversion of hyperglycemia in diabetic immunodeficient mice

Animals. Male Lewis rats (150–170 g body weight) were purchased from Charles River (Wilmington, MA) and used as donors of pancreatic islets. Recipient animals were immunodeficient male CB17-*scid* mice at an average body weight of 29 g (Jackson Labs). Animals were rendered diabetic by a single intravenous injection of alloxan 90 mg/kg. The islet transplantation procedure and graft function monitoring were performed as previously described [11].

Results and discussion

Generation and purification of recombinant TAT/PTD–HO-1 fusion protein

To generate a TAT/PTD–HO-1 recombinant protein we inserted the coding region of the murine heme oxygenase gene in-frame with the 11-amino-acid PTD of the HIV/TAT protein in the pTAT-HA expression

vector. Bl 21 *Escherichia coli* was used to produce the recombinant TAT/PTD–HO-1 protein. A green coloration of the bacterial culture was consistently observed, suggesting that the recombinant TAT/PTD–HO-1 metabolized the heme from *E. coli* into biliverdin, that accumulated due to the lack of biliverdin reductase in the bacteria (Fig. 1B). The fusion protein has additional six consecutive histidines to allow for purification of the recombinant protein by affinity chromatography. The result of the purification procedure is shown in Fig. 1C. The fusion protein was expressed at relatively high levels (8 mg/L of culture) and was more than 95% pure as assessed by SDS–PAGE. Previous expression of the rat HO-1 in *E. coli* [27] had produced two forms of the protein, the membrane bound 32 kDa native protein and a 30 kDa form, representing the non-membrane bound HO-1 protein, lacking the hydrophobic C-terminal segment responsible for membrane insertion [28]. Both forms were found to catalyze the breakdown of heme to biliverdin [27]. Similarly, once the purified TAT/PTD–HO-1 shows a band of 39 kDa, the expected size of the fusion protein TAT/PTD–HO-1, and a band of approximately 37 kDa, representing the non-membrane bound recombinant protein. Both bands were determined by Western blot analysis using an anti-histidine antibody (data not shown). The specific activity of the purified TAT/PTD–HO-1, assayed as bilirubin formation in the coupled assay with biliverdin reductase, was 24×10^3 nmols of bilirubin/mg protein/h, slightly larger than that of heme oxygenase-1 from other sources that were purified to homogeneity [29,30].

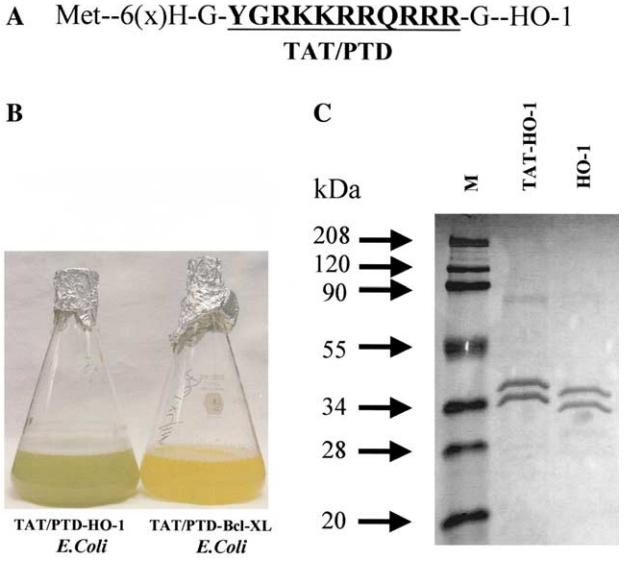

Fig. 1. Generation and purification of heme oxygenase-1 fusion protein. (A) Expression cassette of TAT/PTD–HO-1 fusion protein (TAT/PTD sequence in bold). (B) Culture of BL21 *E. coli* cells was green because of the accumulation of biliverdin. For comparison a culture of *E. coli* expressing TAT/PTD–Bcl-XL fusion protein is shown. (C) Purified fusion protein was analyzed by a 12.5% SDS–PAGE and stained with Coomassie brilliant blue. The gel shows the two forms of TAT/PTD–HO-1 protein; a 39 kDa membrane bound form and the 37 kDa non-membrane bound form.

TAT/PTD–HO-1 efficiently transduces pancreatic β-cells

To test the TAT/PTD–HO-1 transduction capability, pancreatic βTC-3 cells were cultured in the presence of TAT/PTD–HO-1 labeled with fluorescein isothiocyanate (FITC). Cells were simultaneously incubated with propidium iodide (PI) to rule out protein internalization due to damaged cell membranes. Evaluation of TAT/PTD transduction by confocal microscopy was performed with live cells in order to avoid potential artifacts caused by cell fixation [31,32]. PI staining was completely negative. The fusion protein was transduced into all pancreatic β-cells (Fig. 2). Of note, βTC-3 cells have a round shape because transduction was performed with detached cells to avoid high background due to stickiness of TAT–HO-1-FITC.

Transduction of TAT/PTD–HO-1 into islets does not impair insulin secretion capability

Because bioactive peptides were previously shown to damage target cells [33], it was important to verify that TAT/PTD–HO-1 treatment would not damage islet physiology and insulin secretion. For this reason, we assessed their ability to reverse hyperglycemia in chemically diabetic immunodeficient mice. Freshly isolated

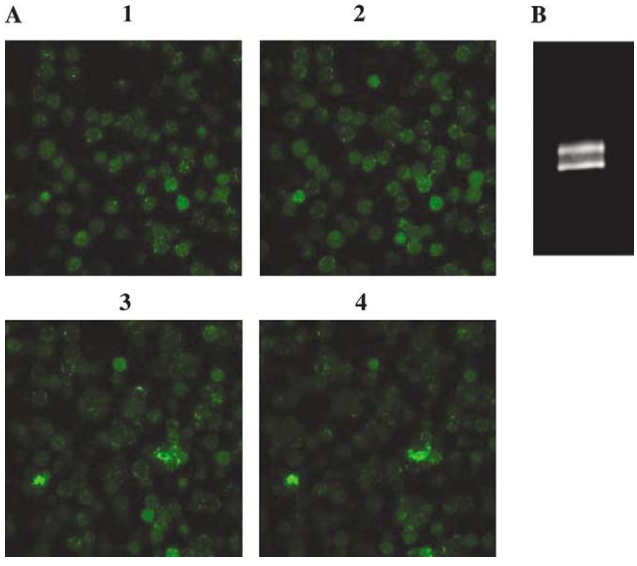

Fig. 2. Transduction of pancreatic βTC-3 cells with TAT/PTD–HO-1-FITC. (A) Transduction of the fusion protein was assessed by confocal laser scanning microscopy of live, non-fixed βTC-3 cells transduced with TAT/PTD–HO-1-FITC for 2 h. Fusion protein fluorescence was analyzed by a 4-step-3.5 μm-Z-position sectional scanning of βTC-3 cells. (B) SDS–PAGE of the fluorescent TAT/PTD–HO-1-FITC labeled protein.

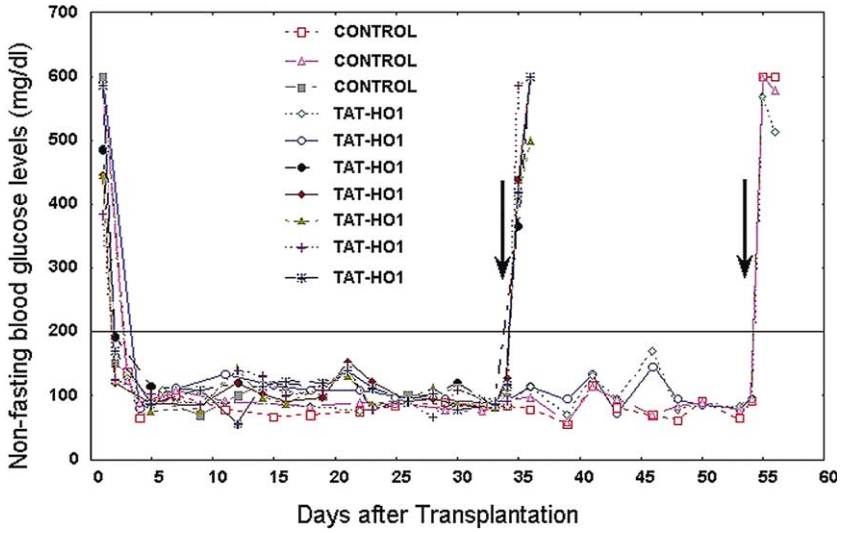

Fig. 3. Transduction of islets with TAT/PTD–HO-1 fusion protein does not affect insulin secretion capability. Control rat islets and TAT/PTD–HO-1 transduced rat isles were transplanted under the kidney capsule of chemically induced diabetic immunodeficient mice. All transplanted islets reversed diabetes and maintained normoglycemia. Removal of the graft (arrows) resulted in prompt return to hyperglycemia.

islets transduced with TAT/PTD–HO-1 protein reversed hyperglycemia as well as control islets (Fig. 3), demonstrating that islet cell performance was not affected by exposure to TAT/PTD fusion protein in vitro for 8 days. After nephrectomy of the kidney with the graft on day 29, hyperglycemia was observed in all animals, confirming that the grafted islets sustained euglycemia. Thus, transduction of TAT/PTD–HO-1 fusion protein did not affect the insulin secretion capabilities of islets.

Assessing the biological effects of transduced TAT/PTD–HO-1 fusion protein

It has been reported that TAT/PTD fusion protein unfolding occurs during the process of internalization into cells, and their refolding assisted by cellular chaperons [34] is subsequently required to regain biological function. Therefore, it is possible for a TAT/PTD fusion protein to efficiently enter cells and lose its biological function if it is not properly refolded inside of the cell.

Pancreatic βTC-3 cell line is highly sensitive to TNF-α toxicity [24,35] and overexpression of HO-1 protects them against TNF-α-mediated cell death [24]. Thus to evaluate whether TAT/PTD–HO-1 was biologically functional upon cell transduction, we investigated the effect of transduced HO-1 fusion protein on βTC-3 cells treated with TNF-α.

Utilizing a viability test combining calcein AM and ethidium homodimer-1 staining, we observed that pancreatic βTC-3 cells transduced with TAT/PTD–HO-1 were more viable than non-transduced cells following TNF-α treatment (Fig. 4). Cells transduced with TAT/PTD–HO-1 fusion protein showed $80 \pm 4\%$ (means $\pm$ SD) of green (viable) cells compared with $98 \pm 0.3\%$ in

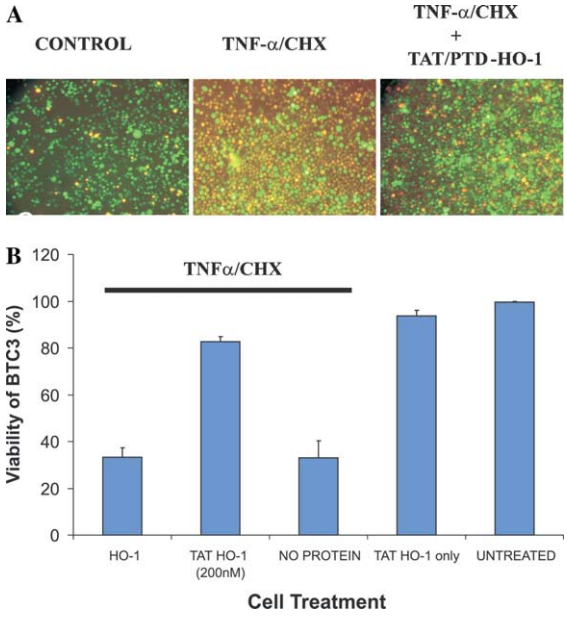

Fig. 4. Transduction of TAT/PTD–HO-1 protects against TNF-α-induced cell death. (A) βTC-3 cells were transduced with TAT/PTD–HO-1. Two hours later cultures were treated with the combination of TNF-α/CHX for 16 h. Control cells remained untreated at all times. Viability of βTC-3 cells was assessed by calcein AM (green/viable) and ethidium homodimer-1 (red-orange/dead) staining. (B) Quantitative histogram assessment of viability from βTC-3 cells incubated with recombinant HO-1 lacking the TAT/PTD, TAT/PTD–HO-1 and or no recombinant protein, subsequently treated with TNF-α/CHX for 18 h. βTC-3 controls cells were not exposed to TNF-α/CHX and included TAT/PTD–HO-1 treated cell and untreated cells incubated during the same period of time as experimental cells.

control cells and only $33 \pm 7\%$ in TNF-α/CHX-treated cells. Percentage of viable cells in βTC-3 cells incubated with a HO-1 recombinant protein without TAT/PTD

($33 \pm 4\%$) was similar to that of non-transduced TNF-α/CHX-treated cells (Fig. 4B). The range of protection conferred by TAT/PTD–HO-1 from different experiments varied between 60% and 80%. This indicates that TAT/PTD–HO-1 fusion protein remains biologically active upon cell internalization and that TAT/PTD is absolutely necessary to mediate HO-1 transduction. These results agree with previously reported results showing that βTC-3 cell sensitivity to cytotoxic effect of TNF-α, can be prevented by induction [26] or overexpression of HO-1 [24].

Enhancement of islet culture conditions

Pancreatic islets are susceptible to culture-induced damage, decreasing in number and losing function over time. This is in part due to cell death induced by apoptosis/oxidative stress during isolation and in vitro culture [36,37]. HO-1 has been reported to have anti-apoptotic and anti-oxidative stress effects on different cell types in vitro and in vivo. Therefore, we investigated whether transduction of TAT/PTD–HO-1 had an effect on preserving islets in culture. We observed that TAT/PTD–HO-1 fusion protein protected islets from deterioration. Significant hypoxia can occur during islet culture, depending on the density at which islets are cultured. Signs of hypoxia are: vacuolization, central necrosis, and DNA release that induces adhesion of all islets into large clumps. After 16 days in culture rat control islets showed clear signs of hypoxia. The islets cultured in the presence of TAT/PTD–HO-1 remained with significantly less signs of necrosis and formation of clumps (data not shown). Consistent with a better morphology, islets cultured in the presence of TAT/PTD–HO-1 also had a higher ratio of glucose-induced insulin secretion response (Fig. 5). After 16 days in culture transduced islets had a stimulation ratio (SI) of 6.1 ± 2.3 or 4.3 ± 2.1 for islets incubated with 100 and 200 nM of TAT/PTD–HO-1 fusion protein, respectively, while control islets were totally unresponsive to glucose stimulation (SI 1.1 ± 0.2). Currently we do not know whether in addition to preservation of the β-cell in culture, transduction in the TAT/PTD–HO-1 also enhances performance of β-cells. Experiments using a marginal mass transplantation model [26] will allow us to shed light on this matter.

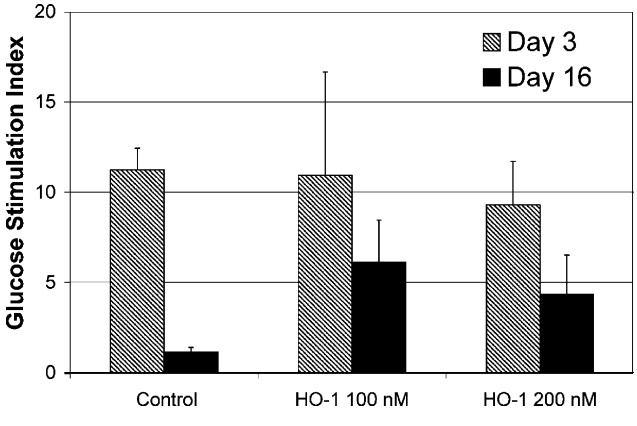

Fig. 5. Preservation of islets in culture by TAT/PTD–HO-1 transduction. Freshly purified TAT/PTD–HO-1 fusion protein (100 and 200 nM) was added to rat islet culture 1 day after isolation. They were kept in culture at 37 °C for 16 days. Media were changed on average every 4 days with an adequate amount of TAT/PTD–HO-1. Rat islets were challenged for glucose stimulation release on selected days.

Conclusions

Our data show that transduction of insulin producing cells with a recombinant HO-1 protein fused to the TAT/PTD confers cytoprotection against TNF-α-cytotoxicity and cell death during culture. Transduction with TAT/PTD–HO-1 protein might be useful to confer transient cytoprotection and therefore enhance the viability of transplantable islets. Prolonged culture of viable islets could help develop immunosuppressive regimes for recipients prior to islet transplantation.

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ANEXO 2

8.2 Ribeiro MM, Ricordi C, Pastori RL. Removal of endotoxin from recombinant proteins after acidic pH treatment in polypropylene tubes. Manuscrito em revisão editorial *Protein Expression and Purification*

Removal of endotoxin from recombinant proteins after acidic pH treatment in polypropylene tubes.

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Abstract

Recombinant proteins are of the utmost importance for therapeutic and research applications. However, when produced in bacteria, contamination with endotoxins is a major hindrance. Endotoxins are powerful pyrogens and have to be removed before parenteral administration and *in vitro* testing to prevent interference with biological assays. We present a method for efficient removal of endotoxin from proteins solutions that come from the simple observation that after storing purified proteins in polypropylene tubes, endotoxin drops with time. This, in combination with pH dropping of proteins bellow it's respective pI, can reduce endotoxin from freshly purified proteins to levels as low as 0.04% with protein recovery ≥70%. Furthermore, the presence of high salt and urea concentrations, commonly utilized in protein purifications, does not significantly affect the efficiency of endotoxin removal. Endotoxin elimination was confirmed by LAL based assays currently available in the market and also by *in vivo* pyrogen testing. This endotoxin removal procedure does not alter the quality of the protein as assessed by Agilent-2100 Bionalyzer. Furthermore, this procedure does not affect the protein's biological activity. The major advantages of our approach are efficiency, simplicity, time-saving and ease of implementation.

Introduction

With the advent of Genomics and Proteomics, many recombinant proteins with commercial and medical applications are produced utilizing genetically modified bacteria. This is the preferred host capable of producing high levels of a given protein in reasonable time frames. However, endotoxin decontamination is a critical step of the protein purification process. Endotoxins are lipopolysaccharides (LPS) responsible for the organization, and stability of bacteria [1]. Endotoxins are associated with part of the outer membrane of gram negative bacteria that often comes as a protein contaminant at the time of bacterial lysis. Adverse reactions like rising body temperature, triggering of coagulation cascade, modified homodynamic and septic shock can occur *in vivo* due to exposure to endotoxin contaminated products [2]. Moreover, in *in vitro* assays the presence of endotoxin can modify significantly the interpretation of results [3]. Therefore, it is critical to remove endotoxins from *E. coli* generated recombinant proteins, in an efficient and economical way.

Endotoxins are considered to be pH and temperature resistant, rendering its elimination one of the most difficult steps in the protein purification process [4]. Several methods are commonly used for endotoxin removal. Ultrafiltration effectively removes endotoxin from water but physical forces necessary for the procedure, can damage protein [5]. Anion exchange chromatography utilizes the negative net charge of endotoxin for binding to the anion exchange resin. Therefore, this method is mostly applied for basic proteins that do not bind anion exchange columns. Notably, competition between ion-exchanger and net positive charged proteins can cause the protein to drag endotoxin along the column [6]. Affinity adsorbents such as histidine, histamine, polymyxin B and

poly-L-lysine facilitate adsorption of endotoxin to matrix by electrostatic and hydrophobic interactions. Even though the interaction between adsorbents and endotoxin is selective, multiple rounds of binding and of protein dilutions decrease product recovery [7]**.** Triton X-114 is an efficient compound for endotoxin removal from recombinant proteins [8, 9]. It can be used either in a phase separation method [9] or as washes on an affinity chromatography columns [10]. However, carryover of the detergent must be removed by repeated adsorption or gel filtration. Each process has an average product loss of 10-20%. Additionally, on industrial scale the detergents are expensive and may potentially change biological activity of the purified proteins [11]. Flammable solvents like ethanol and isopropanol are considered efficient solvents in the removal of endotoxin [12] yet, once used on industrial scale, strict safety procedures are required that significantly increase production cost. Alternative chemicals that are safer and more cost-effective can be used to separate the LPS from proteins immobilized in chromatography columns such as alkanediols [11]. One potential drawback of this method is increasing viscosity dependent on the alkanediol chain length used.

Chemical composition of the sample storage containers can also play an important role in endotoxin measurements. It was previously reported that polypropylene adsorbs most of the endotoxin from samples while polystyrene did so to a lesser extent [13]. Therefore, it was recommended to avoid polypropylene tubes for analytical quantification of endotoxins [11]. Based on these observations we tested the hypothesis that the adsorption of endotoxin to polypropylene could be used as a functional method for removal of endotoxin from freshly purified recombinant proteins.

Here we describe the conditions under which polypropylene associated with acidic pH, eliminates endotoxins from recombinant proteins produced by bacteria. This is a simple, efficient, inexpensive and fast alternative that could be included as a standard procedure for endotoxin elimination in protein laboratories provided that polypropylene container quality is appropriately tested and that the protein in question resists acidic pH treatment.

Materials and Methods

Protein Generation and purification. High density *E.coli* (BL21) pre-inoculum was grown by placing a small aliquot of previously frozen cells (-80°C + glycerol) in 100ml of LB media with 50μg/ml of Ampicillin for 5H at 37°C. Inoculum was made by transferring pre-inoculum to 1L of LB media with same amount of ampicillin. Culture was grown for approximately 16H at 37°C. Pellets was spun down at 3840g and washed with PBS. Pellet was resuspended in approximately 20ml of PBS in 20mM imidazole and protease inhibitor cocktail (Complete, EDTA-free - Roche). Beaker containing cells was placed on ice water for sonication (Fisher Scientific Sonic Dismembrator- Model 500) at 50% amplitude for 3min (21 seconds on and 1 minute off). After centrifuging bacterial extract at 17400g, supernatant was added to Ni-agarose column (Qiagen) previously equilibrated in PBS with 20mM imidazole. Washes were performed first with 20mM imidazole buffer followed by 30mM imidazole. Protein was eluted with 100mM imidazole, desalted in PD-10 column (Amersham-Pharmacia) and filtered in a 0.2μm syringe filter (Acrodisc HT Tuffryn Membrane Low Protein Binding Non-pyrogenic).

Proteins used in this study were: Murine Heme oxigenase 1 (HO1) and a modified version of the same protein (TAT HO-1)[14], human neuroglobin (Ngb) [15], a modified version of the human transcription factor PDX-1(TAT PDX-1) and β-galactosidase [16]. Urea 6M and NaCl 1M were added to TAT HO-1 for experiment of Figure 2. Proteins were quantified by reading samples prepared with Bio Rad protein assay (cat#500-0006), at a wave length of 595 ηm, in spectrophotometer (Beckman –DU 640).

Protein incubation in polypropylene tubes.

5ml of freshly purified protein (0.5mg/ml) at pH 5 (see Table 1 for results with different pHs and different proteins) was added to a sterile, pyrogen-free, DNase and RNase free 50 ml conical from Corning (cat#430828) or Falcon (cat#352098) and rotated at a horizontal position (around its own axial- using a Labquake shaker) at 4ºC for 30 minutes. Protein solution was changed to a new tube and procedure was repeated for another 30 min. Protein was brought back to physiological pH in a new conical. This is the standard protocol for endotoxin removal unless otherwise specified.

Endotoxin quantification.

Endotoxin was quantified using one of two Lymulus Amebocyte Lysate (LAL) methods: QCL 1000 kit from Cambrex/Biowhittaker (cat# 50-647U) reading was performed at a Fluorescent Plate reader FL600 at 405nm. Analyzed curve was done with KC4 software. Endosafe PTS - Charles Rivers Laboratories (cat# PTS 100) using cartridge sensitivity between 10 and 0.1 (cat#PTS 201) or 5 and 0.05 EU/ml (cat# PTS 2005). All dilutions tested were properly spiked to confirm results. Sample pH at the time of endotoxin measurement was between 6.8 and 7.5.

FITC –LPS fluorescent measuring. 5 ml of PBS pH7 with 40KEU of FITC-LPS purchased from Sigma (F3665-Lot# 113k4085) was added to a 50ml Corning conical. Triplicates of 50ul were taken for fluorescent measuring on 1420Multilabel counter model VICTOR 3 from Perkin Elmer (T= 0h). 50ml conical was rotated horizontally at 4ºC and 50 ul (triplicate) samples were taken for fluorescence measuring against PBS at the following time points: 30min, 1h (5ml of FITC-LPS was transferred to a new conical tube and experiment was continued), 2h and 18h.

Assessment of extractable LAL inhibitor from polypropylene.

Corning polypropylene and glass tubes were incubated as previously described with 5 ml of endotoxin-free water at pH5. After standard incubation, both waters were neutralized to pH 7 and used for further dilutions of 1EU/ml standard. Dilutions 0.1, 0.25 and 0.5 EU/ml were made with: Endotoxin-free water from QCL-1000 kit (control), and water that was incubated in polypropylene tube and glass tube. . Endotoxin was quantified in fluorescent plate reader FL 600 as previously described (**Figure 3**).

Statistical analysis

Results were analyzed by with the Wilcoxon signed rank test for paired, non-parametric samples, with 95% confidence intervals. Two-tailed p values < 0.05 were considered statistically significant

(http://www.fon.hum.uva.nl/Service/Statistics/Wilcoxon_Test.html)

Two-tailed student T-test was performed with Excel for Windows whenever applicable.

Insulinoma β-cells (βTC-3) cell viability after induced TNF-α cytotoxicity.

βTC-3 (10^6) cells were transduced for 2h with TAT-HO1 (200nM or 400nM) before adding 1000U of mouse TNFα and 10μg/ml of cycloheximide (CHX). After incubation

for 16H at 37°C and 7.5% CO_2, cells were assayed with Live/Dead viability cytotoxicity kit (Molecular Probes) and analyzed by FACSSTAR (Becton-Dickinson) flow cytometer [14].

***In vivo* pyrogenic study.**

Pyrogenic tests were done with 3 groups each with 3 male rabbits weighing between 2.2 and 3.2 Kg. Group 1:Saline; group 2: TAT-HO1 that underwent endotoxin elimination procedure at pH 5; and group 3: TAT-HO1 that was not treated for endotoxin elimination. A rectal probe was inserted in the rectum of each animal and they were allowed to acclimate to their restrainer position for 1H prior to temperature collection. Four temperature readings were conducted at 30 min intervals beginning 90 minutes prior to injection. The mean temperature of two readings obtained in 40 min time period prior to injection was the initial temperature (base line).

Each of the rabbits on group 1 was injected intravenously via a marginal ear vein with sterile, non-pyrogenic 0.9% sodium chloride solution, warmed to 37°C at a dose of 10ml/Kg. (group 1- Saline). The solution was injected within 4 min period. For all rabbits, temperatures were recorded at 30 min intervals for 3 H after injection. Animals with temperature rise greater than 0.4° C, outside of the 38°C to 39.8°C range or animals with a temperature variation greater than 0.2°C between the 0.5 and 0.0 pre-injection temperatures were excluded from the main test. All procedures were conducted in conformance with good laboratory practice and ISO 17025. To meet non-pyrogenic requirements for United States Pharmacopeia (USP) no single animal shall have increase of more than 0.4°C above its baseline temperature.

For experimental Groups 2 and 3, samples were both equally diluted prior to injection. Dilution was made so the group that had not undergone endotoxin elimination procedure (group 3) would not reach levels of lethal pyrogenicity. Endotoxins concentrations measured by LAL were, 20 EU/ml and 0.3 EU/ml for samples of groups 3 and 2 respectively. Temperatures were recorded at 30 min intervals between 1 and 3 H after injection. All pyrogenic *in vivo* testings were done by NAMSA- Irvine,CA-USA.

Results

LPS-FITC fluorescence decrease after incubation in polypropylene tubes.

In order to confirm that polypropylene and endotoxin interact with each other [13], we incubated commercially available FITC-LPS in 50-ml polypropylene conical tubes. We observed that fluorescence decreased with time (Figure 1), suggesting that polypropylene surface of commercially available tubes (Corning and Falcon) was capable of endotoxin adsorption.

Endotoxin contamination decreases after acidic pH treatment in polypropylene tubes.

To verify if polypropylene plastic could be used to remove endotoxins from recombinant proteins produced in bacteria we used different purified recombinant proteins (as described in material and methods), of various isoelectric points (pI). The experiments were performed across a broad range of pH values (Table 1). Overall, we observed a better endotoxin removal at pH below the protein's respective pI. Interestingly, endotoxin elimination did not occur at the basic pH range (data not shown). Apparently, the efficiency of endotoxin removal also depends on protein type. For example, TAT-HO-1, HO-1, NgB and TAT-PDX all had above 99% of endotoxin removal. Yet, protein

recovery efficiency differed being the most efficient at 97% and the least efficient at 47%.

Endotoxin removal from TAT-HO1 protein solution is not affected by high salt or urea concentrations.

Once a preliminary screening was made with different proteins we focused our studies on TAT-HO-1 by performing endotoxin removal using several different protein preparations at pH 7, 6 and 5 (Table 2.). Endotoxin removal from TAT- HO-1 was confirmed to be highest at pH5 with 0.31 ± 0.11% (n=10; p=0.00018) of remaining endotoxin. Protein recovery was 65.45 ± 3.87 %. Furthermore, conditions frequently used in protein purification protocols, such as high NaCl and Urea concentrations, did not significantly interfere in the removal of endotoxins (Figure 2). After standard endotoxin removal protocol was applied, the endotoxin content of the recombinant protein dropped from 8594 EU/mg to 5.57 EU/mg in standard condition, to 18.03 EU/mg in the presence of 1M NaCl and to 49.6 EU/mg with 6M urea. The average amount of protein loss from all three experiments was 21%. The greatest loss occurred in the presence of 1 M NaCl.

Extractable inhibitors of LAL assay, from polypropylene tubes were not detectable.

It has been previously described that some polypropylene tubes release an extractable substance that inhibits LAL test [17]. Therefore, we investigated if the decrease of endotoxin observed in our experiments were due to inhibition of the LAL test. We performed standard incubation protocol with endotoxin-free water at pH 5 both in polypropylene tubes and control glass tubes. After incubation, the pH of endotoxin-free water from both conditions were adjusted to pH 7 and both samples plus endotoxin free-

water (control 2) were used to further dilute 1 EU/ml standard endotoxin to 0.50, 0.25 and 0.10 EU/ml. Figure. 3 shows that endotoxin standard curve generated with water incubated in polypropylene tubes do not differ from endotoxin curves generated with control waters. Consequently, endotoxin elimination reported in our study was not due to inhibition of the method used to detect endotoxin, but most likely to another phenomena, possibly adsorption of endotoxin to the walls of the polypropylene tubes [13] in combination with treatment at acidic pH.

Endotoxin elimination procedure does not interfere with quality or biological activity of TAT-HO1 protein.

In order to verify that standard endotoxin elimination procedure does not interfere with biological activity of the protein, we decided to perform an *in vitro* test of βTC-3 insulinoma cell viability after induced TNF-α cytotoxicity, described on our previous work [14]. We found that the combination of TNF-α/CHX and TAT-HO1 with endotoxin contamination was much more toxic to cells (86.95 *versus* 60.67 more dying cells) than the same protein with out endotoxin as shown on Table 3.

Protein quality after endotoxin elimination was assessed using the microfluidics-based platform Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Both, membrane bound and non membrane bound forms of TAT HO-1 [14] were present: 41.9% and 58.1% for untreated TAT HO-1 *versus* 40.8% and 59.2% for TAT HO-1 subjected to endotoxin removal treatement at pH 5. These results show no difference in percentage of HO-1 bands before and after endotoxin elimination.

In vivo pyrogenic determination.

In order to confirm that the results obtained with LAL based assays *in vitro* were sustainable *in vivo,* we decided to inject endotoxin elimination treated TAT-HO1 and non treated TAT-HO1 in to rabbits. Figure 4 shows TAT-HO1 non treated protein as fully pyrogenic while TAT-HO1 treated protein has no pyrogenic reaction, confirming *in vitro* LAL based studies.

Endotoxin elimination procedure can be used in proteins other than recombinant proteins purified from bacteria.

We have also tried eliminating endotoxin from proteins produced in sources other than bacteria such as the antibody 5c8 produced by hibridoma clone (ATCC, Manassas, VA catalog # HB-10916). 5c8 antibody was contaminated with low levels of endotoxin (29 EU/mg). We submitted 5ml of antibody to two rounds for endotoxin elimination at pH5. Endotoxin dropped to 1.8EU/mg and protein loss stayed at 14.3% of protein.

Discussion

Endotoxin elimination from protein solutions renders to be one of the most challenging steps of protein purification once the efficiency of the protocol varies with the physical-chemical structure of each protein. Most procedures are expensive, time consuming and with low protein recovery. We here propose the use of a method that is easily and quickly performed, with inexpensive material that most likely are present in every laboratory world wide. The methodology comes from the simple observation that after protein purification and preservation at 4 degrees in Corning or Falcon 50 ml conical, endotoxin dropped with time. After performing experiment with FITC-LPS in the same conicals (Figure 1) we observed similar drop of fluorescence over time leading us to believe that

polypropylene plastic might be adsorbing endotoxin as has been previously described in the literature [13]. We have also noticed that after dropping the pH of proteins in study from physiological to acidic pH, bellow the respective pIs, the efficiency in endotoxin elimination was even greater, reaching levels of 99.9% elimination in some cases. Protein recovery in most cases varied with pH treatment. Proteins here presented, after having performed the standard procedure for endotoxin elimination at different pHs reached recovery levels between 60 and 97%, indicating that it is necessary to try different pHs in order to find an adequate endotoxin removal/protein recovery for each protein in question (Tables 1 and 2).

After performing endotoxin elimination on our proteins we have measured endotoxin levels with LAL based protocols and they showed significant endotoxin elimination was achieved. Extractable LAL inhibitors from polypropylene tubes (Figure 3) as well as β-glucans (data not shown) are know to decrease endotoxin values on LAL assays. Both were not detectable on our experiments

TAT-HO1 quality after endotoxin elimination was analyzed by Agilent 2100 Bioanalyzer and was found to be no different than TAT-HO1 that had not undergone the same treatment. Biological activity was analyzed on βTC-3 insulinoma cells treated with TNF-α and cyclohexamide [14] and noticed better cytoprotection effect of TAT-HO-1 with out endotoxin than with endotoxin contamination (Table 3). This shows not only that we have preserved the biological activity of the protein but also that endotoxin contamination seems to be contributing with toxicity of the cells as previously reported [3].

Because our ultimate goal was achieving endotoxin decontamination levels that would be compatible with *in vivo* studies we decided to perform *in vivo* pyrogenic testing in rabbits

of our treated protein (Figure 4). The protein would be exposed to the immunological system of an animal which is a much more complex system than LAL based technology for endotoxin detection. The endotoxin elimination procedure proved to be efficient in *in vivo* testing in the same way it had been with previous LAL testing; wile the untreated group was highly pyrogenic.

We have not performed any mechanistic studies to further understand how the endotoxin elimination is happening. We believe that polypropylene plastic adsorption and change in physiological pH of proteins play a role in the endotoxin decontamination process. Nevertheless, further experiments need to be done in order to shed light on this matter.

In conclusion, we developed a simple, efficient, fast and inexpensive way to eliminate endotoxin from recombinant proteins that will allow us to proceed with *in vivo* applicability of several proteins produced in our laboratory. It is important to notice that pH adjustment might have to be made for each protein in order to achieve the best endotoxin elimination levels and protein recovery simultaneously.

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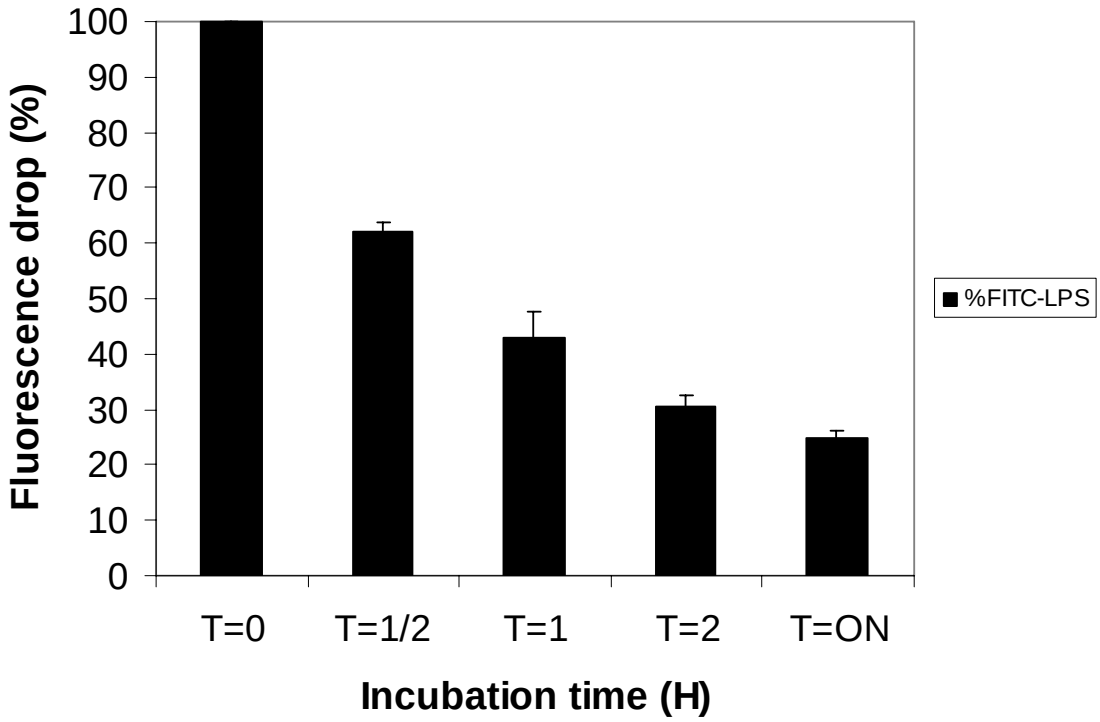
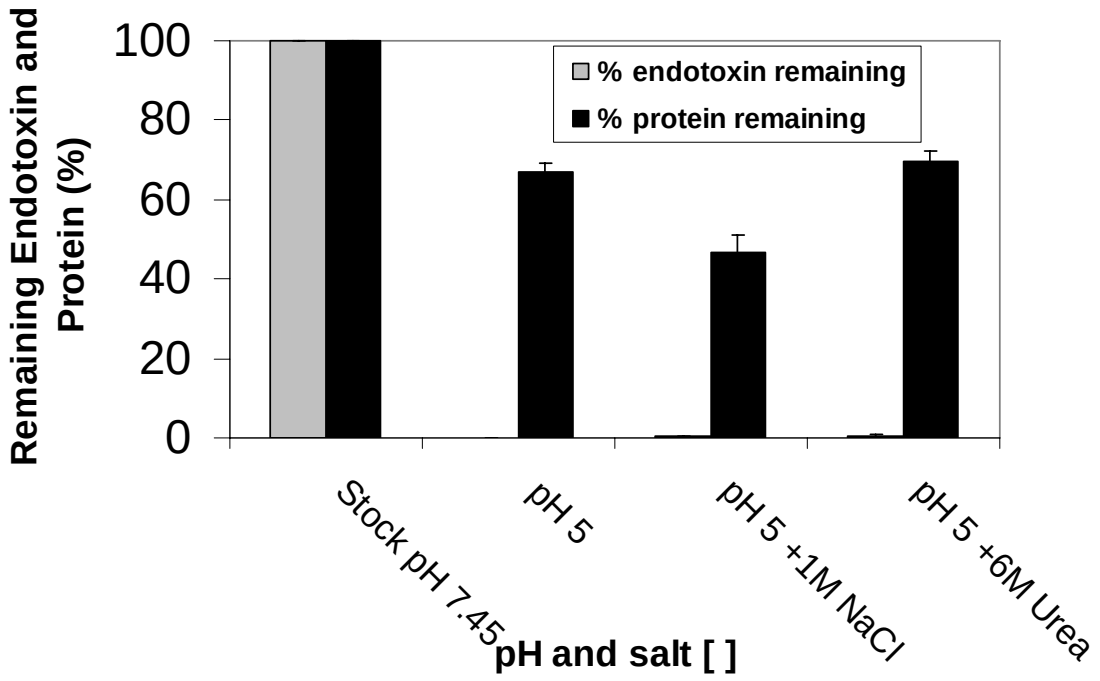


Figure 1. LPS-FITC interaction with polypropylene. 5 ml of LPS-FITC at 40 000 EU/ml was added to a 50ml polypropylene conical and incubated for 1/2 h at 4ºC rotating horizontally. LPS-FITC was changed to a second conical and further incubated for an additional 1/2h. Samples were taken at 1/2H, 1H, 2H and over night (ON). Each reading was performed 3 times at pH 7.4. Graph show fluorescence dropping with time.



F**igure 2**. **Endotoxin removal from TAT HO-1 at high salt concentration.** TAT HO-1 was subjected to standard endotoxin elimination protocol at pH 5 in the presence or not of NaCl 1M or Urea 6M. Stock endotoxin was 19060EU/mg. After standard endotoxin elimination protocol TAT HO-1 pH5 measured 3.2 EU/mg; TAT HO-1 pH5 with 1M NaCl measured 20.3EU/mg and TAT HO-1 pH 5 with 6M urea measured 89.28EU/mg.

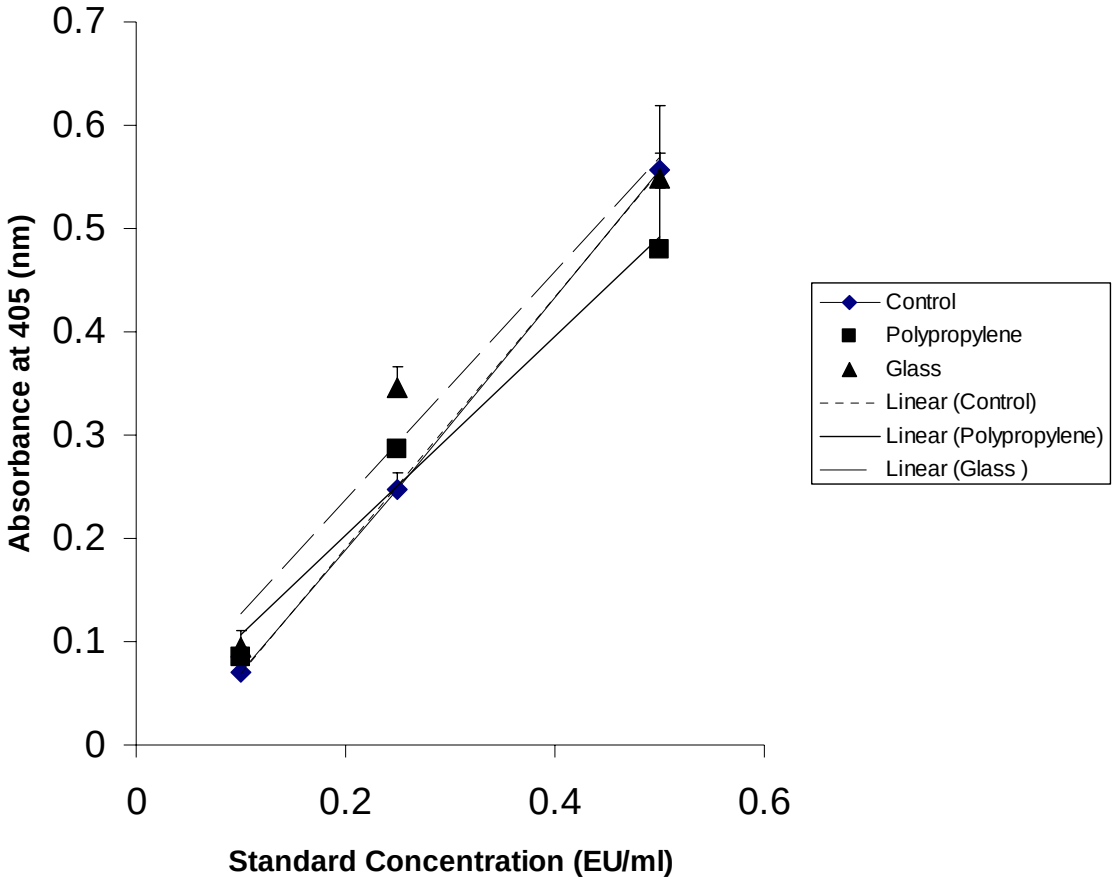


Figure 3. Effect of LAL reagent water (LRW) extracted on polypropylene tube, glass tube and with no extraction. LRW after standard incubation in polypropylene tube, control LRW (provided from LAL kit) and LRW incubated in pyrogen free glass were used to further dilute 1EU/ml stock endotoxin. Each measuring was made in triplicate for 0.1 EU/ml; 0.25 and 0.5 EU/ml. Control LRW $r^2 = 0.999$; Polypropylene LRW $r^2 = 0.9762$; Glass LRW $r^2 = 0.9585$.

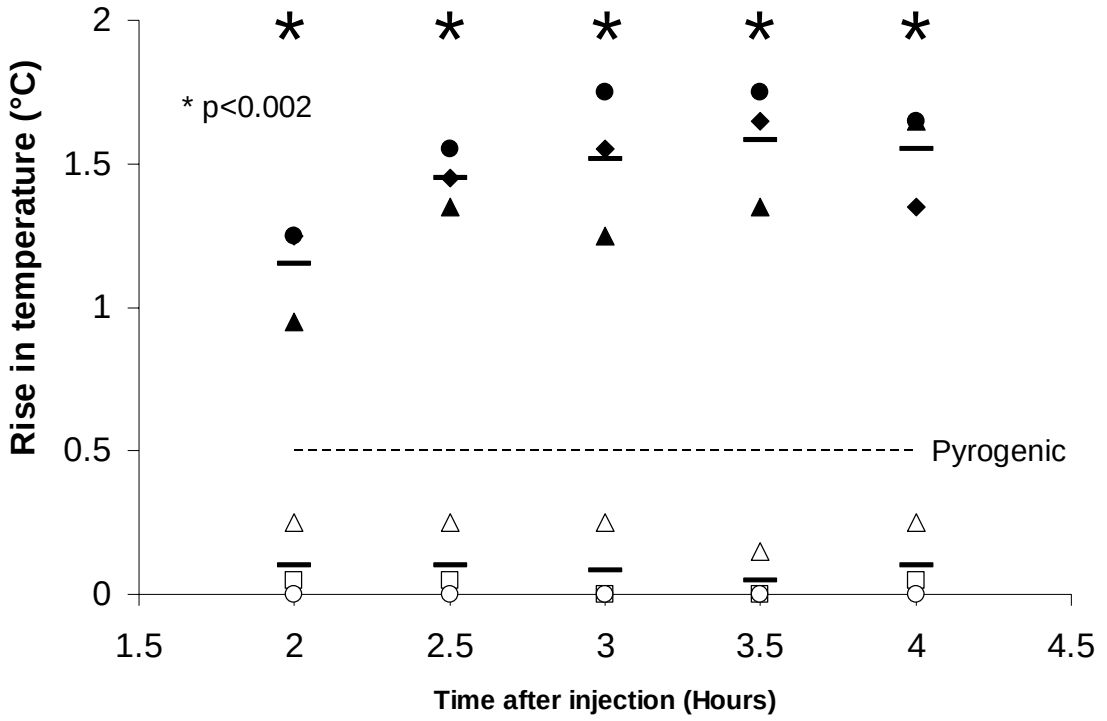


Figure 4. ***In vivo*** **determination of pyrogenicity of TAT-HO1 protein following endotoxin elimination protocol.** Pyrogenicity of TAT-HO1 protein following the endotoxin-reduction protocol (empty symbols) or not (solid symbols) was tested *in vivo* using male rabbits (n=3 per group) that were injected intravenously (marginal ear vein) with 3.5-4 ml/Kg of the indicated protein. Changes in body temperature were measured every 30 minutes after protein injection. The group of animals receiving the unmanipulated protein had pyrogenic reaction higher than 0.5°C above baseline temperature; wile the group of animals receiving the protein that had undergone the endotoxin-reduction protocol had no pyrogenic reaction. Data is relative to 3 animals per group. The broken line indicates the cut-off for pyrogenicity (namely, ≥0.5°C). Unpaired t-test: * = $p<0.002$ at each time point.

Table 1. Endotoxin removal of protein solutions at different pHs in polypropylene tubes.

Protein	Isoelectric point (pI)	pH	Endotoxin elimination %	Protein recovery %	Endotoxin content before purification EU/mg	Endotoxin content after purification EU/mg
TAT-PDX-1	9.3	7.0	18.0	81.8	2,594.5	2,622.22
		5.0	99.2	46.8	3,962.0	157.42
TAT-HO-1	7.9	7.4	39.2	65.2	7,032.0	6,678.00
		6.0	83.1	66.9	29,308.0	4,943.00
		5.0	99.9	72.9	13,886.0	5.65
		3.0	97.7	93.9	3,833.9	93.95
HO1	6.1	5.0	99.9	60.8	40,318.0	25.64
Ngb	5.4	7.0	91.0	97.2	17,112.0	1,875.60
		1.5	99.7	90.4	14,862.0	49.18
β-gal	5.2	5.7	47.9	68.6	11,084.0	7,592.10
		3.0	84.1	80.8	1,263.3	248.45

*pI was calculated using Swiss-Prot tools (http://www.expasy.org)

Table 2. Effect of pH changes on endotoxin reduction and protein recovery of TAT-HO1.

Experimental conditions	Endotoxin Before Treatment (EU/mg)[a]	Endotoxin After Treatment (EU/mg)[a]	Endotoxin After Treatment (%)[a]	Protein After Treatment (%)[a]	Endotoxin p value[b]
pH 7	35570 ± 13820	17755 ± 9143	45.75 ± 11.10	74.34 ± 3.24	0.05031
pH 6	25878 ± 8269	5704 ± 1668	28.66 ± 8.47	69.10 ± 3.17	0.02258
pH 5	22893 ± 9419	34.71 ± 15.73	0.31 ± 0.11	65.45 ± 3.87	0.00018

N=9 (pH 7); N=12 (pH 6); N=10 (pH 5)

[a] = mean ± S.E.

[b] = $p \leq 0.05$ from Wilcoxon Two Sample Test

Table 3.Comparison of βTC3 viability in the presence or absence of

TAT HO-1, before and after submission to endotoxin elimination procedure.

	Live cells (%)	Dead cells (%)	Dying cells (%)
T/C[a] 1000U	7.7	0.08	92.03
T/C[a] H[b] 200nM	39.11	0.12	60.67
T/C[a] HE[c] 200nM	12.87	0.06	86.95
T/C[a] H[b] 400nM	41.28	0.26	58.26
T/C[a] HE[c] 400nM	21.24	0.08	78.44
H[b] 400 nM	93.99	0.06	5.57
HE[c] 400nM	94.97	0.08	4.57

[a] TNFα/CHX

[b]TAT HO-1 after endotoxin elimination procedure

[c]TAT HO-1 before endotoxin elimination procedure

ANEXO 3

8.3 Pastori RL, Klein D, **Ribeiro MM**, Ricordi C. Delivery of proteins and peptides into live cells by means of protein transduction domains: potential application to organ and cell transplantation. Transplantation 2004;77(11):1627-31

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OVERVIEW

DELIVERY OF PROTEINS AND PEPTIDES INTO LIVE CELLS BY MEANS OF PROTEIN TRANSDUCTION DOMAINS: POTENTIAL APPLICATION TO ORGAN AND CELL TRANSPLANTATION

RICARDO L. PASTORI, DAGMAR KLEIN, MELINA M. RIBEIRO, AND CAMILLO RICORDI

Proteins are primary targets in drug discovery. However, with a few rare exceptions, they are unable to cross cell membranes, a limitation that prevents the full exploitation of their therapeutic potential. Major advances have been recently made through a novel approach of protein and peptide delivery into cells known as protein transduction or protein therapy. Proteins and peptides can be directly transferred to cells when covalently linked to protein transduction domains (PTD), small peptides that can freely cross cell membranes with low lytic activity (*1–3*). The mechanism of cellular translocation of PTD are currently poorly understood. Most of the PTD described in the literature have a high content of basic residues. It is believed that the interaction with the negative cell membrane environment has an important role in the translocation process, and the mechanism of cell internalization may differ for each of the PTD. Several PTD have been identified in naturally occurring proteins. The most commonly studied are homeodomain transcription factors such as antennapedia (*4*), the herpes simplex virus type 1 protein VP22 (*5*), and the human immunodeficiency virus (HIV) transactivator TAT protein (*6–7*). In addition, a new gamut of peptides with PTD capabilities have been recently identified. Some of these new peptides are derived from natural proteins, whereas others are synthetic peptides. The PTD included in these groups are described below, with emphasis on the TAT-PTD and its potential application in organ and cell transplantation.

HOMEODOMAIN TRANSCRIPTION FACTORS

Homeodomain proteins have a critical role in tissue architecture and morphogenesis. They exert their regulatory functions by binding to DNA through their homeodomain, a 60-amino acid domain composed of three α-helices separated by two β-turn motifs. Several PTD that form part of homeodomain proteins have been reported. The most widely studied PTD motif of this kind is known as pANTP or penetratin. It corresponds to 16-amino acid (RQIKIWFQNRRMKWKK) residues of the third helix of the antennapedia homeotic transcription factor (ANTP) from *Drosophila* (*4*). It was postulated that the positively charged pANTP associates with the charged phospholipids in the outer side of the cellular membrane. This is followed by destabilization of the membrane and formation of an inverted micelle that somehow penetrates the cytoplasmic compartment (*8*). However, recent studies performed on cells, rather than artificial phospholipid bilayers, indicate that adsorptive-mediated endocytosis has a role in translocation mechanism of pANTP into cells (*9*). pANTP has been mostly restricted to the delivery of small molecules such as peptides (*10*) and peptide nucleic acids (*11–13*). pANTP-mediated transduction of peptides has been successfully used to study the mechanism of RNA transport (*14*). In vivo applications of pANTP-fused molecules include the topical administration of the NH2-terminal peptide of α-smooth muscle actin. As this inhibits the contraction of rat wound granulation tissue, this approach could help develop new therapeutic strategies for fibrocontractive pathological situations (*15*). Moreover, the administration of pANTP fused to a 20-amino acid peptide (amino acids 84–103) from the p16 tumor suppressor protein suppressed pancreatic cancer growth and extended survival in mice (*16*). Inhibitors of the protein kinase C-ϵ fused to the antennapedia cell penetrating peptide were used to confirm the crucial role of this isozyme in the signaling pathway associated with protective heart ischemia preconditioning (*17*). Two other *Drosophila* homeodomain proteins, Fushi-tarazu and Engrailed, have similar transduction properties (*18*). Furthermore, a new PTD, pIsl1, with translocation ability similar to that of pANTP, was recently discovered (*19*). PIsl1 originates from rat protein homeodomain of islet-1, an insulin gene enhancer. It encompasses amino acid residues 45 to 60 (RVIVWFQNKKRCKDKK) from the third helix motif. Similarly, it was recently reported that pancreatic and duodenal homeobox-1, a key transcription factor for pancreatic development and insulin transcription, contains an antennapedia-like PTD (RHIKIWFQNRRMKWKK) in the third α-helix of its homeodomain. Pancreatic and duodenal homeobox-1 is capable of in vitro transduction of pancreatic ducts and islets (*20*).

HERPES SIMPLEX VIRUS TYPE-1 VP22 PROTEIN

The entire VP22 viral protein constitutes a PTD. In contrast to other proteins containing PTD, the short domain capable of cellular transduction when fused to a tandem

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protein has not yet been identified. However, VP22 has an additional feature: it participates in intercellular trafficking. Once expressed in the cell, this protein has the ability to spread to other nontransfected cells. The majority of published studies about transduction with the VP22 PTD have been essentially performed intercellularly. The VP22-fusion protein was generated from a gene transfected into cells and diffused to neighboring untransfected cells (Fig. 1). Conflicting reports have been published on the intercellular transduction of VP22-GFP fusion protein (*21*, *22*). To overcome the difficulties associated with the detection of GFP and potential cell fixation artifacts (*23*), Benett et al. (*24*) demonstrated intercellular transport of VP22 by means of recombinase Flip fused to VP22. COS cells were transfected with a *LacZ* gene that was rendered nonfunctional with a transcription terminator sequence located between the CMV (cytomegalovirus) promoter and the *LacZ* gene. The transcription terminator sequence was flanked by Flip recombinase sites (frt sites). *LacZ*-COS transfected cells expressed β-galactosidase only if they were cultured together with cells expressing a VP22-Flip recombinase fusion protein, indicating that VP22-Flip translocated from one cell to another. Other studies showed biologic activity of several proteins fused to VP22 PTD on intercellular transduction, such as active thymidine kinase (*25*), the tumor suppressor factor p53 (*26*), and the Simian virus 40 large T antigen (*27*). Furthermore, the bystander effect achieved by the intercellular trafficking capability of VP22 has been exploited in a number of in vivo models (*28–30*).

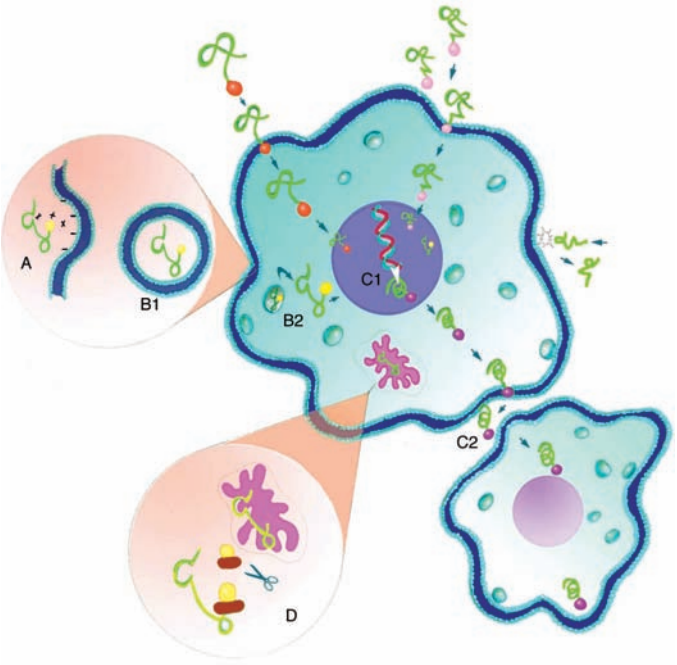

FIGURE 1. (*green cargoes*) Proteins and peptides. (*orange dots*) Generic PTD. (*yellow*, *purple*, and *pink dots*) TAT/PTD, VP22, and pANTP, respectively. Most of the proteins and peptides are unable to enter into cells unless fused to a PTD. The mechanism of PTD entrance remains for the most part unknown. For basic PTD, an electrostatic interaction between positively charged PTD residues and a negative cell membrane environment is apparently necessary as a first step (*A*). Endocytosis (*B1*) and subsequent escape from the endocytosis vesicle (*B2*) has been recently postulated for TAT/PTD and pANTP. VP22-fused cargos are generated from a gene (*C1*) and have intercellular trafficking properties (*C2*). All of the PTD have the potential to localize in the nuclei. TAT/PTD fusion proteins containing a mitochondrial localization signal enter into mitochondria, where the mitochondrial signal sequence is recognized and proteolyzed, resulting in the retention of the fused protein cargo in the mitochondria (*D*).

TAT/HIV-PTD

Green and Loewenstein (*6*) and Frankel and Pabo (*7*) first reported that HIV transactivator TAT protein (TAT/HIV) is capable of crossing cell membranes. Fawel et al. (*31*), later showed TAT-mediated delivery of chemically coupled heterologous proteins into cells. The TAT/PTD consists of 11 amino acids, YGRKKRRQRRR, from the basic domain of the TAT/HIV protein. The basic residues, K and R, have a critical role in transduction properties of TAT/PTD through an interaction with the heparan sulfate chains of cell membrane heparan sulfate proteoglycans (*32*). Although the mechanism of internalization is still poorly understood, there is evidence that endocytosis followed by endosome escape is partially involved (Fig. 1) (*31*, *33*, *34*). Substitution of any of these basic residues with alanine negatively affected internalization into cells (*35*). Furthermore, a 9-mer of arginine was reported to have a significantly greater transduction efficiency than that of the TAT/PTD (*35*). Schwarze et al. described a system to produce and purify recombinant TAT/PTD-fusion proteins that have cell-transducing capabilities in vitro and in vivo and can even cross the hematoencephalic barrier (*36*). Although most of the cells are transduced by TAT/PTD, there are cases in which cell permeability is restricted. For example, extracellular matrix components can interfere with the TAT/PTD transduction to myofibers (*37*). Likewise, TAT/PTD was incapable of transducing in vivo bladder epithelium, as assessed by the lack of transepithelial permeation into other tissues after infusion of TAT/PTD conjugated to technetium-99 in the urinary bladder of living rats (*38*).

It has been disputed that transduction and nuclear localization of TAT/PTD fusion proteins was the consequence of a fixative-induced artifact (*22*, *39*). However, analysis by confocal microscopy of live, nonfixed cells showed efficient internalization of TAT/PTD derivatives (*33*, *40*). Furthermore, the best possible indication of internalization is the biologic function of the full-length proteins and peptides delivered to many different target cells by means of TAT/PTD. They are included to date in more than 70 references in the biomedical database PubMed. A wide variety of full-length proteins fused to TAT/PTD were biologically active on delivery into cells (*1*). Some of the fusion proteins were biologically active in the nuclei such as the cell cycle regulator p21 (*41*) or in the cytoplasmic compartment such as the IκB, the nuclear factor (NF)-κB inhibitory protein. In addition, a significant number of TAT/PTD derivatives were biologically active when administered in vivo. Listed in Table 1 are proteins and peptides that have been delivered in vivo to different animal models by means of TAT/PTD protein transduction (*42–51*).

In another interesting application of this technology, a mitochondrial signal sequence was included in the TAT/PTD fusion protein (*52*). On cell transduction and entrance into

TABLE 1. Proteins and peptides delivered in vivo by means of TAT/PTD protein transduction

Protein or peptide	Description and potential application
Bcl-XL	Antiapoptotic member of the Bcl2 protein family; offers protection against ischemic brain damage and neuronal protection reported in several studies (42–44).
NR2B9c	A peptide that disrupts the interaction of *N*-methyl-D-aspartate receptors with the postsynaptic density protein PSD-95 (45); could lead to a potential antistroke therapy.
Hypoxia inducing factor (HIF)	A transcription factor central to oxygen homeostasis that induces angiogenesis. It has potential for use in ischemic diseases (46).
Glial line-derived neurotrophic factor (GDNF)	A member of the transforming growth factor-β superfamily that promotes survival and differentiation of dopaminergic neurons (47). It has potential as a therapeutic agent in Parkinson disease.
TAT–anti-HDM2	The p53-binding domain to HDM2 (human double minute gene 2), the p53 inhibitory protein (48). Transduction with TAT/PTD–anti-HDM2 could be useful for treating cancers, such as uveal melanoma and retinoblastoma in which p53 is suppressed by overexpression of HDM2.
Delta V1-1 and $\psi\epsilon$RACK	The inhibitory peptide of δ-PKC translocation and the activator peptide of the ϵ-PKC (49), respectively. PKC has an important role in cardioprotection from ischemia-reperfusion injury. Combined treatment with both TAT/PTD derivatives could have a potential application for treating acute cardiac ischemia.
Gp91ds	9-amino acid peptide containing the docking sequence from the NAD(P)H oxidase component, $gp91^{phox}$. Gp91ds is a competitive inhibitor of NAD(P)H oxidase; it has possible application in diseases such as restenosis and atherosclerosis (50).
Catalase	An antioxidant enzyme. Delivery of TAT/PTD catalase to epidermis and dermis of the subcutaneous layer was reported (51). It may have therapeutic applications against skin diseases.

PKC, Protein kinase C.

mitochondria, the mitochondrial signal is recognized and cleaved, releasing the cargo protein, which can be retained in the mitochondria for several days. Furthermore, when administered into pregnant mice, the fusion protein was capable of crossing the placenta and localizing in both the fetus and the newborn pups (*52*).

Interestingly, it has been reported that in addition to transporting proteins through cell membranes, TAT/PTD is capable of transducing nanoparticles into lymphocytes, allowing for the visualization of lymphocytic infiltration in a diabetic mouse pancreas (*53*). If developed for humans, it has the potential for monitoring lymphocyte infiltration caused by autoimmune recurrence in pancreas and islets transplantation.

OTHER PTD

The broad interest in the fields of genomics and proteomics has led to new efforts and strategies to identify and design novel PTD. Examples of novel natural and synthetic PTD are as follows:

- MTS, a 12-residue hydrophobic peptide derived from hydrophobic core region of Kaposi fibroblast growth factor (*54*).
- HIV1 vpr, a 14-kDa protein from the HIV with protein-transducing properties (*55*).
- Syn B peptides, peptide vectors derived from natural peptides known as protegrins. Syn B peptides have an amphipathic structure with hydrophobic and positively charged residues in opposite sides of the structure, and are capable of mediating delivery of the antineoplastic drug doxorubicin to the brain in a mouse model (*56*).
- On the basis of the observation that arginine residues are crucial to the protein transduction capability of TAT/PTD by means of their side chain guanidine groups, Rothbard et al. designed efficient protease-resistant molecular transporters consisting of polyguanidine peptoid derivatives (*35*).
- PTD-4, a synthetic peptide designed to have an optimized Arg alignment on one face of the helix and a strengthened α-helicity (*57*).
- PTD-5, a protein transduction domain selected from the M13 phage peptide display library. PTD-5 was reported to deliver a peptide inhibiting activation of NF-κB in pancreatic islets in vivo (*58*).
- Pep-1, a short amphipathic peptide consisting of three domains: a hydrophobic tryptophan-rich domain required for efficient transduction and interaction with protein hydrophobic pockets, a separator domain, and a nuclear localization signal domain from SV-40 T-large antigen. An advantage of Pep-1 is its capability to deliver proteins that are not bound to it covalently (*59*). Pep-1 was used to deliver active caspase-3 in vivo to simulate a mouse emphysema model (*60*).

POTENTIAL APPLICATION TO CELL AND ORGAN TRANSPLANTATION

PTD technology allows delivering proteins to different cell types and tissues with limited restrictions. These properties make protein transduction a potentially useful application for organ and cell transplantation. In particular, the ability to diffuse across the cellular membrane of most cells independent of specific membrane receptors may be greatly advantageous for ex vivo delivery of proteins to tissues, organs or cells. Another important and distinctive attribute of the PTD protein-peptide delivery system is the transient effect of the transduced protein within the cells. The duration of the expression of the fused PTD protein peptide is limited by its specific protein turnover. This is particularly important in the instance of antiapoptotic proteins, which might be protective and necessary at a particular stage of the transplantable organs or cells, but could be deleterious when expressed for a long period of time in unregulated fashion. Application of protein transduction in transplantation is most suitable for cells and organ preservation. The deleterious effects of

prolonged ischemia after organ harvesting have been closely linked to apoptotic and necrotic events induced by oxidative stress and production of reactive oxygen species. Therefore, preservation of organs could be improved by delivery of cytoprotective, antiapoptotic, or antioxidative proteins or peptides by means of protein transduction at the time of harvesting or immediately after. Encouraging examples have been reported including studies describing reduction of ischemia-reperfusion injury in perfused heart by delivery of the caspase recruitment domain and BH4, the antiapoptotic Bcl-XL domain (*61*, *62*).

Delivery of cytoprotective proteins or peptides by means of PTD could be used to enhance viability of primary transplantable cells that are susceptible to anoikis, a programmed cell death induced by their detachment from the extracellular matrix, such as pancreatic islets and hepatocytes (*63*, *64*). For instance, protein transduction technology has been successfully applied to improve the viability of isolated pancreatic islets (*58*). An inhibitor of NF-κB fused to the novel protein transduction domain PTD-5 was infused into pancreas, by means of the pancreatic duct, before islet isolation.

Pancreatic islets are also susceptible to culture-induced damage, decreasing in number and losing function over time. This in part is caused by cell death induced by apoptosis or oxidative stress during isolation and in vitro culture. TAT/PTD fusion proteins efficiently transduce pancreatic islets (*65*). Inclusion of TAT/PTD derivatives of antiapoptotic-antioxidative proteins, such as heme oxygenase-1, Bcl-XL, and BH4 in the culture media can improve islet viability (*40*, *66*). Enhancing or preserving viability of islets in culture before transplantation could allow for the immunosuppressive preconditioning of recipients.

Watanabe et al. (*67*) recently described an interesting application of protein transduction technology for the development of bioartificial organs. In particular, these investigators applied protein transduction to transiently immortalize human liver stellate cells (HSC), an important part of the hepatic sinusoid responsible for microcirculation and scavenger functions. HSC are capable of preserving hepatocyte liver-specific functions in co-culture. However, HSC have a short lifespan, thus limiting the applicability of this approach. To enhance the limited lifespan of HSC, the cells were transduced with a recombinant virus expressing human telomerase reverse transcriptase (*hTERT*). The *hTERT* gene was flanked by loxP sites, a sequence that is recognized and cleaved by the bacteriophage P1 Cre recombinase. Therefore, control of immortalization was carried out by expression of Cre recombinase. Efficient excision of the retrovirally transferred *hTERT* was achieved by TAT/PTD-mediated expression of the Cre recombinase, thus allowing reversible immortalization, a valuable safety feature for clinical application of genetically modified cells.

CONCLUSION

Currently in its infancy, protein transduction is an area of active research because of its potential to deliver proteins and peptides directly to cells and tissues. Numerous examples of biologically active full-length proteins and peptides have been delivered to cells and tissues, both ex vivo and in vivo. Delivery of cytoprotective proteins or peptides by means of PTD could protect cells or organs during a critical period of time before transplantation, without the requirement for genetic modifications. Growing numbers and a growing variety of PTD offer a great opportunity to delineate the real applicability of this technology in the field of organ and cellular transplantation.

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ANEXO 4

8.4 Klein D, **Ribeiro MM,** Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, Molano RD, Inverardi L, Ricordi C, Pastori RL. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. Biochem Biophys Res Commun 2004;323(2):473-8.

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Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets

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Abstract

Viability of isolated islets is one of the main obstacles limiting islet transplantation success. It has been reported that overexpression of Bcl-2/Bcl-XL proteins enhances islet viability. To avoid potential complications associated with long-term expression of anti-apoptotic proteins, we investigated the possibility of delivering Bcl-XL or its anti-apoptotic domain BH4 to islets by protein transduction. Bcl-XL and BH4 molecules were fused to TAT/PTD, the 11-aa cell penetrating peptide from HIV-1 transactivating protein, generating TAT-Bcl-XL and TAT-BH4, respectively. Transduction efficiency was assessed by laser scanning confocal microscopy of live islets. Biological activity was tested as the ability to protect NIT-1 insulinoma cell line from death induced by staurosporine or serum deprivation. Spontaneous caspase activation in human islets and cytotoxicity caused by IL-1β were significantly reduced in the presence of TAT-Bcl-XL and TAT-BH4. We conclude that both TAT proteins are biologically active after transduction and could be an asset in the improvement of islet viability.

Keywords: Protein transduction; Protein transduction domains; TAT-PTD; Islets; β-cells; Bcl-XL; BH4

Major advances in islet isolation techniques in conjunction with the development of new immunosuppressive regimens have recently established islet transplantation as a realistic alternative to treat patients with a brittle form of Type 1 diabetes mellitus [1,2]. Nonetheless, low viability and impaired physiological function of isolated pancreatic islets increase the total number of islets required for successful outcome, which is generally achieved by sequential transplantations of more than one islet preparations [1,2]. This still remains a significant obstacle limiting the widespread success of the procedure due to the shortage of organs available for transplantation. There is strong evidence that stress originating at the time of organ procurement, during isolation, and in in vitro culture has detrimental effects on islet yield and viability [2]. Cold ischemia at the time of procurement, loss of survival signals when islets are detached from extracellular matrix, and in vitro culture may induce programmed cell death-apoptosis as well as necrosis caused by limited availability of nutrients, growth factors, and oxygen [3,4]. Improving viability of islets in culture prior to transplantation will provide better quality and sufficient number of transplantable islets.

In normal healthy cells the pro- and anti-apoptotic proteins are in equilibrium. In isolated islets this equilibrium has been displaced to advantage of the pro-apoptotic proteins [5,6], thus likely, enhancing the proclivity for cell death. It has been shown that viability of transplantable islets is enhanced by overexpression of Bcl-2 or Bcl-XL [7,8]. These are anti-apoptotic members of the

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Bcl-2 protein family, in control of mitochondrial function and cellular survival. Bcl-XL and Bcl-2 protect cells not only from apoptosis but from some types of necrosis as well [9]. Therefore, transient enhancement of either Bcl-2 or Bcl-XL in islets could be an efficacious way to prevent cell death during isolation and subsequent culture. To avoid potential complications associated with long-term overexpression of anti-apoptotic proteins in cells, we investigated the delivery of Bcl-XL protein to islets by protein transduction. In this technology proteins/peptides are fused to small cationic peptides with cell penetrating properties known as protein transduction domains (PTDs) [10]. Proteins/peptides fused to PTDs can be delivered into a large number of cells including pancreatic islets [11–13]. One of the most characterized PTDs is TAT/PTD, an 11-aa protein transduction domain derived from the basic domain of transcriptional transactivator (TAT) protein of the human immunodeficiency virus-1 (HIV/TAT). TAT/PTD has been reported to deliver full-length proteins and peptides to a great variety of cells and tissues [14].

Conserved homology region 4 (BH4) domain from Bcl-2/Bcl-XL proteins is essential for their anti-apoptotic activity. Protective effects of BH4 have been reported when administered either ex vivo or in vivo [16,17]. BH4 fused to TAT/PTD (TAT-BH4) can bind to mitochondrial voltage dependent anion channel (VDAC), thus regulating the mitochondrial membrane potential and preventing the release of cytochrome *c* [15]. In this study we investigated the potential protective effect of TAT-BH4 and TAT-Bcl-XL in NIT-1 insulinoma cells and in human and non-human primate islets, subjected to a number of different stimuli in vitro: cytotoxicity caused by IL-1β, spontaneous activation of caspase in cultured islets, staurosporine-induced apoptosis, and death by serum deprivation.

Materials and methods

Cell lines and islet isolation. NIT-1 cells were obtained from American Type Culture Collection (ATCC—Manassas, VA) and cultured according to manufacturer's instructions. Human and non-human primate islets were isolated using collagenase (Roche Molecular Biochemicals, Indianapolis, IN) and the automated method, as described previously [18,19]. After isolation, islets were cultured in CMRL medium (Mediatech; Herndon, VA) supplemented with human serum albumin for 24 h at 37 °C followed by 22 °C in humidified incubator with 5% CO_2 until transduction. All transduction experiments were carried out at 37 °C.

Bcl-XL and BH4 TAT derivatives' generation. The recombinant protein TAT-Bcl-XL was generated by subcloning the human coding region cDNA in-frame with the TAT peptide (YGRKKRRQRRR) into the pTAT bacterial expression vector (generous gift from Dr. S.F. Dowdy). TAT-Bcl-XL fusion protein was propagated in BL21 (DE3) pLysE competent bacteria (Novagen, Madison, WI). Bacterial stock was streaked on LB agar plates containing 100 μg/ml of Carbenicillin (Invitrogen, La Jolla, CA). After overnight incubation single colony was inoculated in 1 L of LB medium with 100 μg/ml carbenicillin and grown at 37 °C overnight. For the last hour of incubation IPTG at final concentration of 1 mM (Roche) was added. Bacteria were centrifuged and lysates were prepared using ''Bugbuster'' plus'' Benzonase'' according to manufacturer's instructions (Novagen). Fusion protein was purified by affinity chromatography Ni/NTA column (Qiagen, Valencia, CA) as described previously [13].

HPLC-purified 14-mer TAT peptide control (GYGRKKRRQ RRRGC) was synthesized by Sigma/Genosys (St. Louis, MO) and TAT-BH4 was produced by Calbiochem, La Jolla, CA. TAT-BH4 consists of residues 4–19 from Bcl-XL, fused to TAT peptide (Fig. 2).

Transduction of the islets with pTAT-Bcl-XL—FITC protein. The islets were plated in culture medium supplemented with 300 μM of FITC-labeled pTAT-Bcl-XL protein. After 15 h incubation at 37 °C, transduced islets were washed and transferred to a petri dish fitted with glass coverslip bottom. The presence of fluorescent protein in the islets was assessed using confocal microscopy (Zeiss confocal laser scanning microscope LSM-510).

Protection from cell death induced by lack of nutrition. NIT-1 insulinoma cells (equal number for each condition) were transduced with either TAT-Bcl-XL, TAT-BH4 or control p-TAT peptide and cultured in 6-well plates in F12(HAM) medium containing no serum. Cell death was evaluated by collecting only floating cells after 48 h in culture. The quantity of total floating cells was estimated by total protein in each sample (Bio-Rad protein detection kit; Hercules, CA).

Protection from staurosporine damage. Equal numbers of NIT-1 cells were seeded in 6-well plates in F12 (HAM) medium containing 10% fetal bovine serum. Three hours after transduction with TAT proteins the cells were treated with 500 nM staurosporine and cultured further. The medium was changed and floating cells were collected after 48 h. The number of dead floating cells was estimated by measuring the protein content.

Measurement of total caspase activity in islets by flow-cytometry. Islets were transduced with TAT-Bcl-XL protein, TAT BH4 peptide, and p-TAT control peptide, and cultured at 37 °C for 24 h. At the end of the incubation the islets were collected, dissociated into a single cell suspension by 4-min continuous pipetting in HBSS containing 0.5% trypsin and 0.53 mM EDTA. Activated caspases were evaluated by flow-cytometry (FACS Calibur-Becton–Dickinson). The content of activated caspase was measured using CaspACE-FITC-VAD-FMK in situ marker according to manufacturer's instructions (Promega; Madison, WI).

Measurment of caspase-6 activity. Human or non-human primate islets were transduced with appropriate protective proteins and cultured at 37 °C for 24–96 h. The islets were collected and washed in phosphate buffered solution. Pellets were lysed (0.1% Chaps detergent buffer), and an aliquot of the lysate was used to establish protein content in order to normalize quantitatively all samples. The rest of the lysate was incubated with caspase-6 fluorescent substrate Ac-Veid-AMC (Biomol Research Lab, Plymouth Meeting, PA). Caspase activity of the sample was evaluated by measuring the resulting release of the fluorescence using a plate reader fluorimeter (Wallac Victor, Perkin–Elmer, Boston, MA). In selected experiments, the specific inhibitor of caspase-6-Z-Veid-FMK (BioMol Research Lab) was utilized to confirm its role in mediating apoptosis of islet cells.

Protection from cytokine damage. Transduced islets were treated with 50 U IL-1β (R&D System, Minneapolis, MN) and cultured for 96 h at 37 °C. Islets were harvested and their function was assessed by measuring the glucose-stimulated insulin release, as described previously [13]. Briefly, aliquots of 50 islets in triplicates were incubated at 37 °C for 1 h each sequentially in medium containing 2.8 mM (low), 20 mM (high), and 2.8 mM (low) glucose. Supernatant was collected after each incubation and insulin concentration was assessed with human insulin ELISA kit (Alpco, Salem, NH). The ratio between the insulin output at 20 and 2.8 mM glucose was expressed as stimulation index.

Results and discussion

Analysis of TAT-Bcl-XL transduction efficiency in live islet cells

We investigated the proficiency of TAT-Bcl-XL to translocate into human islets. Human pancreatic islets were transduced with TAT-Bcl-XL fusion protein labeled with fluorescein isothiocyanate (FITC). To avoid potential artifacts due to fixation [20], transduction was assessed by confocal microscopy on live non-fixed, islets (Fig. 1). Propidium iodide staining (red) excluded dead cells and ruled out false positive. Images were collected at 13 different focal planes, demonstrating the ability of TAT-Bcl-XL fusion protein to transduce throughout the entire islet.

Effect of TAT-BH4 and TAT-Bcl-XL transduction on pancreatic β-cells treated with staurosporine and serum deprivation

Bcl-2/Bcl-XL protects numerous cell types against apoptosis induced by cellular stress conditions such as staurosporine (STS) treatment and withdrawal of trophic/growth factors [21–23]. Therefore, we evaluated the protective effect of Bcl-XL and BH4 domain TAT derivatives against these cell death-inducing stimuli in NIT-1 insulinoma cells. Both TAT-Bcl-XL and TAT-BH4 protected NIT-1 cells against STS-induced cell death. After 48 h of culture with STS TAT-Bcl-XL and TAT-BH4-transduced cells showed 20–40% less of apoptotic floating cells than controls: cells transduced with control TAT peptide and untransduced cells (Fig. 2A). We also found that TAT-BH4 and TAT-Bcl-XL could prevent the noxious effect of growth/trophic factors' withdrawal. NIT-1 cells transduced with either TAT-BH4 or TAT-Bcl-XL had an average of 40% and 60% of survival cells, respectively, compared to controls. Untransduced cells and cells transduced with pTAT control peptide under serum deprivation (Fig. 2B).

Transduction with TAT-Bcl-XL or TAT-BH4 partially protects islets against IL-β toxicity

The biological efficiency of TAT-BH4 and TAT-Bcl-XL was evaluated by assessing their ability to inhibit in

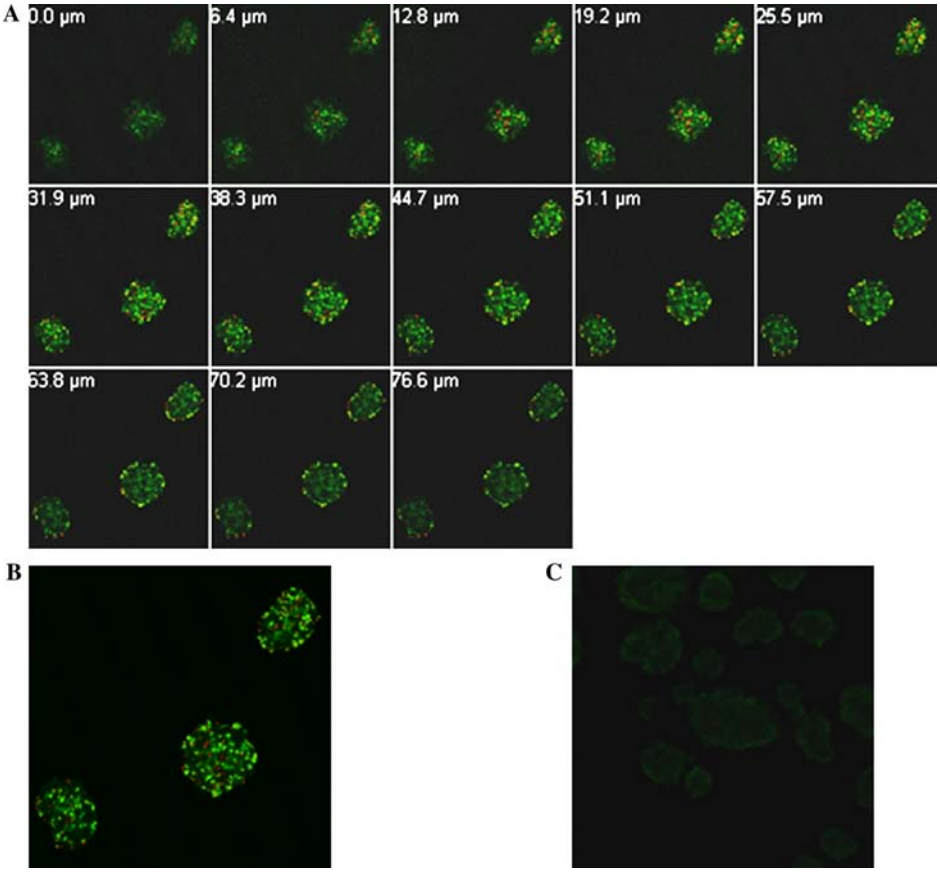

Fig. 1. TAT-Bcl-XL transduction of human islets. Transduction of FITC-labeled TAT-Bcl-XL was assessed by confocal laser scanning microscopy of live, non-fixed isolated islets. (A) TAT-Bcl-XL-FITC fluorescence was analyzed by a 13-step-6.4 µm Z-position sectional scanning of islets, and the 13 sequential scans are shown (10×). Islets were simultaneously stained with propidium iodide to rule out false positives (membrane damaged dead cells). (B) Projection of optical sections from islets transduced via TAT-Bcl-XL-FITC. (C) Autofluorescence of non-treated control islets was negligible compared to transduced islets.

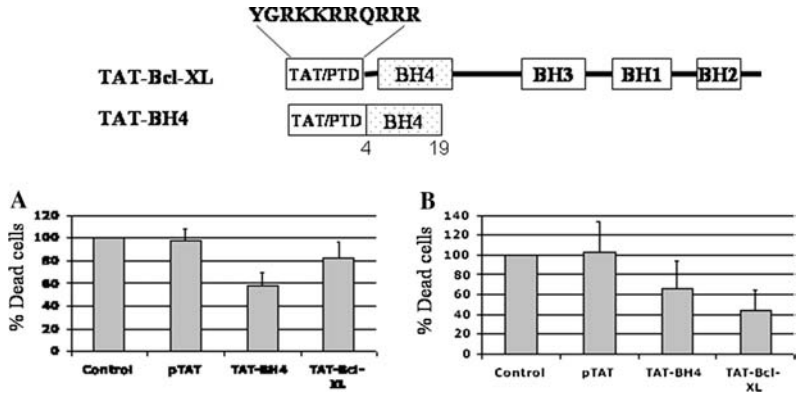

Fig. 2. TAT-Bcl-XL and TAT-BH4 protect NIT-1 cells from staurosporine and serum deprivation. Schematic structure of both TAT-derivatives used in this study. BH4 domain comprises of residues 4–19 from Bcl-XL. (A) Protective effect from staurosporine. Control pTAT and untransduced NIT-1 cells were compared with TAT-Bcl-XL and TAT-BH4-transduced cells. Staurosporine in final concentration of 500 nM was added 3 h after transduction and cells were cultured for a total of 48 h. (B) Protective effect in NIT-1 cells subjected to 48 h of serum deprivation. Untransduced controls represent 100% of dead cells. The data shown are means ± SD of four experiments.

vitro deleterious effect of IL-1β on islet glucose-stimulated insulin secretion. IL-1β affects mouse islets and human islets differently. The cytokine greatly affects viability of mouse islets inducing apoptosis [24]. In human islets IL-1β suppresses the glucose-induced insulin secretion response [25]. Overexpression of Bcl-2 protects mouse islets [8] and several insulinoma cell lines against cytokine-induced apoptosis/necrosis with variable efficiency [26–29]. We observed that human and monkey islets cultured with either TAT-Bcl-XL or TAT-BH4 anti-apoptotic molecules were significantly protected against the deleterious effect of IL-1β, as assessed by in vitro insulin glucose challenge (Table 1). Untransduced islets cultured with IL-1β showed mostly non-physiological glucose-stimulated insulin secretion profiles, whereas TAT-Bcl-XL- and TAT-BH4-transduced islets showed preserved insulin response to glucose challenge. The extent and efficiency of protection achieved by TAT derivative molecules varied from preparation to preparation which reflects in all likelihood the broad variation of islet viability in different islet isolations.

Table 1
Glucose stimulation index (SI) of non-human primate islets (NHP) and human primate islets (HP)

Islet preparation	IL-1β	TAT-Bcl-XL	TAT-BH4	SI
NHP-1	–	–	–	3.7
	+	–	–	NF
	+	+	–	3.2
NHP-2	–	–	–	3.3
	+	–	–	NF
	+	–	+	1.95
	+	+	–	1.4
HP-1	–	–	–	1.9
	+	–	–	NF
	+	+	–	1.35
HP-2	–	–	–	3.3
	+	–	–	1.2
	+	–	+	2.8
	+	+	–	2.4

Two human (HP-1 and HP-2) and non-human primate islet preparations (NHP-1 and NHP-2) islets were used. NF stands for non-functional insulin secretion profile, with a glucose stimulation index (SI) less than 1.

Inhibition of caspase activation in cultured islets by transduction with Bcl-Xl and BH4 TAT/PTD derivatives

Next to necrosis-induced cell death due to factors such as hypoxia, apoptosis is a major cell death pathway occurring in cultured islets. [3,6,30]. In that context, we have assessed the activation of caspases in cultured islets transduced either with TAT-BH4 or TAT-Bcl-XL. Apoptotic occurrence leading to caspase-6 activation in non-human primate islets in culture has been reported previously [31]. To investigate the effect of Bcl-XL and BH4 TAT/PTD derivatives in regard to caspase activation, we measured the enzymatic activity of caspase-6 in cultured islets. Depending on islet availability, we have used either human islets or non-human primate islets for different sets of experiments. After transduction, islets were incubated at 37 °C for various lengths of time. We observed a significant activation of caspase-6 that could be substantially reduced by transduction with either TAT fusion molecule (Fig. 3A) but not with the control pTAT. Further, addition of the specific caspase-6 inhibitor Z-Veid-FMK to the culture resulted in inhibition of caspase-6 activation. Therefore, the protective effects of TAT-Bcl-XL and TAT-BH4 act through inhibition of caspase-6 in isolated islets (data not shown).

Inhibition of caspase-6 activation by TAT/PTD derivatives was observed in our study at minimum of

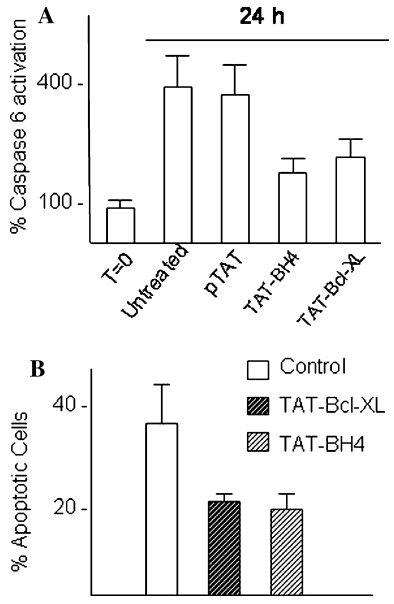

Fig. 3. Transduction of TAT-Bcl-XL or TAT-BH4 inhibits caspase activation in cultured islets. (A) Inhibition of caspase-6 activation in non-human islets by transduction with either TAT-Bcl-XL or TAT-BH4 fusion molecules. Islets were transduced with either pTAT, TAT-BH4 or TAT-Bcl-XL (200 nM) and incubated at 37 °C for 24 h. Activation of caspase-6 was determined enzymatically as described in Materials and methods. Caspase-6 activity of islets at time 0 was taken as 100%. Data shown are representative of four different experiments. (B) Percentage of cells with total activated caspases as assessed by binding of the pan caspase inhibitor zVAD-FMK-FITC. Islets were incubated 24 h at 37 °C in the presence or absence of TAT/PTD derivatives. After dissociation, islets were treated with zVAD-FMK-FITC and evaluated by flow-cytometry. Representative data from three independent experiments are shown.

24 h and up to a maximum of 96 h of culture, depending on the quality of islet preparation. The reasons for caspase-6 activation in isolated islets are still unknown. The literature presents contradictory data: one report suggests that treatment with TNF-α-related apoptosis-inducing ligand (TRIAL) activates caspase-6 in cultured islets [31], while another recent report stipulates that primary islets are resistant to TRIAL mediated cytotoxicity [32].

We assessed the activation of total caspases in dispersed islet cells by measuring the proportion of cells binding the FITC-conjugated pancaspase inhibitor VAD-FMK by flow-cytometry (Fig. 3B). Both TAT-BH4 and TAT-Bcl-XL fusion peptide/protein were able to inhibit up to 50% of total caspase activation in islets.

The transient nature of overexpression and the efficient delivery of proteins/peptides by protein transduction throughout the islet cell cluster may be utilized to achieve cytoprotection and preserve the mass of viable islets for transplantation. Our results indicate that TAT-Bcl-XL and TAT-BH4 could improve the viability and preserve the function of islet cells in culture. This approach may represent a viable strategy to minimize islet loss after isolation and possibly allow for reduction of subsequent islet transplants required for successful achievement of insulin independence.

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ANEXO 5

8.5 Pastori RL, **Ribeiro MM,** Klein D, Ricordi C. Protein Transduction Domain as a Novel Tool for Delivery of Proteins, Peptides and Nucleic Acids. In*: "Biomaterials for Delivery and Targeting of Proteins* and *Nucleic Acids"* RI Mahoto Editor. CRC Press 2005

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17 Protein Transduction Domain as a Novel Tool for Delivery of Proteins, Peptides and Nucleic Acids

Ricardo L. Pastori, Melina M. Ribeiro, Dagmar Klein, and Camillo Ricordi

CONTENTS

17.1 INTRODUCTION

Proteins have a primordial role in mammalian cell functions and are the most abundant cellular macromolecules. They have a key role in cellular structure, regulation of signal transduction, discharge of genetic information and cellular

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metabolism. Therefore, delivery of proteins and peptides into cells would be an extremely valuable approach for therapeutic purposes as well as for cell biology studies.

The cell membrane restricts protein and peptide translocation into the cytoplasm and so limits their use as pharmaceutical and research tools. The phospholipid bilayer structure of the cell membrane efficiently controls inward and outward flow of molecules. Only gases and a few small molecules are able to passively diffuse through the cell membrane. Internalization of other proteins and peptides is severely restricted by their size and polarity.[1]

Several methods for introduction of proteins/peptides into cells are explored and described in the literature. The most studied and utilized are electroporation, microinjection, entrapment within liposomes and polymeric delivery systems.[2–4] Nonetheless, factors such as toxicity, limited cell transduction efficiency and reproducibility have restricted their application.

A rapidly evolving technology known as protein transduction has been recently developed to deliver proteins and peptides into cells and tissues.[5] This technology is based on the ability of certain small peptides known as protein transduction domains (PTD) to cross the cell membranes. Thus, fusing proteins or peptides to PTDs, either chemically or genetically, allows direct delivery of the PTD-fused molecules into cells. The most studied and described PTDs originate from naturally occurring proteins. However, synthetic PTDs have been recently developed as well.

PTDs can be divided into three groups based on their structures: the first group consists of PTDs with cation residues as a common feature, especially with arginines, that have a key role in transduction of PTD-fused molecules. The second group is PTDs that have an amphiphilic or amphipathic structure with hydrophobic and positively charged residues in opposite sides of the structure. The third group is of non-polar PTDs that are derived from the hydrophobic core region of peptide leader sequences, of secreted or cell surface proteins. Although the detailed mechanism of cellular uptake is still not well known, it is well possible that PTDs differ in their mechanism of internalization. Several in depth, comprehensive reviews have been published on this subject.[5–7] Table 17.1 compiles PTDs, derived from naturally occurring proteins as well as synthetic PTDs that have been characterized and described in the literature.[8–22] We will now focus on molecular description and protein/peptide/nucleic acid transduction capabilities of the most researched and best-characterized PTDs.

17.2 PTDs DERIVED FROM NATURALLY OCCURRING PROTEINS

The most extensively characterized PTDs in this group are the transactivating transcriptional transactivator (TAT) protein (86-mer polypeptide) of the human immunodeficiency virus-1 (HIV-1/TAT), *Drosophila*–Antennapedia transcriptional factor (ANTP) and VP22 transcriptional factor from herpes simplex virus type-1 (HSV-1).

TABLE 17.1
List of naturally occurring and synthetic protein transduction domains (PTDs)

PTDs from naturally occurring proteins

Protein/PTDs name	Sequence	Reference
HIV-TAT/PTD	YGRKKRRQRRR	8,9
Antennapedia/ Penetratin	RQIKIWFQNRRMKWKK	10
PDX-1/	RHIKIWFQNRRMKWKK	11
HSV-VP22/	NAATATRGRSAASRPTERPRA PARSASRPRRPVE	12
Protegrins/Syn B1	RGGRLSYSRRRFSTSTGR	13
Kaposi FGF/MTS	AAVALLPAVLLAAP	14
HBV-PreS2/TLM	PLSSIFSRIGDP	15
Vascular endothelial cadherine/pVec	LLILRRRIRKQAHAHSK	16
Prion protein	N-terminal (1–28)	17
Synthetic PTDs		
PTD name	**Sequence**	**Reference**
PTD-4	YARAAARQARA	18
PEP-1	KETWWETWWTEWSQPKKKRKV?	19
PTD-5	RRQRRTSKLMKR	20
Transportan	GWTLNSAGYLLGKINLKALAA LAKKIL	21
PolyArginine	RRRRRRR	22
Polyguanidine peptoids	7 to 9 guanidine head groups of arginine on a glycine backbone.	22

17.2.1 HIV-1/TAT Protein

The first two pioneer studies reporting the transducing capabilities of the HIV/TAT protein were published back to back more than fifteen years ago in 1988.[23,24] Subsequently, heterologous proteins fused to the TAT protein, either chemically or generated as a recombinant protein, were delivered into cells.[25,26] Recently, Dowdy et al. have developed a bacterial expression-based system to produce proteins fused to residues 47–58 (TAT/PTD). This is the TAT protein basic domain, rich in arginine residues, that constitutes the minimal sequence required for transduction.[8,9] The prokaryotic expression vector contains the TAT/PTD-fusion protein under the control of bacteriophage T7 promoter (Figure 17.1). This method allows for the generation of sufficient amount of TAT/PTD-fusion recombinant proteins to perform in vitro and even *in vivo* experiments. TAT/PTD fusion proteins are capable of transducing wide variety of cells and tissues including the hemato-encephalic barrier, which shows resiliency to most methods of exogenous molecules introduction.[27] However transduction of TAT/PTD derivative molecules is not without restrictions. Indeed, some tissues or cells, such as bladder epithelial cells cannot

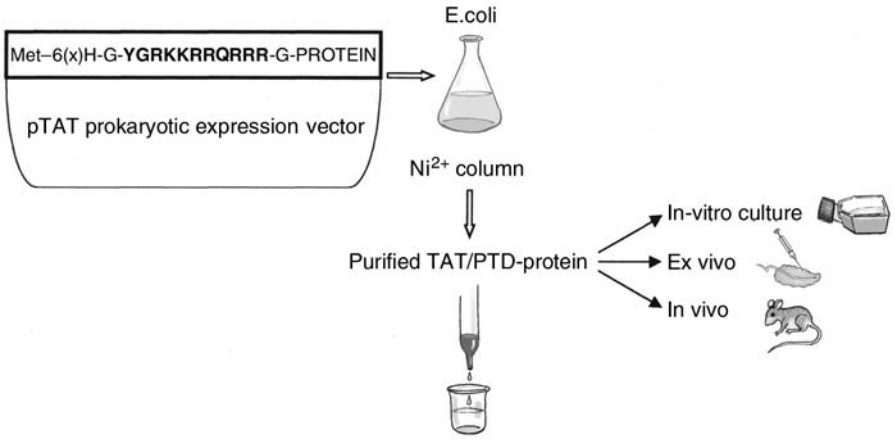

FIGURE 17.1 Generation of TAT/PTD-fusion recombinant proteins. The bacterial expression cassette of TAT/PTD fusion proteins consists of the ATG (Metionine) initiator codon and a N terminal stretch of six histidine residues to allow for binding to a Ni^{2+} column and purification of the fusion protein. The 11-amino-acid TAT protein transduction domain (in bold) contains a potential nuclear localization signal (underlined).

be transduced.[28] Similarly, binding of TAT/PTD fusion proteins to extracellular matrix components can interfere with their cell transduction capability.[29]

It has been historically argued that the process of translocation of peptide/proteins fused to TAT/PTD into cells, known as transduction, was independent of cellular receptors and of temperature, suggesting that energy-dependant endocytosis was not involved. This concept has been now disproved as a cell fixation artifact[30] and several groups have recently shown that cargo molecules fused to TAT/PTD enter cells through endocytosis mechanism.[31–34] Collectively, new investigations suggest that a first step in the process of transduction is binding of the positively charged TAT/PTD to negatively charged components of the cell membrane. Substitution of the positively charged arginine residues by alanines completely inhibits transduction capability.[22] Subsequently, the TAT/PTD fusion molecule enters into cells via energy-dependent endocytosis (Figure 17.2). Both, conventional clathrin-endocytosis and nonclassic caveolar endocytosis have been reported taking place in the translocation process. In the first type of endocytosis the TAT/PTD derivative is internalized in a clathrin-coated pit. Caveolae endocytosis, on the other hand, utilizes caveolae, 50–80 nm plasma membrane invaginations, rich in cholesterol and sphingolipids.[35] Once the caveolae endosome is formed the TAT/PTD molecule is internalized in the cytoplasm. Regardless of the endocytosis mechanism used for translocation, it has been suggested that endosome or caveolosome internalized TAT/PTD must be released into the

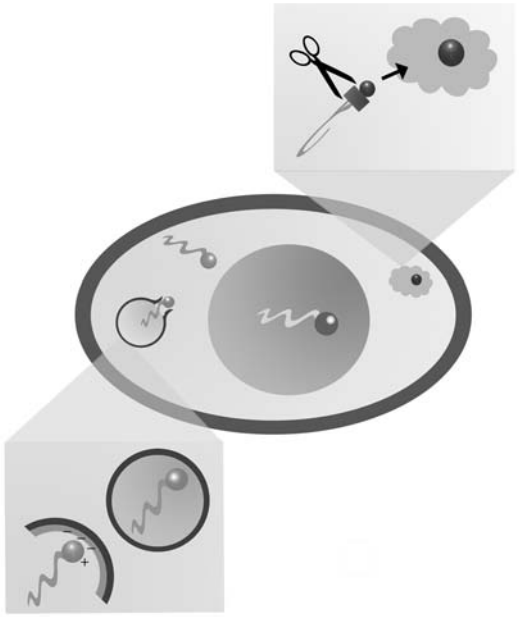

FIGURE 17.2 Transduction of cells by the PTD from the HIV-1/TAT protein. Protein/peptides fused to TAT/PTD are represented as cargoes linked to dots. The mechanism of entrance of TAT/PTD fusion proteins involves endocytosis followed by a subsequent escape from the endocytosis vesicle, after which the fused protein could stay in the cytoplasm or could go to the nuclei. It is believed that the first step involving the interaction of the positively charged TAT/PTD with negative cell membrane environment has an important role in the translocation process. TAT/PTD fusion proteins can be targeted to specific organelles. For example, TAT/PTD engineered with a mitochondrial localization signal (MLS) enters the mitochondria and is recognized and cleaved. The fused protein cargo stays anchored in the mitochondria.

cytoplasmic compartment in order to achieve full biological function (Figure 17.2). The subcellular location of transduced protein depends on the cell type, nature of the protein, and method of delivery.[36]

Wide variety of biologically active peptides and full-length proteins, of molecular weight up to 120 kDa, have been delivered to cells, tissues and organs in vitro, ex vivo and *in vivo*,[5,6,37] offering exciting therapeutic applications. For example, *in vivo* administration of the anti-apoptotic member of Bcl2 protein family, Bcl-XL confers neuronal protection and reduces ischemia brain damage.[38,39] Furthermore, TAT/PTD-fusion proteins have been generated to specifically target cell organelles. For instance, engineering

a mitochondrial recognition sequence (MRS) between the protein cargo and the TAT/PTD allows for anchoring proteins in the matrix of mitochondria. Once the PTD-fusion protein is transduced into the cell and specifically into mitochondria, the MRS is cleaved away from the fusion protein resulting in transduced protein being anchored in the mitochondria (Figure 17.2). TAT/PTD-MRS-fusion protein has remarkable property. When administered into pregnant mice the fusion protein is capable of crossing the placenta and localizing in both the fetus and the newborn pups.[40] Schwarze et al. (1999)[27] demonstrated that the intraperitoneal injection of TAT-β-galactosidase into mice results in detectable β-gal activity in bone marrow as well as in all regions of the brain. Moreover, TAT-β-gal did not disrupt the blood-brain barier as assayed by co-injection with Evan's blue dye.

It has not been determined yet what size of molecules can be efficiently cargoed by TAT/PTD. Among others TAT protein transduction technology has been applied to introduce ions, such as $^{99\,m}$Tc. This may be applied in imaging medicine.[41] Additionally, TAT/PTD has been successfully used to deliver macromolecules that could be used for therapeutic purposes.[42,43] In particular, it allowed delivery of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer to both cytoplasm and nuclei of human ovarian carcinoma cells.[43] This technology holds great promise for the development of polymer-based systems for the delivery of therapeutic molecules. Using TAT protein supramagnetic particles and nanostructures were delivered into cells.[44,45] Supramagnetic particles fused to TAT/PTD were delivered to hematopoetic cells in order to investigate homing profile of leukocytes *in vivo* and to develop new pharmacological transporters.[44] The surface-functionalization of shell cross-linked nanoparticles with the TAT/PTD peptide sequence YGRKKRRQRRR allowed cell binding and internalization of nanobioconjugates to CHO and Hela cells.[45]

TAT delivery system is particularly useful when the expression of a particular gene is only transiently needed as it often happens in cell transplantation. For example, due to the development of new immunosuppressive regimens, transplantation of pancreatic islets has become a therapeutic treatment for type I diabetes.[46] However, despite the great advances achieved in islet technology, a substantial number of islets die during culture due, partly, to programmed cell death, particularly apoptosis. Pancreatic islets are efficiently transduced by TAT protein transduction domain (Figure 17.3). We tested the transduction capability of TAT/PTD in live nonfixed islets to avoid potential fixative artifacts. Human pancreatic islets were transduced with TAT/PTD labeled with fluorescein isothiocyanate (FITC). Transduction was assessed by confocal microscopy in nonfixed, live islet cells, and propidium iodide staining excluded dead cells. Images were collected on five different focal planes demonstrating the ability of TAT protein domain to transduce throughout the entire islet. Furthermore, transduction of islet with anti-apoptotic proteins can significantly enhance the viability of islets in culture with the advantage that islets remain genetically unmodified, thus avoiding undesirable long-term effects.[47,48]

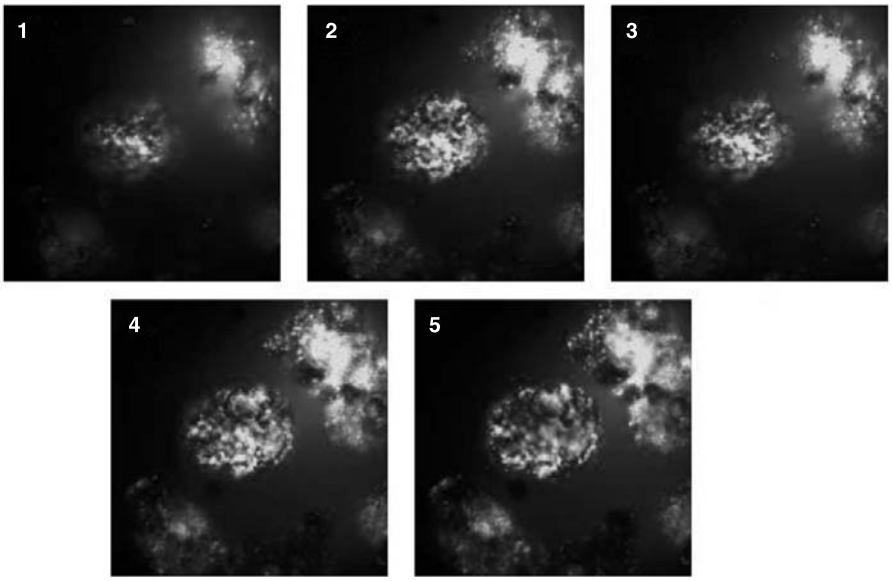

FIGURE 17.3 (See color insert following page 512) Transduction of pancreatic islets in culture with TAT/PTD. Sectional scanning using confocal microscopy of live non-fixed islets. To test the transduction capability of TAT/PTD, human pancreatic islets were transduced with a 14-mer TAT peptide control labeled with fluorescein isothiocyanate (GYGRKKRRQRRRGC-FITC). In order to avoid potential fixation artifacts, transduction was analyzed by confocal microscopy in non-fixed live cells. Islets were simultaneously stained with propidium iodide to rule out false positives (membrane damaged dead cells).

17.2.2 Homeobox Proteins

Homeobox proteins are a family of transcription factors which have a key role in regulation of many developmental genes. The homeobox or homeodomain that characterizes the members of this family is a region of 60 aa forming three alpha-helixes connected by β-turns. Prochiantz's group first reported that the entire homeodomain of the *Drosophila*–Antennapedia transcription factor, was internalized by cells in culture.[49] The same group, later found that the16 aa, of homeodomain's third α-helix, (residues 43–58) (RQIKIWFQNRRMKWKK) known as penetratin, had the same capability as the entire homeodomain.[50] Similar to TAT/PTD, the mechanism of cell membrane translocation is currently unknown. Previous studies performed on artificial phospolipid bilayers suggested a possible mechanism of translocation involving formation of inverted micelles.[10] However, recent work performed on live, nonfixed cells indicated, that similar to TAT/PTD, penetratin could enter into cells via endocytosis-like mechanism.[51] Penetratin has been mostly applied to delivery of small peptides. Table 17.2 shows peptides that have been delivered *in vivo* via penetratin, that could have therapeutic potential.[46–49]

TABLE 17.2
In vivo delivery of protein/peptides through penetratin

Protein/peptide	Description and potential application
P16	The administration of penetratin fused to a 20 amino acids peptide (aa 84–103) from p16 tumor suppressor protein suppressed pancreatic cancer growth and extended survival in mice.[46]
α-smooth muscle actin	The NH_2-terminal sequence EEED of α-smooth muscle actin, is a marker of fibroblast–myofibroblast modulation. Topical administration of Penetratin–EEED inhibits the contraction of rat wound granulation tissue.[47] This approach could help to develop new therapeutic strategies for fibrocontractive pathology.
Caveolin	The scaffolding domain of caveolin-1, primary coat protein of caveolae endosome, fused to penetratin successfully suppressed acute inflammation and vascular leak in mice. This could have potential to develop new therapeutic approach against endothelial inflammation.[48]
NEMO	An amino terminal-helical region of NEMO (Nuclear factor kappa B essential modifier) containing the NEMO binding domain fused to penetratin inhibited inflammatory responses in two experimental mouse models of acute inflammation.[49]

Other homeobox transcription factors have also been reported to carry a protein transduction capability sequence embedded in their homeodomain:

- Fushi-tarazu and Engrailed, two *Drosophila* homeodomain proteins have similar transduction properties.[52]
- the rat protein islet-1, an insulin enhancer containing pIsl1, a PTD encompassing amino acid residues 45–60 (RVIVWFQNKKRCKDKK) of the third helix motif.[53]
- PDX-1 (pancreatic and duodenal homeobox-1), transcription factor that regulates insulin transcription and has a critical role in pancreatic development, contains penetratin-like PTD, (RHIKIWFQNRRMKW KK), in the homeodomain's third α-helix and can be delivered in vitro to pancreatic ducts and islets.[11]
- HOXB4, the third helix of human homeobox B4 protein, contains the sequence identical to the Antennapedia-PTD. Human stem cells, cultured on stromal cells genetically engineered to secrete HOXB4, have been expanded by more than 20-fold over their input numbers. This expansion was associated with enhanced stem cell repopulating capacity *in vivo* and maintenance of pluripotentiality.[54]

17.2.3 HSV/VP22 Protein

It has been reported that the translocation capability of the 38 kDa structural protein from the herpes simplex virus was based on the entire protein. Unlike the other PTDs, VP22 did not have a shorter domain capable of cellular transduction. A unique feature of VP22 is the capability to mediate intercellular trafficking. Most of the published literature comprises studies in which the production of VP22 fused protein was genetically engineered and diffused to nontransfected neighboring cells via intercellular trafficking. However, a recent study showed that VP22 C-terminal 34 amino acids were sufficient for import of proteins into cells.[12] In vivo application of transduction via intercellular trafficking of VP22-fused derivatives was achieved with tumor suppressor p53[55,56] and the enzyme thymidine kinase.[57] VP22-protein transduction was reported to be successful in the delivery of therapeutic proteins into cells of the central nervous system.[58] VP22 protein translocation capability has also been utilized through a rather unusual technology. The soluble subdomain of VP22 (residues 105–300) induces formation of spherical particles when incubated with small oligonucleotides. The particles termed vectosomes are taken up by a number of cell types, remain in the cytoplasm for several days until disrupted by light activation. Using this phenomenon a short peptide originating from pro-apoptotic BH3 domain of Bak protein was delivered into cells via vectosomes and subsequently released into the cytoplasm by light activation.[59]

17.2.4 Other PTD Derived from Naturally Occurring Proteins

A group of less characterized and documented PTDs originating from natural occurring proteins includes PreS2 protein[15] from hepatitis B virus, membrane translocation sequence (MTS) from Kaposi fibroblast growth factor[14] and peptide SynB1 from protegrins family of peptides.[13] PreS2 protein expressed on the surface of hepatitis B virus (HBV) contains amphipathic peptide residues 41–52 with membrane translocation properties. The peptide was used to express the HBX regulatory protein from HBV.[60] The nonconserved hydrophobic region of signal peptides has the capability of crossing cell membranes. In particular, the 12 residues of Kaposi's fibroblast growth factor, a hydrophobic domain, termed MTS, has been utilized to deliver several peptides and proteins.[14]

The SynB1 peptide is derived from protegrins, a family of natural small peptides with antimicrobial capability.[61] It has an amphipathic structure, capable of crossing cell membranes and blood–brain barrier without lytic activity. It has also been used to deliver anti-neoplastic drugs into brain. For instance, SynB1 coupled to doxorubicin significantly enhances the extent of doxorubicin penetration through the blood–brain barrier.[13]

17.3 SYNTHETIC PTDs

Reflecting the significant increase in number of laboratories interested in studying delivery of proteins and peptides through PTDs, several synthetic PTDs have been recently reported. The most promising PTD designed rationally to increase their cell translocation capabilities are:

- *PTD-5*. This protein transduction domain was discovered by screening an M13 phage display library for cationic peptides. PTD-5 has a high content of Arg residues (Table 17.1). Fused to an antimicrobial peptide induced apoptosis and tumor reduction of human head and neck tumor in mice.[62] In addition, when fused to PTD5 a peptide derived from NEMO protein (nuclear factor kappa B essential modifier) inhibited activation of nuclear factor kappa B in pancreatic islets *in vivo*.[63]
- *PTD-4*. Based on the observation that the arginine-rich TAT/PTD has a strong amphipathic structure, a synthetic peptide known as PTD-4 was designed to acquire an optimized amphipathic structure with arginine residues alignment on one face of the helix and an Ala residues to strengthen the alpha-helix structure of the peptide on the other.[18] PTD-4 was utilized to deliver the SH3 domain of an adapter protein.[64]
- *Polyarginine and polyguanidine peptoid derivatives*. Arginine residues not only have a critical role in the transduction capability of TAT/PTD, but was also shown to have a polyarginine containing seven arginine residues (R7) that was more efficient in translocating through cell membranes.[22] Moreover, it was observed that the side chain guanidine group from arginine residues, was ultimately responsible for cell transduction.[22] Based on this fact an efficient protease-resistant molecular transporters consisting of polyguanidine peptoid derivatives were developed.[22]
- *Pep-1*. This is a short amphipathic peptide engineered by combining three domains: a hydrophobic-tryptophan rich domain required for efficient transduction and interaction with protein hydrophobic pockets, a separator domain and a nuclear localization signal domain from SV-40 T-large antigen. Its main feature is the capability of delivering proteins that are not covalently bound.[19] A mouse emphysema model was generated by delivery of active caspase-3 *in vivo* by PEP-1.[65]

17.4 PROTEIN TRANSDUCTION DOMAINS FOR DELIVERY OF NUCLEIC ACIDS

Gene therapy is presently considered to be a promising way to cure a broad spectrum of diseases. Because of low biomembrane permeability and rapid degradation of DNA and oligonucleotides, they are believed to have limited therapeutic value of their own. Although viral gene transfer technologies have greatly improved during the last decade, basic concerns regarding their safety still remain. Limited targeting of cells, integration with potential oncogenes and unwanted immunoresponse are the greatest drawbacks of this method.

Therefore, PTD-mediated delivery of genes could potentially offer new possibilities for gene therapy in medical research.[66]

17.4.1 Delivery of DNA

PTDs are efficient DNA condensating agents that protect it from anionic proteoglycans disruption and degradation,[67] allowing for intracellular transport across mammalian and prokaryotic cells in vitro and *in vivo* with no toxic effect. However, methods that do not involve condensation can also be used for DNA delivery. For example, engineering an λ phage to display the TAT peptide on its surface, greatly facilitates transfection of encapsulated DNA into mammalian cells.[68]

Several results indicate that TAT peptide may become a useful component of synthetic gene delivery vehicles, applicable in the *in vivo* transfer of therapeutic genes. In order to facilitate membrane transduction TAT peptides can be bound directly to DNA or to DNA carriers, or even be just present in the DNA mixture. For this purpose different strategies have been described. Electrostatic based complexes of plasmid DNA with monomeric[2] or oligomeric TAT peptides[69] (2 to 4 molecules) facilitated the transduction into mammalian cells and expression of genes. Similarly, branching TAT peptides, containing eight TAT moieties were able to deliver DNA into cells.[70] TAT peptide conjugated to cationic liposomes efficiently transduced cells in vitro and tumor *in vivo*.[71] This could be effective strategy for therapeutic gene delivery. Furthermore, it was reported that TAT/PTD or Antennapedia complexed with recombinant retrovirus increased viral mediated gene delivery and protein expression in vitro and *in vivo*.[72]

An efficient gene delivery agent with low cytotoxicity was generated by enzymatic digestion of the arginine-rich low molecular weight protamine (LMWP) with thermolysine.[73] LMWP showed similar intracellular localization and kinetics to those of TAT peptides and efficiently transduced and expressed the Lac Z gene. Compared to either, naked DNA or DNA/polyethyleneimine (PEI) complex, the LMWP/DNA showed significantly enhanced gene transfer while exhibiting markedly reduced cytoxicity.

A synthetically engineered PTD, named CFIS-R, was successfully designed to deliver DNA in vitro and *in vivo*.[74] CFIS-R consists of an arginine core sequence that facilitates DNA transduction. It is flanked with terminal cysteines to stabilize DNA condensation and histidine-glycine-histidine triplet interspersing every arginine residue completes the design. Spacing of the arginine core enhances gene delivery probably by protonation of the histidine groups thus allowing the plasmid to exit to the cytoplasm.[75] While six arginines are required for optimal in vitro transfection only three arginines are sufficient for *in vivo* gene transfer. The transfection is inhibited at 4°C which suggests that endocytosis is the mechanism of internalization with subsequent plasmid DNA endosomic escape. CFIS-R increases gene expression in both murine and human tissue *in vivo*.

17.4.2 Delivery of Oligonucleotides and Peptide Nucleic Acids

The use of PTDs for delivery of oligonucleotides and peptide nucleic acid (PNA) has not been investigated as extensively as delivery of peptides and proteins. It is basically limited to TAT/PTD and Antennapedia delivery systems. Antisense oligonucleotides to the amyloid precursor proteins (APP) gene, successfully transfected cells and decreased the neosynthesis of APP expressed in glial and neural cells.[76] The change caused by the antisense oligonucleotide was transient but adequate to decrease axon and dendritic outgrowth in embryonic cortical neuron development. Enhancement of oligonucleotide delivery via TAT/PTD or Antennapedia conjugation, inhibited expression of Luciferase reporter gene.[77] TAT/PTD was utilized to deliver phosphorodiamidate morpholino oligomers (PMO) as well.[78] TAT-PMO conjugate targeted *c*-myc RNA and achieved nearly 100% inhibition of the expression at high doses, which lead to toxic side effects.

PNA is a molecule similar to DNA except an amino acid backbone replaces the sugar-phosphate present in DNA.[79] The amino acid backbone stabilizes PNA/DNA and PNA/RNA duplexes and protects them from degradation. PNAs have great potential for antisense applications because of their biological resistance to degradation and ability to bind complementary mRNA with stability and specificity. However, PNAs are not capable of crossing cell membranes. Several PNA molecules directed against oncogene PM1 RNA, or telomerase and galanin receptor RNAs have been described.[80–82] PTD conjugated PNAs have been generated to study *in vivo* characterization and to measure biological processes at the cellular and molecular level (molecular imaging).[83] Intracellular MRI contrast agents were developed based on a PNA directed against oncogene c-myc conjugated to Antennapedia. An increased signal intensity in the tumor cells was observed in vitro and *in vivo*, using a *c*-myc-specific PNA compared to a non-specific PNA control.

A novel antibacterial therapy was developed based on PNA delivery by PTDs. The entry of foreign molecules in the bacteria is restricted by the lipopolysaccharide (LPS) layer and the inner and outer bacterial membranes. However, the delivery of a PNA against an essential fatty acid biosynthesis protein (acp) was achieved by conjugation to a peptide, KFFKFFKFFK, that was able to cross the bacterial membranes.[84,85] Treatment with anti-acp PNA-KFFKFFKFFK completely suppressed bacterial growth. Moreover, cell cultures, noninvasively infected with *Escherichia coli* K12, were decontaminated by transduction with anti-acp PNA.

17.5 CONCLUDING REMARKS

The discovery of PTDs and their ability to translocate cellular membranes, with high efficiency and low toxicity, opened the possibility for intracellular delivery of proteins and peptides, DNA, antisense oligonucleotides, PNA and even nanoparticles in vitro and *in vivo*. Basic issues such as mechanism of cell

membrane translocation, immunogenicity and distribution within cells, have to be more deeply understood in order to have an optimal design of PTD molecules. Nevertheless, this technology already points out in the direction of a new and exciting way of generating novel molecules for research and therapeutic applications.

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ANEXO 6

8.6 Mendoza V, Klein D, Ichii H, **Ribeiro MM**, Ricordi C, Hankeln T, Burmester T, Pastori RL. Protection of islets in culture by delivery of oxygen binding neuroglobin via protein transduction**.** Transplant Proc 2005;37(1):237-40.

ELSEVIER

Protection of Islets in Culture by Delivery of Oxygen Binding Neuroglobin via Protein Transduction

V. Mendoza, D. Klein, H. Ichii, M.M. Ribeiro, C. Ricordi, T. Hankeln, T. Burmester, and R.L. Pastori

ABSTRACT

Islet transplantation has become an accepted method to treat type 1 diabetes. To succeed and achieve normal levels of glucose in transplant recipients, the quality of the transplanted islets is of the utmost importance. Lack of oxygen during organ procurement, islet isolation, and subsequent culture triggers apoptosis or necrosis and loss of islet function, causing the yield and quality to diminish. A promising candidate for cytoprotection against oxygen deprivation is neuroglobin (Ngb). Ngb is a recently described member of globin family and is expressed in neurons, retina, and pancreatic islets. To overexpress this protein in the islets and study its ability to protect them, we utilized protein transduction. Protein transduction is achieved by fusing Ngb to the TAT/PTD transduction domain, a peptide originated from the HIV transcriptional transactivator protein. Our study proved that TAT-Ngb is an efficient fusion protein capable of protecting the human islets in culture from loss of cell mass and function, thus increasing the quality of transplantable islets. If the islets could be cultured for a longer period of time without suffering harmful effects, it would be possible to precondition the recipient and there would be more time to assess their quality and function before transplantation.

IMPROVEMENTS IN IMMUNOSUPPRESSIVE REGIMENT have made clinical islet transplantation a feasible choice to treat type I diabetes.[1] The low quality and viability of the transplantable islets is one of the major obstacles to successful outcome of the transplantation. Hypoxia/ischemia at the time of organ harvesting, the devascularisation and the removal of extracellular matrix at the time of isolation, and ficol toxicity at purification are all factors contributing to an early death of insulin-producing cells either by apoptosis or necrosis.[2] During culturing, oxygen deprivation is one of the leading causes of islet mass loss. A promising candidate for cytoprotection against oxygen deprivation is neuroglobin (Ngb), a recently discovered member of the globin vertebrate super family.[3] Globins are intracellular heme-Fe-proteins that bind reversibly to oxygen and other gaseous ligands. The two best-characterized members of this group are hemoglobin (Hb) and myoglobin (Mb). Hb transports oxygen to tissues, while Mb facilitates the diffusion of oxygen to mitochondria in muscle tissue. Ngb is a cytoplasmic protein expressed predominantly in tissues that have a high O_2 consumption rate such as neurons, retina and endocrine tissues, including the islets of Langerhans.[3,4–6] Ngb expression in neurons is upregulated at mRNA and protein level, in acute phase of hypoxia.[7] Overexpression of Ngb has been proved to have protective role in cerebral ischemia.[8]

We hypothesize that overexpression of Ngb in cultured islets could protect them from noxious effects of hypoxia and other insults that inevitably lead to loss of cell mass and function.

Therefore, we investigated delivery of Ngb in the form of recombinant fusion protein utilizing a protein transduction (PT). In this technology cell penetrating peptides such as TAT/PTD, an 11 amino-acids long peptide from HIV transcriptional transactivator (TAT) protein, freely cross cell membranes, including the hemato-encephalic barrier[9]

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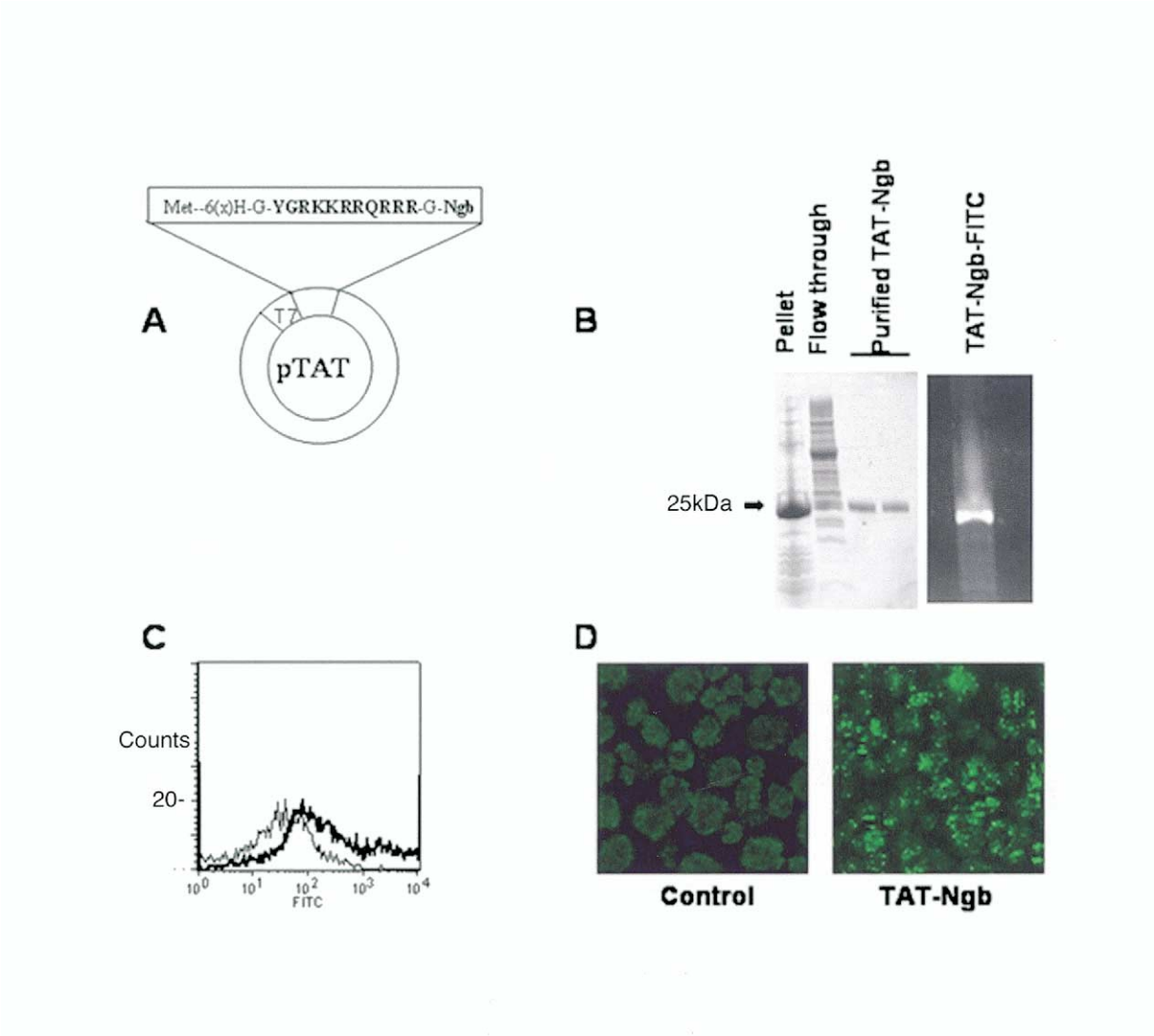

Fig 1. Generation of a recombinant TAT-Ngb and transduction of human islets. **A** Cloning of recombinant human TAT-Ngb. The TAT-Ngb was generated by inserting the DNA coding region of human neuroglobin in frame with TAT into the pTAT expression vector. **B** Purification of TAT-Ngb. Protein was purified by HIS-tag affinity chromatography. The purity of the protein was assessed by SDS-Page gel and coomassie blue stain. The size of the obtained protein is approximately 25 kDa. From left to right the gel depicts separation of the pellet, the flow through, and purified desalted TAT-Ngb protein. The pure TAT-Ngb protein was labeled with FITC. The labeling efficiency was assessed by SDS-Page gel. **C** Flow cytometry of human islets transduced with TAT-Ngb-FITC. The bold line represents fluorescent intensity of TAT-Ngb-FITC. The standard line represents untransduced control. **D** Confocal microscopy evaluation of human islets transduced with TAT-Ngb-FITC. The fluorescence of transduced islets was analyzed by Z-position 10- step sectional scanning 2 μm optical sections of live unfixed islets. Average of optical sections is shown. Original magnification is 10×. Note the punctuated fluorescence pattern observed within the islets suggesting localization of labeled molecules in endosomic cytoplasmic vesicles prior to their release into cytoplasm.

and pancreatic islets.[10] In this study, we have characterized a functional recombinant protein TAT-Ngb and studied its cytoprotective function in human islets.

MATERIALS AND METHODS

Cloning, Purification, and Labeling of Human TAT-Ngb

The TAT-Ngb was generated by inserting the coding DNA sequence of human Ngb in frame with TAT in the pTAT expression vector (gift from Dr S.F. Dowdy). The recombinant fusion protein TAT-Ngb was propagated in BL21(DE3)pLysE competent bacteria (Novagen, Madison, Wisc) and purified by His-tag affinity chromatography Ni/NTA column (Qiagen, Valencia, Calif). Obtained TAT-Ngb protein was 95% pure as assessed by SDS-Page gel (BioRad, Hercules, Calif). To evaluate efficiency of transduction, TAT-Ngb was labeled with fluorescein isothiocyanite (FITC) following the manufacturer's instructions (Invitrogen, La Jolla, Calif) (Fig 1A–B).

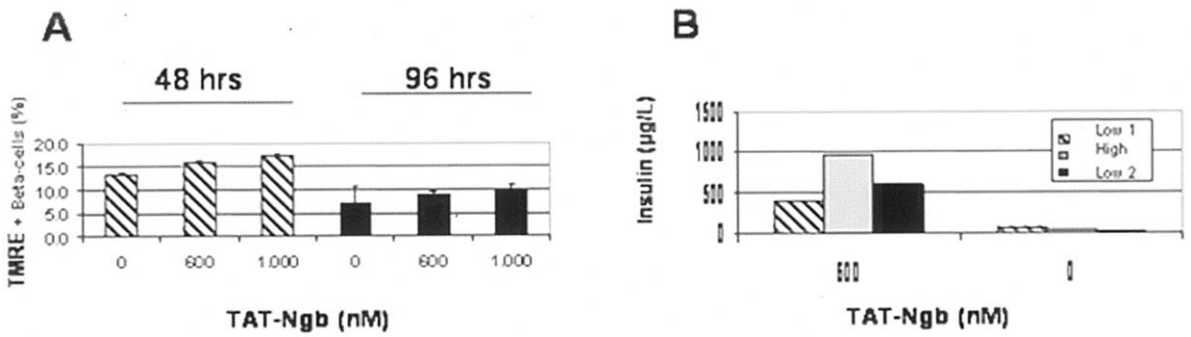

Fig 2. Transduction with TAT-Ngb protects islets in culture. Flow cytometry analysis of human islets cultured for 48 to 96 hours withTAT-Ngb. **A** Percentage of potentiometric dye TMRE stained β-cells. (TMRE is a dye staining live mitochondria in the cells). **B** Glucose index challenge of islets cultured with TAT-Ngb for 120 hours and their control. Black striped bar represents Low 1 (insulin concentration in the sample after incubation with low glucose buffer). Gray bar is High (insulin concentration after incubation with high glucose buffer). Black filled bar is Low 2 (the second incubation with low glucose buffer). The islets cultured with TAT-Ngb (600 nM) retained their physiological function and are able to respond to different concentrations of glucose in the buffer while the controls no longer respond.

Human Isolation Culture and Transduction With TAT-Ngb

Islets were isolated using collagenase (Roche Molecular Biochemicals, Indianapolis Ind) and the automated method, as described previously.[11] The islets were cultured in CMRL medium (Mediatech, Herndon, Calif) supplemented with human serum albumin in humidified incubator with 5%CO_2. All transduction experiments were carried out at 37°C. Islets were plated in nontissue culture treated 6-well plates (VWR, Bristol, Conn). Sterile TAT-Ngb protein was added at final concentration of 600 nM or 1 μM. Protein remained in designated wells throughout the experiment. Controls were untransduced islets.

Flow Cytometry Analysis of Islets Transduced With TAT-Ngb, TAT-Ngb/FITC, and Controls

Islets were assessed for cell viability using 7AAD dye exclusion test. Insulin producing beta-cells were identified by binding of Zn sensitive probe Newport Green (Invitrogen, Carlsbad, Calif) to insulin[12] and mitochondrial membrane potential was assessed by fluorescent probe tetra-methyl rhodamine ethyl ester (TMRE). Islets (500–1000 IEQ) were dissociated into single-cell suspension with 1 mL Accutase solution (Innovative Cell Technologies Inc, San Diego, Calif) for 5–15 min at 37°C, subsequently dispersed by gentle pipeting and immediately analyzed by flow cytometry (FACS Calibur, Becton-Dickinson, Mountain View, Calif).

Confocal Microscopy of Live Human Islets

Human islets transduced and incubated for 24 hours with TAT-Ngb-FITC were washed in PBS-suplemented with 0.5% BSA and plated on a glass-bottom plate (MatTek Corp, Ashland, Mass) for confocal microscopy analysis. Analysis was carried out with Zeiss confocal laser scanning microscope LSM-510.

Static Glucose Challenge of Human Islets of Langerhans

Islets cultured for an extensive period of time at 37°C lose their physiological function, specifically response to glucose and ability to secrete insulin. TAT-Ngb transduced islets and controls were subjected to static glucose challenge. Aliquots of 50 IEQ islets were cultured for 120 hours then transferred to column loaded with 1 mL Sephadex G-10 containing Krebs low glucose buffer (40 mg/dL) plus complete protease inhibitors (Roche) and pre-incubated for 45 minutes at 37°C. After pre-incubation the buffer was changed for a fresh 1 mL low glucose Krebs buffer and columns incubated 1 hour at 37°C. Collected samples were named L1. The Krebs low glucose buffer was replaced with Krebs high glucose (400 mg/dL) and incubated 1 hour at 37°C. Samples collected after incubation were named Hi. The high glucose buffer was washed out with low glucose buffer and incubation repeated. Samples collected at this time were named L2. Insulin content of the samples was determined with the "Insulin Elisa kit" (Alpko, Winham, NH). Stimulation index (SI) was calculated as a ratio of Hi/L1. The SI is valid only if the value of L2 returns to similar levels as L1 proving the ability of the islets to respond to glucose concentrations in the buffer.

RESULTS AND DISCUSSION

TAT-Ngb-FITC Efficiently Transduces Human Islets

Human islets were transduced with fusion protein TAT-Ngb labeled with FITC. To avoid potential artifacts associated with fixation, the transduced islets and untransduced controls were examined live, nonfixed using confocal microscopy and flow cytometry. Confocal images were taken at 10 focal points generating optical sections of 2-μm thickness going throughout the islets and confirming that every islet cell was transduced, including the cells in the most inner core of the islet (Fig 1 D).

The same islets were later dissociated and analyzed by flow cytometry. Shift of medium fluorescence intensity of TAT-Ngb-FITC transduced cells compared to untransduced cells suggests that the entire cell population contains recombinant fusion protein TAT-Ngb-FITC (Fig 1C).

TAT-Ngb Protects Islets in Culture

Next, we studied whether transduction with TAT-Ngb affects viability of cultured islets. Permeabilization of mitochondrial membranes is characteristic of apoptotic and necrotic cell damage and can be indirectly determined by a

reduction in the mitochondial potential ($\Delta\psi$). Using flow-cytometry we evaluated the percentage of beta-cells having a normal potential ($\Delta\psi$) through binding of potentiometric dye TMRE. We found that transduction of islets with TAT-Ngb protected them in culture for 48 as well as 96 hours (Fig 2).

After 48 hours of culture the percentage of beta-cells with a normal mitochondrial potential (TMRE positive cells) was 13.2% and 17.2% for control and islets treated with 1 μM TAT-Ngb, respectively. For 96 hour culture percentage of viable beta-cells were 7.1% control and 9.9% for Ngb-treated cells. Therefore, there are 30% more viable beta-cells in islets transduced and cultured with TAT-Ngb for 48 hours and 39% more viable beta-cells in 96 hours-long culture. We have consistently observed Ngb protection in islet preparations of different quality.

The preservation of function was assessed by glucose static insulin secretion assay. The transduced islet cells retained the ability to react to changing concentrations of glucose in the medium by producing insulin at appropriate concentrations even after being cultured for 120 hours, while their untransduced counterparts clearly lost the valuable function (Fig 2D). After 120 hours in culture, transduced islets had a stimulation ratio (SI) of 2.5, while control islets were totally unresponsive to glucose stimulation (SI < 1).

In conclusion, at the present time we do not fully understand the mechanism by which TAT-Ngb protects islets in culture. Several possible biological functions of TAT-Ngb have been proposed, including oxygen storage, elimination of inducible NO produced by hypoxia conditions, and scavenging of free radicals.[13] Any of these properties could provide the beta cells with broader resistance to oxidative stress. Future experiments will allow us to shed light on this matter. Because of its ability to protect the viability and function of beta cells in long-term cultures, the TAT-Ngb could be an asset in preparation of better quality islets for transplantation. The possibility of extended time of culturing islets would allow for the preconditioning of recipients for transplant.

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ANEXO 7

8.7 Domínguez-Bendala J, Klein D, **Ribeiro M**, Ricordi C, Inverardi L, Pastori R, Edlund H. TAT-Mediated Neurogenin 3 Protein Transduction Stimulates Pancreatic Endocrine Differentiation In Vitro. Diabetes 2005;54(3):720-6

TAT-Mediated Neurogenin 3 Protein Transduction Stimulates Pancreatic Endocrine Differentiation In Vitro

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Stem cell technologies hold great potential for the treatment of type 1 diabetes, provided that functional transplantable β-cells can be selectively generated in an efficient manner. Such a process should recapitulate, at least to a certain extent, the embryonic development of β-cells in vitro. However, progress at identifying the transcription factors involved in β-cell development has not been accompanied by a parallel success at unraveling the pattern of their instructive extracellular signals. Here we present proof of principle of a novel approach to circumvent this problem, based on the use of the HIV/TAT protein transduction domain. Neurogenin 3 (ngn3), a factor whose expression is essential for pancreatic endocrine differentiation, was fused to the TAT domain. Administration of TAT/ngn3 to cultured pancreatic explants results in efficient uptake, nuclear translocation, and stimulation of downstream reporter and endogenous genes. Consistent with the predicted activity of the protein, e9.5 and e13.5 mouse pancreatic explants cultured in the presence of TAT/ngn3 show an increased level of endocrine differentiation compared with control samples. Our results raise the possibility of sequentially specifying stem/progenitor cells toward the β-cell lineage, by using the appropriate sequence and combination of TAT-fused transcription factors.
***Diabetes* 54:720–726, 2005**

Islet transplantation has proven successful for the treatment of type 1 diabetes (1,2), but the shortage of donor pancreata has hindered the widespread clinical implementation of this therapy. Therefore, it is essential to find additional sources of islets. Human embryonic stem cells may present one promising alternative for the in vitro generation of islet cells. For this prospect to be realistic, however, we need to identify the appropriate conditions that will favor differentiation of islet cell types. Ideally, such conditions should reproduce as accurately as possible the sequence of events that results in islet formation during embryogenesis. Although little is known about the first of such events (endodermal specification), subsequent steps in pancreatic development have been associated with the timed expression of key transcriptional factors, such as insulin promoter factor-1 (Ipf1)/pancreatic and duodenal homeobox factor-1 (pdx1), Ptf1a, neurogenin 3 (ngn3), Pax4, Pax6, and Isl1 (3–8). During murine pancreatic development, endocrine differentiation occurs through a lateral inhibition process, mediated by Notch signaling. Cells in which Notch is activated by the ligands delta or serrate express high levels of *HES-1*, which in turn represses the proendocrine gene *ngn3*. However, in ligand-expressing cells, *HES-1* expression is not upregulated, thus allowing robust *ngn3* expression and differentiation toward the endocrine lineage (5–8).

ngn3 encodes a class B basic helix-loop-helix factor, which has been shown by loss-of-function studies to be required for the development of the four endocrine cell lineages of the pancreas (5). The pro-endocrine role of *ngn3* has also been demonstrated in gain-of-function studies. Ectopic *ngn3* expression (6–9), as well as lineage tracing experiments (10), indicates that ngn3 is a cell-autonomous determinant and true marker of endocrine progenitor cells. The adoption of each endocrine fate within the islet (α-, β-, δ-, and PP cells) occurs preferentially at specific time points during embryogenesis, suggesting that *ngn3*-positive cells adapt their responses to an evolving milieu of signals. Premature expression of the *ngn3* gene in early pancreatic progenitor cells (e8.5–e9) results in their differentiation into glucagon-producing cells (6). Adenovirus-mediated expression of *ngn3* in adult human duct cells induces neuroendocrine differentiation (11). Finally, ectopic expression of *ngn3* in the chick gut leads to the differentiation of endodermal cells into endocrine cell types that form clusters in the mesenchyme (12). Taken together, these studies suggest that ngn3 could be used as a molecular agent to induce endocrine differentiation in islet neogenesis protocols.

Although gain-of-function studies are essential for the elucidation of gene function and regulation, genetic manipulation is not desirable for clinically oriented differentiation approaches. The unpredictability of both the site of integration and the number of gene copies, as well as the

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β-gal, β-galactosidase; Ipf1, insulin promoter factor-1; ngn3, neurogenin 3; pdx1, pancreatic and duodenal homeobox factor-1.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

side effects often observed when using viral vehicles (13,14), are just a few of the drawbacks of conventional gene transfer strategies. Furthermore, terminal endocrine differentiation is invariably associated with *ngn3* silencing, which is only transiently expressed in cells that are about to exit the cell cycle (8). Therefore, any possible use of ngn3 as an in vitro pro-endocrine agent should ideally be transient and not involve the transfer of the gene itself. The use of protein transduction domains would circumvent such restrictions by providing a versatile transduction system, where the protein of interest could be added to the culture medium at the appropriate concentration and only for the time its function is required. The protein transduction domain of the HIV/TAT protein has been extensively used because of its effectiveness and small size (11 amino acids) (15). Many TAT-fused full-length functional proteins have been transduced into cells and tissues (16–24,25). When systemically administered to rodents in vivo, TAT-protein hybrids have been shown to freely diffuse across all tissues, crossing the hemato-encephalic barrier (26) and even the placenta (27). Recently, TAT has been used to deliver the homeobox HOXB4 protein to human hematopoietic stem cells, which resulted in rapid expansion without loss of normal in vivo potential for differentiation or long-term repopulation (28). Intriguingly, many homeobox proteins already have protein transduction domains embedded in their amino acid sequence. In fact, IPF1/PDX1 has its own antennapedia-like protein transduction domain, which has been used to successfully deliver native IPF1/PDX1 protein to islets and cultured duct cells, where it enhanced insulin expression (29). However, ngn3 lacks such intrinsic domain.

Here we report that a TAT/ngn3 fusion protein is effectively taken up by cells and functions in vitro in a manner consistent with the reported activity of native ngn3. These results raise the possibility of using protein transduction domain technology to sequentially introduce critical transcription factors to stem and progenitor cells in vitro as a way of promoting their differentiation into functional cell types in a controlled and reproducible manner.

RESEARCH DESIGN AND METHODS

Vector construction and protein purification. The TAT/ngn3 construct (online appendix available at http://diabetes.diabetesjournals.org) was generated by inserting the coding region of the mouse *ngn3* in the *Nco*I/*Age*I sites of a pTAT expression vector (provided by Stephen Dowdy, University of California San Diego, San Diego, CA) in frame with the TAT/protein transduction domain peptide (YGRKKRRQRRR). The *ngn3* cDNA inserted into the pTAT vector was generated by PCR amplification of the *ngn3* cDNA (6). The oligonucleotides CCATGGCGCCTCATCCCTTGG and ACCGGTTCACAAGAA GTCTGAGAAC were used as forward and reverse primers, respectively. The ngn3 bacterial expression vector was generated by removing the TAT domain from the TAT/ngn3 construct. The TAT/β-galactosidase (β-gal) expression vector was also generously provided by Stephen Dowdy. TAT expression vectors feature a 6(x)His-affinity tag, which allows the purification of the fusion proteins by affinity chromatography using the nickel/nitryloacetic acid system (Qiagen, Valencia, CA). Then, 100-ml LB/Amp overnight culture of BL21(DE3)LysS bacteria expressing the protein of interest were inoculated into 1 l of LB/Amp and grown overnight at 37°C. Next, 0.4 mmol/l IPTG (isopropyl β-D-1 thiogalactopyranoside) was added 2 h before harvesting. Cells were centrifuged and washed with 50 ml PBS. Pellets were resuspended and combined in 10 ml of buffer Z (8 mol/l urea, 100 mmol/l NaCl, 20 mmol/l HEPES, pH 8.0) and 20 mmol/l imidazole. Cells were sonicated on ice and centrifuged at 12,000 rpm for 25 min. The supernatant was applied to a 5-ml nickel/nitryloacetic acid column pre-equilibrated with 20 mmol/l imidazole. The column was washed with 50 ml of imidazole (20 mmol/l) in buffer Z, and the protein was eluted with 250 mmol/l imidazole in buffer Z. Fractions were monitored by colorimetric determinations using a protein assay kit (Bio-Rad). The protein was desalted on a PD-10 column (Amersham), and final protein concentration was determined spectrophotometrically using the Bio-Rad protein assay kit. The TAT peptide was custom made by Sigma.

Western blot. Protein aliquots (15 μl) were diluted in 2× protein loading buffer (National Diagnostics) and run in a 15% polyacrylamide gel (Bio-Rad). For in situ staining, GelCode blue stain reagent (Pierce) was used. Transfer to Amersham enhanced chemiluminescence membranes was performed using the semidry method. Membranes were probed with rabbit anti-ngn3 antibodies (30) at 1:500 dilution.

Cell and tissue culture. Mouse ES cells and fibroblasts were cultured as previously described (31). β-TC3 cells were cultured at 37°C (5% CO_2) on opaque 96-well plates (Nunclon) and fed daily with Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 0.1 mmol/l minimum essential medium nonessential amino acids (Invitrogen), sodium pyruvate, 5% (vol/vol) newborn bovine serum, 5% (vol/vol) fetal calf serum, 0.1 mmol/l 2-mercaptoethanol, penicillin (100 units/ml)/streptomycin (100 μg/ml), and L-glutamine (250 μmol/l) from Invitrogen. Pancreata from e12.5–e13.5 embryos resulting from CBA × B6 crosses (where 12:00 P.M. of the day a vaginal plug is found is considered 0.5 days of gestation) were isolated, microdissected in ice-cold L-15 medium (Invitrogen), and cultured in explant medium (199 minimum essential medium, 10% calf serum, penicillin/streptomycin, and Fungizone) on top of 12-mm Millicell culture plate inserts. Whole guts of e9.5 embryos were dissected and cultured as above. Purified protein was added freshly every day to the culture medium.

Immunostaining and image analysis. Explants were grown as above for 48 h and then fixed with 4% paraformaldehyde (30 min.), washed with PBS (30 min), and frozen in optimal cutting temperature compound (Sakura). Pancreatic rudiments were sectioned in their entirety (5 μm) and mounted with DAPI (4,6-diamidino-2-phenylindole)-Vectashield (Vector). Guinea pig anti-insulin and rabbit anti-glucagon antibodies (ready-to-use solution; BioGenex) were used for double staining. Rabbit anti-ngn3 antibodies (30) were used at a 1:500 dilution. Metamorph imaging was used to quantify relative amounts of insulin and glucagon staining in each section. This software allows the detection and precise quantification of any given fluorescent signal in biological samples. Positive areas were calculated as a percentage relative to the total area of individual histological or confocal sections and then averaged for the entire sample.

TAT/ngn3 in vitro reporter system. β-TC3 cells were transiently transfected with the vector pBETA2(1.0)-Luc (kindly provided by Ming-Jer Tsai, Baylor College of Medicine, Houston, Texas) using an Effectene transfection kit (Qiagen). Protein was added to the medium 24 h later and maintained for another day. Luciferase measurements were performed with a Promega Bright-Glo luciferase reporter kit and a Molecular Diagnostics luminometer.

RT-PCR. β-Actin primers were: ATGGATGACGATATCGCT (forward) and ACCTGACAGACTACCTCAT (reverse), with 568 bp. beta2/neuroD primers were: CTTGGCCAAGAACTACATCTGG (forward) and TTCCCGGTGCATC CCTACTCC (reverse), with 228 bp. A Lightcycler instrument (Roche) was used for real-time RT-PCR analyses (primers as above).

RESULTS

Transduction of TAT/ngn3 into mammalian cells and pancreatic buds. TAT-fusion proteins are known to effectively transduce mammalian cells (15). Using a TAT/β-gal reporter protein, we observed that the uptake efficiency of TAT-fused proteins by ES cells is concentration dependent (Fig. 1*A–D*). This was further confirmed by ngn3 immunostaining of TAT/ngn3-transduced ES cells (data not shown). Neither protein was toxic to the cells within the concentration range used in our experiments (100 nmol/l to 5 μmol/l).

After 24-h incubation with 1 μmol/l TAT/ngn3, UV microscopic examination of mouse ES cells shows that virtually 100% of the cells stain positively for ngn3. Immunostaining is evident throughout the cell, although it appears to concentrate in granular structures. This is consistent with the prevailing view that TAT promotes cellular uptake via endocytosis (18,32,33). Confocal analysis confirmed the presence of ngn3-positive vesicles in the cytoplasm of the transduced cells, as well as diffuse nuclear staining (Fig. 1*E*). Incubation with ngn3 alone did not result in cellular uptake, as detected by immunohistochemistry (Fig. 1*F*).

To test whether TAT/ngn3 would be able to evenly

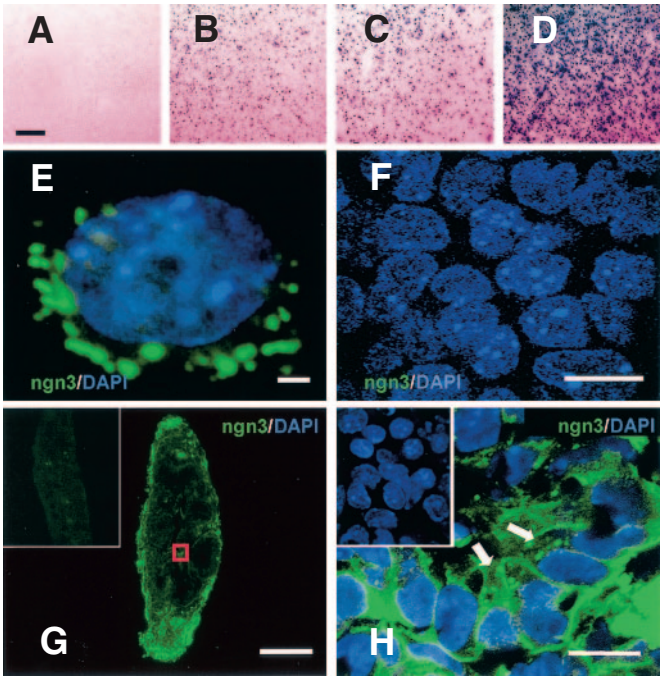


FIG. 1. Uptake of TAT/ngn3 by mammalian cells. TAT/β-gal uptake (X-gal staining) after incubation of ES cells (24 h) with the protein: nontransduced control (*A*), 100 nmol/l (*B*), 300 nmol/l (*C*), and 500 nmol/l (*D*). *E*: CARV confocal image of an ES cell incubated with TAT/ngn3 (2 μmol/l) for 24 h (ngn3 staining). *F*: Control. Cells were incubated with ngn3 protein (2 μmol/l). *G*: Ngn3 immunostaining of an e13.5 bud section cultured with 2 μmol/l TAT/ngn3 (12 h). *Inset*: TAT-treated bud stained for ngn3 (negative control). *H*: A region in the center of the bud (red square) is shown at a higher magnification (confocal). Ngn3$^+$ vesicles (white arrows) can be observed in virtually 100% of the cells. Nuclear staining (blue) of DAPI (4,6-diamidino-2-phenylindole) is shown. *Inset*: TAT-treated bud stained for ngn3 (negative control). Original magnification and scale bars: 100×, 150 μm (*A–D*); 400×, 1 μm (*E*); 400×, 10 μm (*F* and *H*); and 100×, 150 μm (*G*).

transduce structures thicker than a cell monolayer, e13.5 pancreatic buds were cultured with 2 μmol/l TAT/ngn3 for 12 h. Confocal analysis of ngn3-immunostained samples shows, as expected, an uptake gradient from the surface to the core of the bud (Fig. 1*G*). However, cells located in the center of the explant display the typical ngn3-positive vesicles observed in TAT/ngn3-transfected monolayers (Fig. 1*H*). This observation shows the ability of TAT/ngn3 to efficiently transduce cells in a three-dimensional structure.

Exogenously administered TAT/ngn3 activates a beta2/neuroD reporter system in vitro. To demonstrate that transduced TAT/ngn3 functions at the nuclear level in vitro, we used the reporter vector β-*luc*, where expression of luciferase is driven by a 1.0-kb fragment of the *beta2/neuroD* promoter. *beta2/neuroD* is a downstream target of ngn3 during endocrine differentiation (34). Therefore, nuclear import of active TAT/ngn3 would result in stimulation of the *beta2/neuroD* promoter and expression of luciferase in our system. First, we examined the inducibility of the reporter system upon ectopic expression of *ngn3*. β-TC3 cells, which do not express detectable levels of endogenous *ngn3* (Fig. 2*A*), were sequentially transfected with 250 ng of β-*luc* (day 1) and either 500 or 1,000 ng of a CMV (cytomegalovirus)-*ngn3* expression vector at day 2. We observed two- and threefold increases in luciferase activity, respectively, compared with mock controls (Fig. 2*B*).

Next, β-TC3 cells that had been transiently transfected with β-*luc* were incubated for 24 h in the presence of TAT/ngn3 (2 and 5 μmol/l). Two control groups were treated with PBS or native ngn3 protein. As shown in Fig. 2*C*, luciferase activity was increased 2.5- and 3.5-fold in cells that had been treated with TAT/ngn3 (2 and 5 μmol/l, respectively) compared with the basal level of luciferase expression observed in PBS and ngn3 controls.

Nonlinearized vectors tend to remain episomal after transfection (35). We reasoned that the effectiveness of TAT/ngn3 at inducing expression of an episomal promoter might not necessarily correlate with its ability to regulate the expression of endogenous genes. To test whether TAT/ngn3 was able to enhance the expression of the endogenous *beta2/neuroD* gene, we incubated β-TC3 cells with 2 μmol/l TAT/ngn3 and obtained RNA samples for quantitative RT-PCR analysis at different time points. Figure 2*D* shows that there is a sharp increase in *beta2/neuroD* expression 5 h after addition of the protein. The signal decreases to noninduced levels after 16 h. These results are consistent with the observed half-life of the protein in vitro (Fig. 2*E*). Collectively, these data demonstrate that physiologically active TAT/ngn3 does migrate to the nucleus after uptake and is able to activate a downstream target gene in vitro in a manner similar to that expected of native ngn3.

Treatment of early pancreatic explants with TAT-ngn3 results in preferential differentiation into glucagon-producing cells. During murine development, the first endocrine cell type (glucagon positive) is observed as early as e9. Premature differentiation of pancreatic progenitor cells caused by forced expression of *ngn3* under the control of the *Ipf1/Pdx1* promoter results primarily in the generation of glucagon-expressing cells (6). We predicted that TAT/ngn3 would have a comparable effect on pancreatic progenitors in cultured e9.5 whole-gut explants.

In *Ipf1/ngn3* transgenic animals, the premature differentiation of pancreatic progenitors occurs at the expense of pancreatic progenitor cell expansion and later differentiation of other pancreatic cell types (6), such as insulin-expressing cells and exocrine cell types, that effectively appear first around e13. To see whether TAT/ngn3 would promote the generation of glucagon-positive cells at the expense of insulin-positive cells when applied to early pancreatic anlagen, e9.5 whole-gut explants were cultured for 2 or 6 days in the presence or absence of TAT/ngn3 (2 μmol/l). After 2 days, all four explants in the control group, but only one of five in the TAT/ngn3 group, had insulin-positive cells (Fig. 3). After 6 days, four of seven (57%) explants in the control group, but none (of seven) in the TAT/ngn3 group showed insulin expression (Fig. 3).

Although the above experiment suggests that TAT/ngn3 promotes the differentiation of glucagon-expressing cells at the expense of insulin-expressing cells, the scarcity of insulin-positive cells in the control explants leaves open the possibility that their reduced appearance rate is not a direct consequence of enhanced glucagon cell differentiation. To further explore this issue, we next determined the amount of glucagon-producing cells in TAT/ngn3-exposed explants compared with that found in controls. Then, e9.5 entire guts were dissected and cultured for 48 h in the presence of TAT/ngn3 or TAT peptide alone. Explants were subsequently fixated and immunostained for glucagon. Confocal planes of each embryonic pancreas were ob-

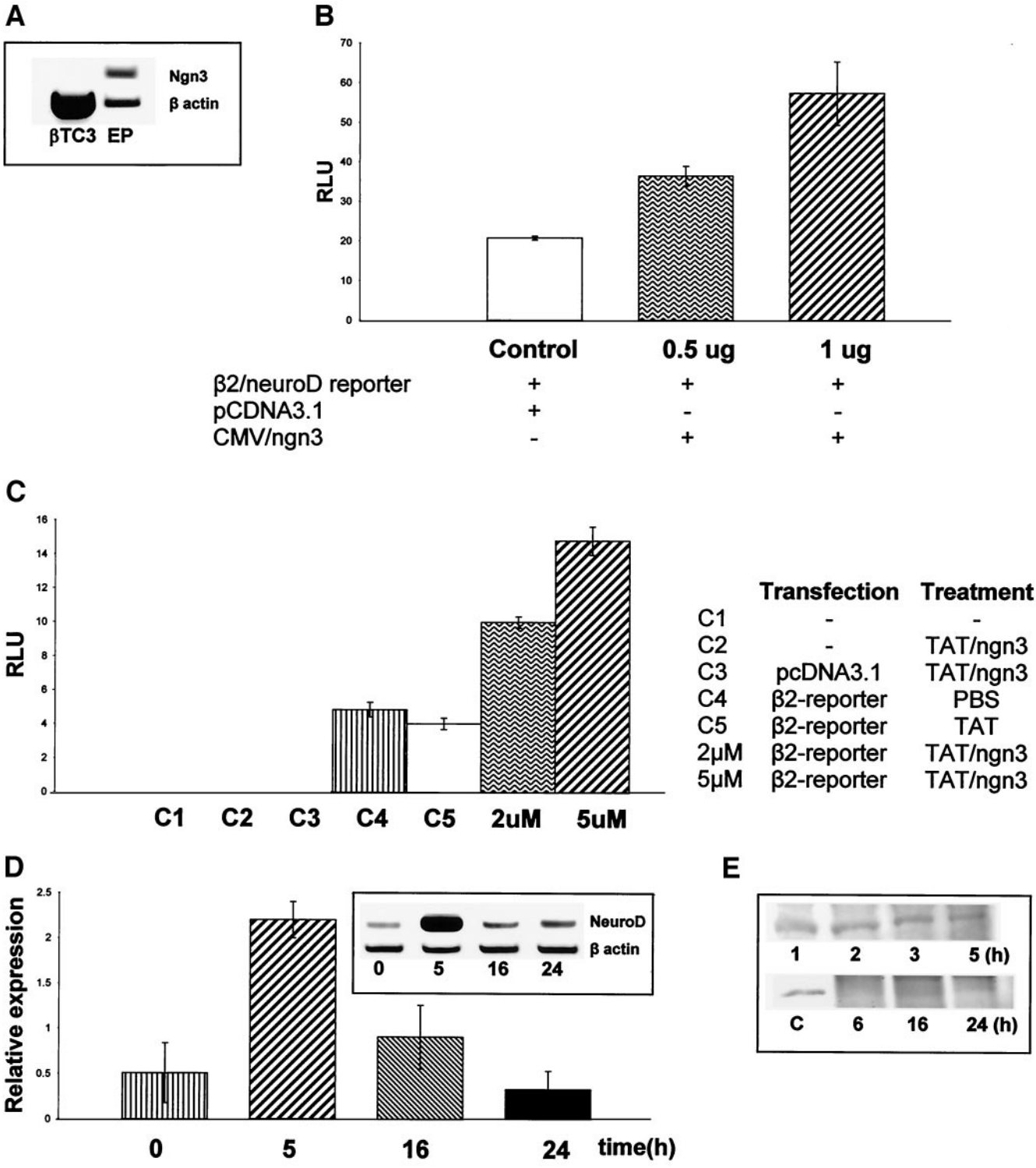


FIG. 2. TAT/ngn3 activates a nuclear reporter gene in vitro. *A*: RT-PCR shows that *ngn3* is not actively expressed in β-TC3 cells. Positive control, e13.5 embryonic pancreas (EP). Loading control, β-actin. *B*: Cytomegalovirus (CMV)-*ngn3* activates a *beta2/neuroD2* reporter gene. Transfection of β-TC3 cells with a *beta2/neuroD2*-luciferase reporter gene (day 1) and a ngn3 expression vector (CMV-*ngn3*; day 2) induces luciferase expression. Y bars: SE for each group. Control, cells transfected with a mock plasmid (pcDNA 3.1; day 2); RLU, random luminescence units. *C*: TAT/ngn3 activates a *beta2/neuroD* reporter gene. Incubation of β-TC3 cells with TAT/ngn3 stimulates luciferase expression under the control of the *beta2/neuroD2* promoter. Cells were transiently transfected with a *beta2/neuroD2*-luciferase reporter vector and then incubated for 24 h with 2 or 5 μmol/l of TAT/ngn3. C1, control 1, cells untransfected and untreated; C2, control 2, cells untransfected and treated with TAT/ngn3; C3, control 3, cells transfected with a mock DNA molecule and then treated with TAT/ngn3 (2 μmol/l); C4, control 4, cells transfected with the reporter vector (*beta2/neuroD2*-luciferase) and then treated with PBS; C5, control 5, cells transfected with the reporter vector and then with TAT peptide. Y bars: SE for each group. *D*: TAT/ngn3 induces expression of endogenous *beta2/neuroD*. TAT/ngn3 (2 μmol/l) induces expression of the endogenous *beta2/neuroD* gene, as evidenced by real-time and conventional (*inset*) RT-PCR of samples taken at time points 0 (before addition), 5, 16, and 24 h. Y bars: SE for each group. All values were normalized to β-actin expression. *E*: GelCode-stained polyacrylamide gel showing TAT/ngn3 band at different time points of incubation in serum-containing medium at 37°C. The protein is stable for up to 5 h and then is progressively degraded. *C*: Fresh protein control.

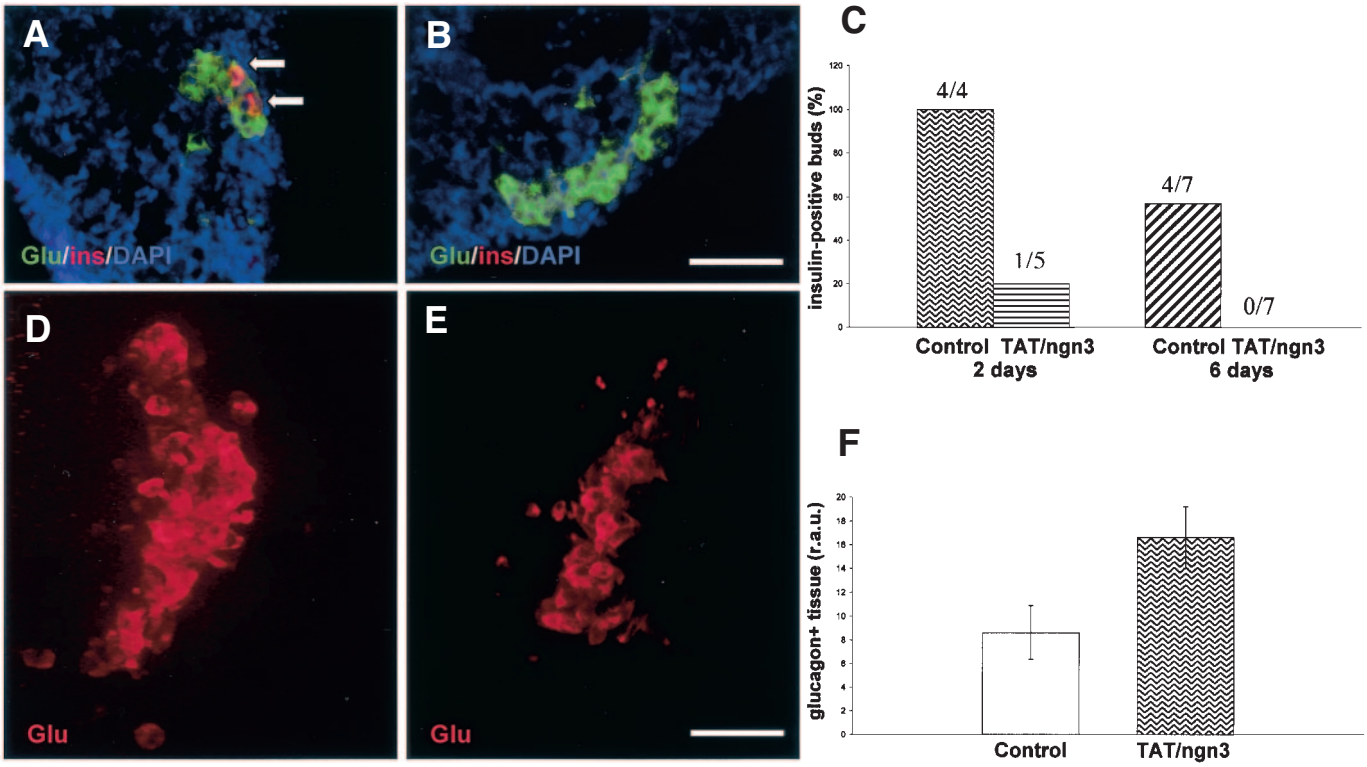


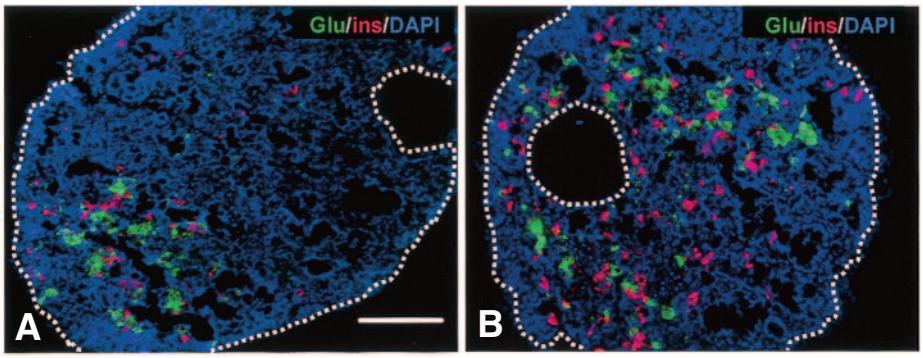
FIG. 3. Effect of TAT/ngn3 on e9.5 pancreatic buds in vitro. *A*: Glucagon (green) and insulin (red, arrows) in e9.5 embryonic gut cultured with native ngn3 (6 days). DAPI (4,6-diamidino-2-phenylindole)-stained nuclei (blue) are shown. *B*: Insulin$^+$ cells are normally absent from e9.5 embryos cultured for 6 days with TAT/ngn3. *C*: TAT/ngn3 on e9.5 embryos: incubation experiments (2/6 days). *y*-axis: percent of explants with insulin-positive cells. Insulin-positive embryos/embryos per group are indicated above each column. *D* and *E*: Three-dimensional reconstruction of glucagon-positive clusters (red) in e9.5 guts cultured with (*D*) or without (*E*) TAT/ngn3. *F*: TAT/ngn3 on e9.5 embryos. TAT/ngn3-treated embryos had a twofold average increase in glucagon-positive cells compared with their TAT-treated counterparts. Original magnification: 320×. Size bars: 50 μm. r.a.u., random area units.

tained every 25 μm, from the first (top) to the last (bottom) sections positive for glucagon. We observed that in TAT/ngn3-treated embryos ($n = 6$), clusters of glucagon-producing cells were generally thicker and denser than in control guts (Fig. 3). Metamorph image analysis was used to quantify the overall amount of glucagon-positive cells in each embryo. As shown in Fig. 3, TAT/ngn3-treated guts contained approximately twice as much glucagon-producing tissue as controls ($n = 5$). An ANOVA test indicated that this increase was statistically significant ($F = 5.16$; $P = 4.95 \times 10^{-2} < 0.05$). These results are consistent with our hypothesis that TAT/ngn3 stimulates α-cell differentiation at the expense of other pancreatic cell types in e9 explants.

Treatment of e13.5 pancreatic explants with TAT-ngn3 enhances endocrine differentiation. Although glucagon-producing cells appear throughout development, it is thought that the inductive microenvironment found in the pancreatic bud at e12–e13 favors the differentiation of insulin-producing cells (6). Therefore, ectopic expression (or administration) of ngn3 at this time is likely to result in enhanced differentiation of cycling progenitors, preferentially into insulin-expressing cells. To test this hypothesis, e13.5 dorsal pancreatic buds were cultured for 48 h in the presence of TAT/ngn3 (2 μmol/l, $n = 23$). A control group ($n = 14$) was treated either with TAT peptide (2 μmol/l, $n = 8$) or native ngn3 protein (2 μmol/l, $n = 6$). At termination, each bud was individually fixed, frozen, sectioned (5 μm), and immunostained for insulin and glucagon. Metamorph image analysis software was used to quantitate relative amounts of insulin and glucagon cells in each section, and values were averaged for each single pancreatic bud. Because the mean values obtained in each of the control subgroups (TAT peptide and native ngn3 protein) were statistically similar, we combined them into one single group for the sake of simplicity. As shown in Fig. 4, there is a 1.93-fold increase in the overall number of endocrine cells (insulin + glucagon) in the study group compared with the control group (ANOVA $F = 7.42$, $P = 1.9 \times 10^{-2} < 0.05$). Although the number of glucagon-positive cells is higher in the study group than in the controls, the increase in insulin-expressing cells is markedly superior (2.07-fold, ANOVA $F = 6.45$, $P = 2.27 \times 10^{-2} < 0.05$). The ratio of insulin- to glucagon-expressing cells is also enhanced in the TAT/ngn3 group, but such an increase is not statistically significant. The use of TAT/ngn3 in buds explanted at a slightly earlier developmental stage (e12.5) also resulted in a similar enhancement in endocrine differentiation (data not shown). Together, these results indicate that TAT/ngn3 stimulates overall endocrine differentiation, especially that of insulin-producing cells, in explanted e12–e13 pancreatic buds.

DISCUSSION

Although TAT-mediated transportation of proteins is a well-established technology (15,36), its application to deliver transcription factors is less well documented (28). The observation that TAT-fused proteins are internalized



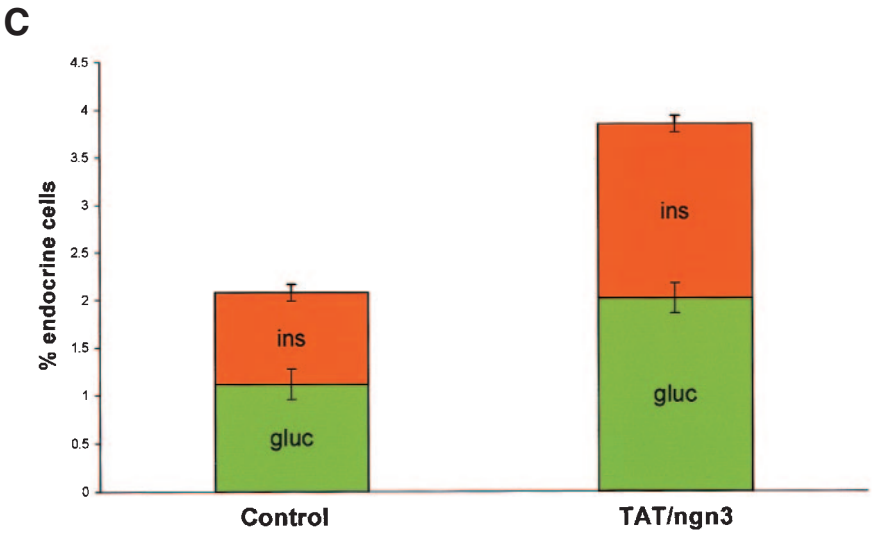


FIG. 4. Effect of TAT/ngn3 on e13.5 explants in vitro. Dorsal buds were explanted and cultured for 48 h with TAT-ngn3 (2 μmol/l) and either TAT peptide or native ngn3 protein (both at 2 μmol/l). *A*: Section of an e13.5 bud, representative of the average percentages of glucagon (green) and insulin (red)-expressing cells found in control (TAT-treated) cultures (~2% of endocrine cells). *B*: Section of an e13.5 bud with the average percentage of endocrine cells (~4%) after incubation with TAT/ngn3. *C*: Effect of TAT/ngn3 on e13.5 pancreatic buds (insulin and glucagon). Y bars: SE for each group. Original magnification: 100×. Size bars: 100 μm.

by endocytosis, a mechanism commonly associated with cytoplasmic degradation (36), as well as the need for the protein to translocate across several cellular membranes (outer, vesicular, and nuclear), have been cited among the theoretical concerns for the use of TAT to transport nuclear factors. Indeed, there is evidence suggesting that TAT-mediated membrane translocation might require unfolding and subsequent renaturation of the protein (36,37), which might decrease the overall efficiency of the process and therefore the amount of protein in the nuclear compartment available for immunodetection. However, nuclear translocation itself is probably not a rate-limiting step here because native nuclear factors are naturally transported to the nucleus after they are synthesized in the cytoplasm. TAT/ngn3 seems to accumulate preferentially in the cytoplasm, but some diffuse staining can also be detected in the nucleus. Our observation that TAT/ngn3 enhances expression of both a reporter gene placed under the control of the *beta2/neuroD* promoter (a natural downstream target of the native protein) and the endogenous *beta2/neuroD* gene confirms that the recombinant protein reaches the nucleus in a biologically active conformation. Our experiments in embryonic explants further support this conclusion. It is known that early expression of *ngn3* under the *Ipf1/Pdx1* promoter in transgenic mice results in a premature differentiation of progenitor cells into glucagon-expressing cells (6). Such an increase in the number of glucagon-producing cells occurs at the expense of other terminally differentiated cell types, including insulin-expressing cells. In our experiments, insulin-producing cells were rarely spotted in TAT/ngn3-treated whole-gut explants compared with controls. Although there is some variability in the appearance of β-cells in vitro, the increased amount of glucagon-producing cells observed in the explants exposed to TAT/ngn3 is consistent with an effect of TAT/ngn3 at promoting endocrine differentiation. Treatment of e12 and e13 pancreatic explants with TAT/ngn3 also results in a net increment of endocrine cells. Although glucagon-expressing cells still appear (and will keep differentiating throughout development), our data suggest that the progenitor cells activated by TAT/ngn3 are preferentially recruited toward the β-cell lineage.

The half-life of the recombinant protein is short, which explains why a TAT/ngn3-induced gene (*beta2/neuroD*) recuperates original levels of expression in β-TC3 cells 16 h after the protein was added to the medium. In contrast, exposure of embryonic explants to TAT/ngn3 has a permanent effect, consistent with the irreversible induction of endocrine differentiation in predisposed progenitor cells. Our approach, therefore, seems uniquely suited to mimic in vitro the natural pattern of expression of genes that are only transiently expressed.

In summary, our data demonstrate that TAT/ngn3 promotes endocrine differentiation in vitro, in a manner consistent with the predicted biological function of the native protein. The use of protein transduction domains to deliver transcription factors at specific time points potentially represents a powerful tool for gain-of-function developmental

studies, circumventing the need for time-consuming and often unpredictable methods such as transgenesis or conditional gene targeting. This work is also the first study, to our knowledge, in which protein transduction domain–fused transcription factors are used to aid in the directed differentiation of progenitor cells. The results presented here suggest a novel way to design islet differentiation protocols, which would involve the precise in vitro recapitulation of islet development by means of the sequential administration of key transcriptional factors to stem cell cultures. Such an approach would be more advantageous and flexible than those based on gene transfer because it would allow for the precise timing of protein administration and removal when its function is no longer required.

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