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ORIGINAL ARTICLE

Liraglutide protects Rin-m5f β cells by reducing procoagulant tissue factor activity and apoptosis prompted by microparticles under conditions mimicking Instant Blood-Mediated Inflammatory Reaction†

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Conflict of interest
The authors have declared no conflict of interest.

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Summary

Instant Blood-Mediated Inflammatory Reaction (IBMIR) occurs at the vicinity of transplanted islets immediately after intraportal infusion and is characterized by cytokine secretion, tissue factor (TF) expression, and ß cell loss. Microparticles (MPs) are cellular effectors shed from the plasma membrane of apoptotic cells. Modulation of the properties of ß cell-derived MPs by liraglutide was assessed in a cellular model designed to mimic IBMIR oxidative and inflammatory conditions. Rin-m5f rat β cells were stimulated by H_2O_2 or a combination of IL-1β and TNF-α. Cell-derived MPs were applied to naive Rin-m5f for 24 h. Apoptosis, MP release, TF activity, P-IκB expression, and MP-mediated apoptosis were measured in target cells. Direct protection by liraglutide was shown by a significant decrease in the oxidative stress-induced apoptosis (18.7% vs. 7.6%, $P < 0.0001$ at 1 μM liraglutide) and cellular TF activity (−40% at 100 nM liraglutide). Indirect cytoprotection led to 20% reduction in MP generation, thereby lowering MP-mediated apoptosis (6.3% vs. 3.7%, $P = 0.022$) and NF-κB activation (−50%) in target cells. New cytoprotective effects of liraglutide were evidenced, limiting the expression of TF activity by ß cells and the generation of noxious MPs. Altogether, these data suggest that liraglutide could target pro-apoptotic and pro-inflammatory MPs in transplanted islets.

Introduction

Pancreatic islets transplantation is a highly promising procedure with advantages over whole pancreas transplantation, predominantly because it is a noninvasive and diabetogenic effects pertaining to the immunosuppressive treatment are lowered [1,2]. Furthermore, improvements in islets isolation and therefore enhanced engraftment efficacy can be foreseen in clinical practice on a short term [3]. According to data from the Collaborative Islet Transplant Registry in 2012, one-year insulin independence was reached in 80% of transplanted patients, and 44% maintained a three-year insulin independence [4]. Despite these encouraging data, the destruction of a proportion of the islets at the early stages of transplantation remains a major issue. Indeed, engraftment and/or survival of a sufficient β cell mass are mandatory for long-term graft function. Ischemia/reperfusion may occur as a result of the isolation procedure when islets are disconnected from their vascular bed and immediately after portal injection when they are again in contact with the recipient blood flow. In particular, cell apoptosis during the isolation procedure or in the conservation medium before infusion [5,6], and the Instant Blood-Mediated Inflammatory Reaction (IBMIR) immediately after transplantation are thought to be early causes of the loss of graft function [7]. IBMIR is prompted by the contact of infused islets with portal blood [8]. It is characterized by a drastic cytokine secretion and a high expression of tissue factor (TF) at islets' vicinity [9]. TF, a member of the type-1 cytokine receptor superfamily and a trigger of the coagulation cascade [10], has previously been associated with transplantation failure, particularly because of its prothrombotic properties that are enhanced after plasma membrane remodeling at the surface of stressed cells [11–13]. In islet transplantation, the loss of graft function 7 days after infusion has been associated with the degree of activation of the blood cascade immediately after islet infusion [9]. Indeed, factor VIIa antithrombin complexes (FVIIa-AT) and thrombin–antithrombin complexes (TAT) are readily elevated in the plasma 50 min after infusion. Furthermore C-peptide levels 7 days after infusion are inversely correlated with levels of FVIIa-AT and TAT, indicating the major role of TF and its ligand factor VIIa in the coagulation events triggered by IBMIR [9].

In the vessel, TF is a plasma membrane protein expressed by endothelial cells and monocytes. The active form of TF is conveyed by microparticles (MPs) [14,15]. MPs are submicron plasma membrane fragments shed by stressed or apoptotic cells and their release in cell supernatant is proportional to the degree of apoptosis [16]. MPs contribute to cell cross-talk because they bear active proteins and lipid mediators from the parental stimulated cells that they can deliver to naïve target cells [17]. In addition to phosphatidylserine, the anionic phospholipid exposed by stressed membranes, TF constitutes another procoagulant entity borne by MPs. When delivered by MPs, TF increases the procoagulant surface potential of target cells at early and later stages. Recent data suggest that TF-bearing MPs could contribute to a sustained procoagulant activity at endothelial cell surface through the recycling of TF^+-MPs [18].

In transplantation, MPs have been scarcely studied. Endothelial circulating MPs were shown reliable markers of patients' recovery after renal transplantation and promising tools for the monitoring of immunosuppressive treatment [19,20]. Furthermore, they were associated with the occurrence of acute myocardial graft rejection [21]. Circulating MPs could also prove useful to detect graft loss when biopsies are not informative or impossible. Indeed, an early rise in plasma MPs was evidenced in islet-transplanted patients undergoing rejection [22,23].

It was recently proposed that liraglutide could be of benefit in islet transplantation through the cytoprotection of the graft by islet preconditioning combined with peri-operative treatment. Liraglutide is a GLP-1 analog that belongs to the incretinomimetics new class of drugs. In the treatment of type 2 diabetes, beneficial effects of incretinomimetic rely on their ability to enhance insulin secretion and to promote β cell survival. Recently, their anti-apoptotic properties and ability to favor the proliferation of insulin-secreting β cells was evidenced. *In vitro*, liraglutide protects isolated islets from apoptosis [24], and several animal studies have already shown an improvement in islet engraftment and functions by liraglutide treatment [24,25]. Nevertheless, information on the cellular actors that could mediate liraglutide cytoprotective effects at the early stages of islet transplantation is still lacking.

The present *in vitro* study investigates the effects of liraglutide on MPs with a particular emphasis on their role on β cell function and survival. Cells were submitted to cytokine and oxidative stress conditions to mimic IBMIR and the stress generated by ischemia /reperfusion during islet transplantation. TF activity at cell surface, properties of MPs on the target cell survival, and their modulation were assessed using a MP-mediated cell cross-talk model.

Material and methods

Cell culture

Rin-m5f, rat β cells line (CRL-11605™, ATCC, Manassas, VA, USA), was seeded at 125 000 cells/cm^2 in RPMI 1640 (PAN™ Biotech GmbH, Aidenbach, Germany) medium containing 4.5% glucose, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate and supplemented with 10% of bovine fetal serum (Gibco, Saint Aubin, France) and 20 μg/ml of gentamicin (Lonza, Basel, Switzerland). Cells were cultured at 37 °C and 5% CO_2 in a humidified atmosphere.

Cellular model of cytokine and oxidative stress and pharmacological modulation

Stress was applied when cells had reach 70% of confluence as reported elsewhere [26]. For each stress procedure, a specific pharmacological modulation was applied. Cytokine stress was induced by the addition of 50 U/ml of IL-1β (Sigma, St. Louis, MO, USA) and 1000 U/ml of TNF-α (Sigma) for 24 h in the presence or absence of 1 μM of liraglutide (Novo Nordisk, Bagsvaerd, Denmark) as proposed by other investigators [27–30]. Oxidative stress was induced by the sequential application of H_2O_2 (Sigma) mimicking the conditions of the islet graft procedure: Rin-m5f cells were first pretreated by 1 μM liraglutide for 4 h, a concentration proposed in clinical settings for the preconditioning of islets maintained in culture medium 24 h before transplantation [24]. Medium was removed at the end of the first incubation time, and 100 μM of H_2O_2 in fresh medium was added for 6 h to mimic the oxidative stress in the islet graft prompted by ischemia/reperfusion. MPs were collected in the supernatant at the end of the 6-h incubation and kept at 4 °C until measurement. Cells were further incubated with fresh medium containing 1 μM liraglutide for 14 h to mirror the treatment of the transplanted patients. Cell supernatant was finally collected and kept at 4 °C.

MP generation, collection, and quantification

Microparticles (MPs) were collected from the supernatant by differential centrifugation. Detached cells and debris were discarded (13 000 *g* for 5 min) and MPs were further isolated and washed by two centrifugation steps (13 000 *g* for 1 h). Total MP concentration was determined by prothrombinase assay after capture onto insolubilized Annexin V. In this assay, blood clotting factors (FXa, FVa, FII) and calcium concentrations were determined to ensure that phosphatidylserine (PhtdSer) borne by MPs is the rate-limiting parameter in the generation of soluble thrombin from prothrombin. Immobilized MPs were incubated with factor Xa (11.2 pM, Biogenic, Perols, France), factor Va (33.3 pM, American Diagnostica, Stamford, CT, USA), prothrombin (1.2 μM, Hyphen BioMed, Paris, France), and 2.2 mM $CaCl_2$ in Tris buffer saline for 10-min incubation at 37 °C. Conversion of prothrombin to thrombin was revealed after 15 min by the cleavage of a specific chromogenic thrombin substrate (1.52 mM final concentration, paranitroaniline peptide (pNAPEP), CRYOPEP, Montpellier, France) using a microplate spectrophotometric reader set in kinetic mode, at 405 nm. Results were expressed as nanomolar PhtdSer equivalent (nM PhtdSer eq.) with reference to a standard curve constructed using liposomes of known concentration and PhtdSer eq. proportion [31].

MP-mediated cell cross-talk

Microparticles (MPs) generated by oxidative stress (MPox) and MPs generated by cytokine stress (MPcyt) were applied to naïve Rin-m5f cells at a final concentration of 10 nM PhtdSer eq. for 24 h with or without 1 μM liraglutide.

Assessment of MP transfer to target cells

Collected MPs were labeled by 2 μM of a red fluorescent lipid probe PKH26 (Sigma, L'isle d'Abeau Chesnes, France), washed twice by centrifugation (13 000 *g*, 60 min), and 10 nM PhtdSer eq. finally applied to Rin-m5f for 24 h. Accumulation of the probe on target cell surface was observed by fluorescent confocal microscopy and quantified by flow cytometry with a FACS-scan cytometer (Becton Dickinson, Pont de Claix, France) set at logarithmic gain. 10 000 events were recorded for each sample

Quantification of apoptosis

After stimulation, cells were washed and permeabilized by a 70% ethanol solution at 4 °C for at least 24 h. After three washing steps, cells were resuspended in a solution containing I-A RNase A (Sigma) for 15 min at 37 °C. Saturating concentration of propidium iodide (Sigma) was applied (0.1 mg/ml) and the degree of apoptosis evaluated by the quantification of hypodiploid DNA by flow cytometry (Becton Dickinson).

Measurement of TF activity

After 24 h of stimulation, TF activity at cell surface was evaluated by Tenase enzymatic assay. TF activity was measured on washed cells through its ability to promote the activation of factor X (150 nM, Hyphen Biomed, Neuville, France) by factor VII(a) (5 nM, Novoseven, Denmark) in the presence of 1 mM $CaCl_2$. The reaction was allowed to proceed for 15 min at 37 °C. CS11 (65), a chromogenic substrate for factor Xa (Hyphen Biomed), was then added at a final concentration of 0.1 mM and variations in absorbance were recorded at 405 nm after 20-min incubation. Results were expressed as fM TF activity per 50 000 living cells reference to a standard curve established with known amounts of highly purified lipidated recombinant human tissue factor (ADF Biomedical, France).

Western blot analysis

After treatment, cells were washed twice with PBS and then lysed in TRIS buffer containing protease inhibitors (5 μg/mL leupeptin, 5 mM benzamidine) and 2% Triton® X-100

on ice. Total proteins (30 μg) were separated by electrophoresis on 10% SDS-polyacrylamide (Sigma) gels as previously described [32]. Blotting membranes were incubated with the primary mouse antibody for p-IκBα (US Biological, Swampscott, MA, USA; 1:1000 dilution) or total IκBα (Millipore, Molsheim, France, 1:1000 dilution) overnight at 4 °C. Detection of β-tubulin protein was used for normalization and quantification. After washing, membranes were incubated with the anti-mouse IgG antibody (Cell Signaling Technology, Danvers, MA, USA, 1:10 000 dilution) at room temperature for 60 min. Prestained markers (Invitrogen™, Carlsbad, CA, USA) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham, GE Healthcare, Velizy-Villacoublay, France). Density analysis was performed using ImageQuant LAS 4000 imager (GE Healthcare). For each specific labeling, values were expressed as the ratio of protein to β tubulin densities.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM) and were analyzed using GraphPad Prism5®. Statistical analysis between the two groups was carried out using unpaired Student's test, and all experiments were per-

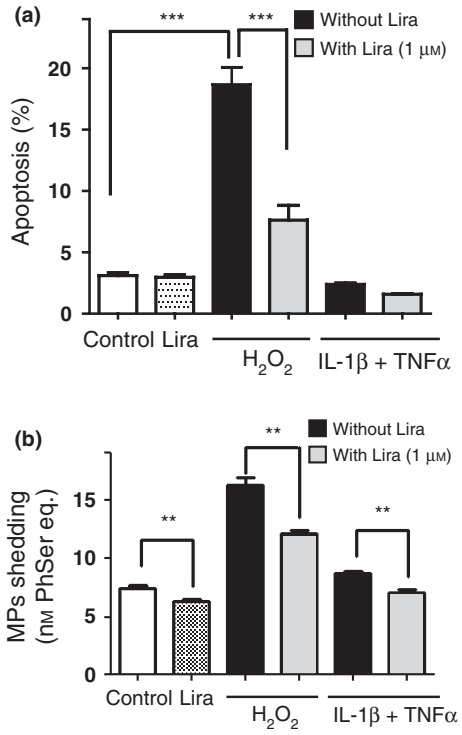

Figure 1 Liraglutide decreases ß cell apoptosis (a) and microparticle (MP) shedding (b). Cell stress was achieved by H_2O_2 or IL-1β + TNF-α combination in the presence (gray bars) or absence (black bars) of 1 μM liraglutide. MPs generated in the supernatant were measured by solid-phase prothrombinase enzymatic assay under conditions where phospholipids are the rate-limiting parameter of the conversion of prothrombin into thrombin. Data are expressed as nM PhtdSer eq. by reference to a standard curve constructed with known amounts of synthetic phospholipid vesicles. Empty bars represent unstimulated cells, and dotted bars represent cells treated by liraglutide alone. Data are presented as the mean ± SEM ($n = 6$; Lira: liraglutide; **: $P < 0.01$; ***: $P < 0.001$). Untreated cells: 3.1 ± 0.06%.

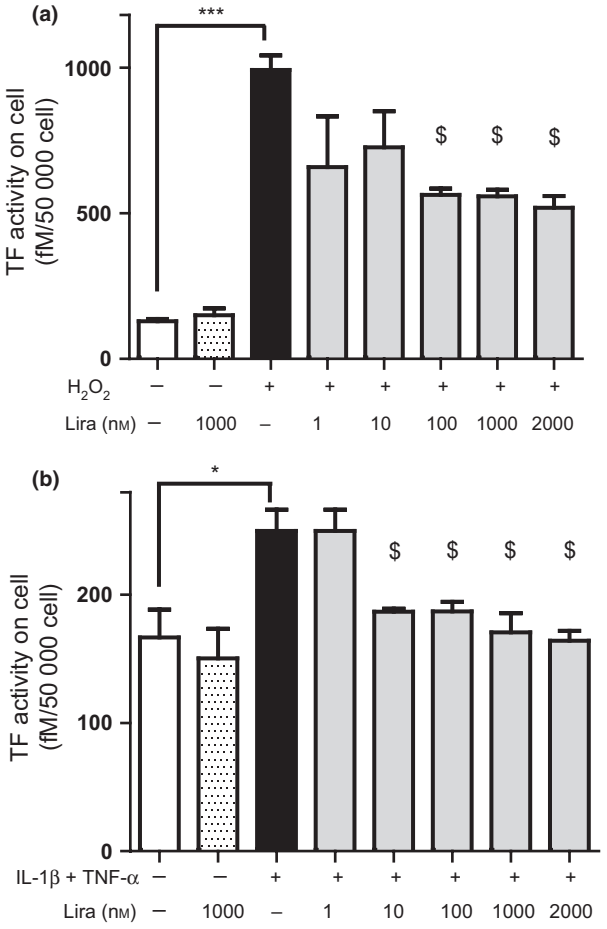

Figure 2 Liraglutide decreases tissue factor (TF) activity at β cells surface after oxidative (a) and cytokine (b) stress. After stimulation by H_2O_2 or IL-1β + TNF-α in presence (gray bars) or absence (black bars) of 1 μM Liraglutide, the supernatant was withdrawn. TF activity was assessed by Tenase assay in which TF catalyzes the formation of the Tenase complex in the presence of exogenous factor X and factor VIIa, calcium. The conversion of factor X into factor Xa was measured by chromogenic assay using a specific substrate. Values are expressed as fM of TF activity by reference to a standard curve established with known amounts of highly purified lipidated recombinant human TF. Data are normalized as fM of TF per 50 000 cells. Empty bars represent unstimulated cells, and dotted bars represent cells treated by liraglutide alone. Data are represented mean ± SEM ($n = 6$; Lira: liraglutide; $: comparison with the negative control *: $P < 0.05$; ***: $P < 0.001$).

formed at least three times; a P value < 0.05 was considered significant.

Results

Liraglutide protects β cells from apoptosis and limits the release of MPs

Liraglutide reduced the degree of apoptosis prompted by the oxidative stress from 18.69 ± 1.9% to 7.63 ± 1.2% ($P < 0.0001$). Modulation of apoptosis by liraglutide in cells submitted to IL-1β+TNF-α was hardly detectable due to the very low values of apoptosis generated by the cytokine stress (Fig. 1a). A significant increase in MP shedding was, however, detectable after both treatments (16.17 ± 0.7 nM PhtdSer eq. in H_2O_2-treated cells $P = 0.0042$ and 8.83 ± 0.1 nM PhtdSer eq. in IL-1β+TNF-α-treated cells $P = 0.0026$ vs. 7.58 ± 0.1 nM PhtdSer eq. in untreated cells) and was limited by 1 μM liraglutide with MP values decreasing to 12.13 ± 0.2 nM PhtdSer eq. ($P = 0.0064$) in H_2O_2-treated cells and to 7.20 ± 0.2 nM PhtdSer eq. in IL-1β+TNF-α-treated cells ($P = 0.0049$; Fig. 1b)

Liraglutide limits TF activity at β cell surface

Tissue factor (TF) activity at cell surface was increased under both conditions of stress with values that reached 992.1 ± 49.4 fM TF ($P = 0.0014$) in H_2O_2-treated cells and 249.9 ± 16.5 fM TF ($P = 0.0431$) in IL-1β+TNF-α-treated cells compared with 129.0 ± 8.3 fM TF in untreated control cells. Liraglutide counteracted the induction of TF activity under both conditions of stress. Significant reductions were evidenced at low liraglutide concentrations after cytokine stress (10 nM liraglutide) and from 100 nM after oxidative stress (Fig. 2). In addition, a significant difference in TF activity generated by cytokine stress could be observed between 10 nM and 2000 nM liraglutide treatment ($P = 0.047$), suggesting a possible dose-dependent effect.

MPs interaction with target Rin-m5f cells

Red fluorescent PKH26 lipid probe was used to assess MP transfer. Confocal microscopy revealed optimal staining after 24 h indicating that PKH26-MPox and target cells

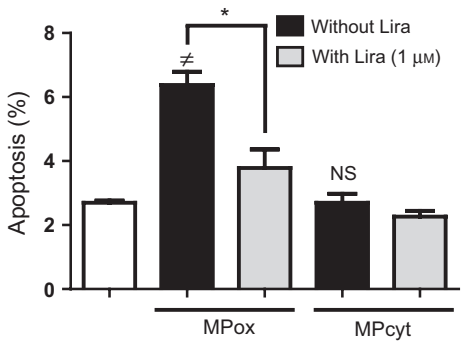

Figure 4 Microparticules (MPs) induce β cell apoptosis. Cells were incubated with 10 nM PhtdSer eq. MPs generated by oxidative or cytokine stress in the presence (gray bars) or absence (black bars) of 1 μM liraglutide. Apoptosis was assessed by IP labeling in ethanol-permeabilized cells using flow cytometry. Empty bars represent unstimulated cells. Data are presented as mean ± SEM. ($n = 3$, Lira: liraglutide; ±: comparison with the positive control *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.0001$).

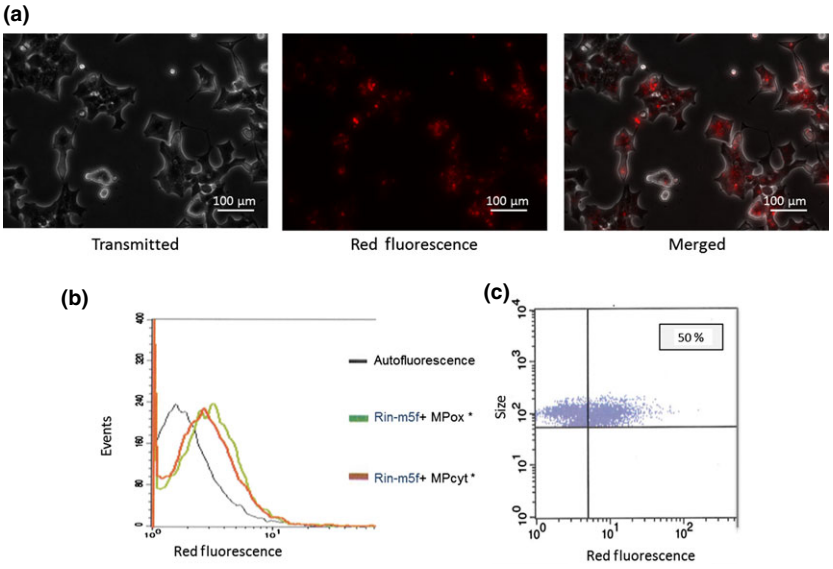

Figure 3 MPox and MPcyt are equally transferred to target RIN-m5F cells. Cells were incubated with 10 nM PKH26-labeled microparticles (MPs) for 24 h and observed by confocal microscopy. A representative observation of MPox transfer is shown (a). A shift in cell fluorescence brought by red fluorescence-labeled MPs was evidenced by flow cytometry (b) and plotted using 10 000 recorded events per sample (c).

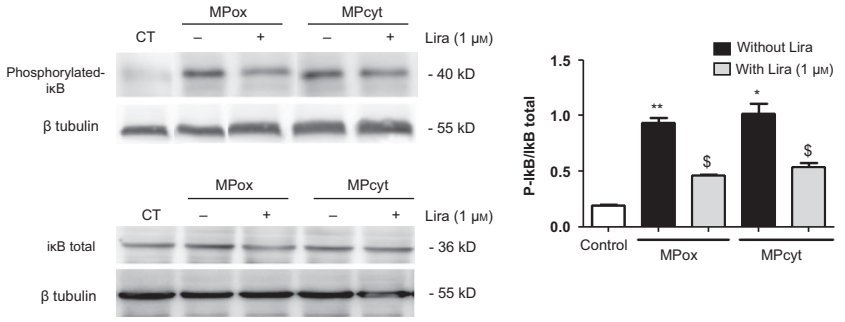

Figure 5 Microparticle (MP) transfer activates NF-κB pathway. IκB phosphorylation and total IκB-α expression in Rin-m5f cells treated for 24 h by 10 nM PhtdSer eq. MPs were assessed by Western blotting (Left panel). Analysis was performed by calculating the P-IκB/ total IκB density ratio as described in M&M (right panel). MPox: MPs generated by oxidative stress; MPcyt: MPs generated by cytokine stress; Lira: liraglutide.

were co-localized (Fig. 3a; MPcyt: data not shown). Quantitative assessment by flow cytometry revealed that MPox and MPcyt were captured to the same extent by targeted cells as shown by similar shifts of fluorescence (Fig. 3b). Statistical analysis by the cytometer CellQuest software revealed that 50% of cells had integrated MPs (Fig. 3c). Liraglutide did not modify the proportion of MPs transferred to target cells (data not shown).

MPs induce the activation of apoptosis in β cells

In target cells, a 24-h incubation with 10 nM MPox induced a significant increase in apoptosis (6.37 ± 0.4% in MPox-treated cells compared with 2.69 ± 0.06% in untreated cells) to an extent close to the range observed after treatment by H_2O_2 alone (18.69% in H_2O_2-treated cells compared with 3.1 ± 0.06% in untreated cells; Figs 1a and 4). Liraglutide was able to counteract the MP-driven apoptosis down to 3.78 ± 0.5% ($P = 0.022$). Interestingly, treatment by 10 nM PhtdSer eq. MPs generated from cytokine stress did not induce any significant variation of apoptosis (Fig. 4), in accordance with the above observation on cytokine-treated cells (Fig. 1a).

Higher phosphorylation of IκB, an indicator of NF-κB activation, was observed in MP-treated cells and was limited by the addition of liraglutide (Fig. 5). Because results were normalized with respect to total IkB-α, the specificity of this phosphorylation pattern is confirmed.

Discussion

Our cross-talk model was set to investigate the effects of MP of endocrine origin under apoptotic conditions. For this purpose, endocrine Rin-m5f cells were considered because of their susceptibility to apoptosis as previously reported [33,34]. Our data confirm other observations of β cell cytoprotection by liraglutide, predominantly through the reduction in apoptosis [35–37]. Indeed, they clearly evidence a reduction in the Rin-m5f cell response to oxidative stress. Importantly, procoagulant MPs shed from apoptotic Rin-m5f cells were identified as a new target of liraglutide. In our MP-mediated cell cross-talk model, liraglutide modulated the noxious action of MPs. Most interestingly, we evidenced TF activity as a new marker of ß cell damage that was also modulated by liraglutide.

Our data question the mechanism by which liraglutide exerts its control on MP generation. In view of the oxidative stress response, one tempting explanation would be a direct anti-apoptotic effect, thereby lowering MP release. Nevertheless, although apoptosis was not observed after cytokine treatment, liraglutide was still able to reduce MP shedding, indicating a specific effect. Possible downstream events mediated by liraglutide could involve p-38 MAP kinase phosphorylation, already identified in MP release processes in endothelial cells submitted to TNF-α [28].

Tissue factor (TF) activity has been proposed as a major contributor to early coagulation events at the vicinity of the islet and thereby to the loss of graft functions [9]. Because TF expression can be induced by cytokine stress and its activity enhanced by the presence of PhtdSer in the outer leaflet of the plasma or MP membranes [38,39], we hypothesized a possible role of TF on ß cell damage. Indeed, liraglutide could contribute to diminished TF activity through two nonexclusive mechanisms: a decrease in PhtdSer exposure due to its anti-apoptotic properties and the reduction of TF expression at the cell surface. Further investigations are needed to examine the role of liraglutide on TF mRNA in our model of pro-inflammatory and pro-apoptotic stress.

The MP-mediated cross-talk model points at MPs as contributors to the amplification of the inflammatory endocrine cell response (see Fig. 5). Previous data have already shown the delivery of a pro-inflammatory signal by MPs from patients with diabetes to endothelial cells [40]. We have also reported that MPs, shed by pancreatic exocrine

cells isolated from a patient with cystic fibrosis, are able to activate the NF-κB pathway under pro-apoptotic conditions [26]. In the present model, because we could evidence MP recapture by by-stander cells after 28-h treatment (data not shown), we decided to focus on the variations of MP release that were already significantly elevated after 24 h, while recapture was still undetectable. These conditions allowed the discrimination between cell responses to cytokine and oxidative stress after 24 h. Only the oxidative stress-induced apoptosis seems to generate MPs able to impair β cell survival. Conversely, cytokine stress did not lead to early apoptosis but rather promoted early MP-mediated pro-inflammatory NF-κB response. Indeed, we evidenced IκB phosphorylation in Rin-m5f target cells, while the level of the total protein remained unchanged.

In the present work, kinetics of β cell apoptosis were different with respect to the treatment, and the apoptosis elevation was significant only after 32 h of stimulation by cytokines. Our observations are in accordance with other reports of delayed apoptosis under cytokine treatment [27]. We cannot, however, exclude that PhtdSer exposure leading to MP generation in Rin-m5f cells could result from both cytokine stimulation and apoptosis processes [16].

Altogether, the present work underlines the feasibility of a pharmacological modulation of MP shedding from endocrine cells by liraglutide and its beneficial effects through the reduction in the expression of TF activity. Moreover, liraglutide was efficient in the circulating concentration range reported in treated type 2 diabetes patients, or even lower when it was applied in the cross-talk model. In clinical settings, one could anticipate an early blunting of TF activity and of the deleterious pro-inflammatory signals disseminated by MPs at the vicinity of transplanted islets. Further *in vivo* investigations are, however, needed to establish the optimal liraglutide concentrations for graft preconditioning and for recipients' treatment to reach an efficient limitation of the IBMIR and preservation of graft function.

Authorship

CG: performed research and wrote the paper. AC: performed important part of the cell research. MA: performed important part of the Western blots. HB: performed part of the pharmacological study and Wester blot. FZ: performed microparticle measurements. LK and FT: designed research and corrected the paper.

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