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Phenolic excipients of insulin formulations induce cell death, pro-inflammatory signaling and MCP-1 release

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ABSTRACT

Skin reactions at the infusion site are a common side effect of continuous subcutaneous insulin infusion therapy. We hypothesized that local skin complications are caused by components of commercial insulin formulations that contain phenol or *m*-cresol as excipients. The toxic potential of insulin solutions and the mechanisms leading to skin reactions were explored in cultured cells.

The toxicity of insulin formulations (Apidra, Humalog, NovoRapid, Insuman), excipient-free insulin, phenol and *m*-cresol was investigated in L929 cells, human adipocytes and monocytic THP-1 cells. The cells were incubated with the test compounds dose- and time-dependently. Cell viability, kinase signaling pathways, monocyte activation and the release of pro-inflammatory cytokines were analyzed.

Insulin formulations were cytotoxic in all cell-types and the pure excipients phenol and *m*-cresol were toxic to the same extent. P38 and JNK signaling pathways were activated by phenolic compounds, whereas AKT phosphorylation was attenuated. THP-1 cells incubated with sub-toxic levels of the test compounds showed increased expression of the activation markers CD54, CD11b and CD14 and secreted the chemokine MCP-1 indicating a pro-inflammatory response.

Insulin solutions displayed cytotoxic and pro-inflammatory potential caused by phenol or *m*-cresol. We speculate that during insulin pump therapy phenol and *m*-cresol might induce cell death and inflammatory reactions at the infusion site *in vivo*. Inflammation is perpetuated by release of MCP-1 by activated monocytic cells leading to enhanced recruitment of inflammatory cells. To minimize acute skin complications caused by phenol/*m*-cresol accumulation, a frequent change of infusion sets and rotation of the infusion site is recommended.

1. Introduction

Patients suffering from insulin-dependent diabetes mellitus have two main options for insulin administration: multiple daily injections or continuous subcutaneous insulin infusion (CSII) using insulin pumps. It is well documented that many patients benefit from CSII therapy by achieving a better glycemic control [1]. Although CSII is a safe and efficient therapy, it may be hampered by metabolic and non-metabolic complications. Among these are technical problems with the infusion sets (occlusion,

Abbreviations: APC, allophycocyanin; CCL2, chemokine ligand 2; CD, cluster of differentiation; CSII, continuous subcutaneous insulin infusion; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; IgG, immunoglobulin G; IL, interleukin; JNK, Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; Mip-1α, macrophage inflammatory protein-1alpha; PE, phycoerythrin; TNFα, tumor necrosis factor alpha; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

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kinked tubes, leakage), lipohypertrophy, and local skin reactions at the infusion site (inflammation, infection, scarring) [2,3]. Inflammation at the infusion site has high prevalence, especially among pediatric patients (25–42%) [4–6]. The occurrence of these adverse events increases with longer indwelling time of the insulin catheter, which is reflected by the limited application time of 3–4 days found in clinical studies or surveys [7–10] and the fact that most manufacturer recommend to change the infusion set every 2–3 days. Infusion sets have been optimized to reduce skin reactions by means of material selection and design. Using modern infusion sets the occurrence of true infections at the infusion site is very low compared to what was seen in clinical trials in the 1980ies [11,12]. However, skin irritation and inflammation is a persistent problem for patients on CSII therapy and are a major reason for premature catheter replacement and even for giving up on CSII [13,14]. However, the underlying mechanism for acute skin complications has not been resolved.

Preparations of insulin or rapid-acting insulin analogs contain phenolic excipients that serve two purposes: maintaining sterility of the solution and stabilization of the insulin molecule in the hexameric form to avoid aggregation [15,16]. All insulin formulations for pump use contain either phenol, *m*-cresol or a mixture of both in considerable amounts (29–32 mM or 2.7–3.2 mg/ml). These compounds are problematic since they are known to be toxic and irritant, and are suspected sensitizers and carcinogens [17,18]. There is limited knowledge on the toxicity of insulin solutions when administered to subcutaneous tissue. One clinical study reported that skin irritation at the infusion site are more common in patients using insulin preparations with *m*-cresol as compared to insulin containing methyl *p*-hydroxybenzoate as preserving agent [19]. However, this study was conducted with a small number of patients and it is not clear if the results can be transferred to current insulin formulations.

The aim of the study was to investigate the toxic potential of current pump insulins *in vitro* using cultured cells. We hypothesized that *m*-cresol or phenol in relevant concentrations could lead to cell death and trigger pro-inflammatory responses. The murine fibroblastic cell line L929 was selected as a model, because it is a well-established tool for cytotoxicity testing generating highly reproducible results. Although not of human origin, L929 cells are commonly used for risk assessment of medical devices like infusion catheters. This cell line was preferred for cytotoxicity testing of insulin formulations to obtain comparable results. Besides fibroblasts, adipose tissue mainly consists of adipocytes. Therefore, we have chosen human adipocytes to confirm our results in a second model that is closer to human physiology. In addition, we tested whether phenol or *m*-cresol would stimulate the secretion of pro-inflammatory cytokines by immune cells. For this purpose, human monocytic THP-1 cells were preferred, as cell activation by pro-inflammatory stimuli as well as cytokine secretion can be monitored in this cell line.

2. Materials and methods

2.1. Chemicals

Phenol (CAS No.108-95-2) and *m*-cresol (CAS No. 108-39-4) were obtained from Sigma–Aldrich. Jun N-terminal kinase (JNK) inhibitor SU3327 (10–20 μM in dimethyl sulfoxide (DMSO)) and p38 Inhibitor SB202190 (1–2 μM in DMSO) were from Tocris Bioscience.

The following formulations of insulin or insulin analogs were used, each at 100 I.U./ml: Apidra (3.15 mg/ml *m*-cresol, Sanofi-Aventis), Insuman Infusat (2.7 mg/ml phenol, Sanofi-Aventis), Humalog (3.15 mg/ml *m*-cresol, Eli Lilly), NovoRapid (1.72 mg/ml *m*-cresol and 1.5 mg/ml phenol, Novo Nordisk). Recombinant human insulin (Roche Diagnostics GmbH) was dissolved in phosphate buffer at 3 mg/ml (equivalent to 100 U/ml).

2.2. Cell culture and viability assay

The murine fibroblast cell line L929 and the human monocytic cell line THP-1 were cultured in RPMI1640 (Sigma–Aldrich) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO_2.

Cell viability assay was performed using the Cell Proliferation kit II (XTT) (Roche Diagnostics GmbH). Briefly, the tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) is cleaved by viable cells to form an orange formazan dye that can be quantified photometrically at 492 nm. Before the assay, 1×10^4 L929 cells were cultured in 96-well plates for 24 h. The culture medium was replaced by medium containing the desired concentration of insulin solutions, phenol or *m*-cresol. The cells were incubated for 24 h or otherwise as indicated. The XTT reagent was added and absorption was measured after 2 h using the Synergy4 plate reader (BioTek Instruments). Data analysis was performed using the Gen5 Data Analysis Software (BioTek Instruments). The threshold for toxicity was set to <70% cell viability.

2.3. Cytokine Immunoassay

5×10^5 THP-1 cells were incubated with insulin solutions or phenol or *m*-cresol (0.2 mg/ml) for 24 h. The release of the cytokines interleukin (IL)-1β, IL-6, IL-8, IL-12, tumor necrosis factor alpha (TNFα), macrophage inflammatory protein-1 alpha (Mip-1α) and monocyte chemotactic protein-1 (MCP-1) was determined in cell-free supernatants obtained by centrifugation. Immunodetection was performed using cytometric bead assays (CBA, BD Bioscience) and a BD FACSarray flow cytometer (BD Bioscience) as described by the manufacturer. Data analysis was performed using FCAP array software v1.0.1 (Soft flow Hungary Ltd.).

2.4. CD54, CD11b and CD14 immunostaining

Monocyte activation was determined by cluster of differentiation (CD)54, CD11b and CD14 expression. THP-1