IN VITRO DISSECTION OF ANTI-DIABETIC EFFECTS OF COMPOUND A, A DISSOCIATING GLUCOCORTICOID RECEPTOR LIGAND

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Abstract: Compound A (CpdA), or 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethyl-ammonium chloride, is a stable analog of the hydroxyl phenyl aziridine precursor found in the Namibian shrub Salsola tuberculatiformis Botschantzev. It belongs to the group of so-called “dissociated” GC receptor ligands that downmodulate pro-inflammatory gene expression via the transrepression mechanism, but without physically binding to DNA. We have recently reported that the in vivo administration of CpdA exerts a strong protective effect in a pharmacological model of type 1 diabetes in mice. The goal of this study was to investigate in more detail the effects of CpdA on multiple immune system components, as well as on target pancreatic beta cells in direct in vitro exposure. The utility of CpdA in diabetes prevention was evaluated through its addition to mitogen-activated spleen, lymph node and peritoneal cells of C57BL/6 mice, and to murine pancreatic islets and INS-1 and RINm5F beta cell lines. CpdA modulated immune cell-derived cytokine production in vitro by restraining the pro-inflammatory M1/Th1/Th17 response and switching it towards an anti-inflammatory Th2 profile. However, it did not preserve beta cells from the cytotoxic action of inflammatory cytokines. Thus, the anti-diabetic properties of CpdA are mediated through the modulation of immune cell differentiation pathways rather than through rescue of target cells from autoimmune attack.

Key words: Compound A; inflammation; macrophage; lymphocyte; beta cell

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INTRODUCTION

Type 1 diabetes is organ-specific, autoimmune disease that arises as a result of progressive destruction of insulin-producing pancreatic beta cells (Bluestone et al., 2010). This leads to the loss of normal insulin secretion and development of hyperglycemia (Eizirik et al., 2009). The disease begins with a loss of tolerance to self-antigens derived from pancreatic islets. Both innate and adaptive arms of the immune system take a part in the events of autoimmune cascade (Herold et al., 2013). The nature of the infiltrate surrounding pancreatic islets is determined by the fine balance between pro- and anti-inflammatory macrophages and T-helper (Th)-subsets. When this balance is lost, the infiltrate becomes aggressive and insulitis occurs (Bettelli et al., 2007; Wang, et al., 1997; van den Brandt et al., 2010). The inflammatory lesion within the islets of those with type 1 diabetes is typically characterized by infiltrates composed mainly of pathogenic Th1/Th17 lymphocytes, M1 macrophages, and smaller numbers of other immune cells (Herold et al., 2013). Each of these subsets expresses a unique set of cytokines. Aside from the balance of inflammation versus regulation, the inherent shortage of defense mechanisms makes beta cells extremely sensitive to the activation of the apoptotic program by cytokines (Acharya and Ghas-kadbi, 2010). A number of pathological stimuli have been identified to induce beta cell apoptosis. The most important are pro-inflammatory cytokines IFN-γ and IL-17 (signature cytokines of Th1 and Th17 cells, respectively), as well as M1-derived IL-1β and TNF-α (Padgett et al., 2013).

Current strategies in diabetes management are directed toward treating the pathological consequences
of diabetes, rather than its causes. Therefore, tremendous efforts are made for the discovery of novel modalities of prevention and/or reversal of type 1 diabetes. To exhibit their functions, immune cells must undergo a series of cellular processes including activation, proliferation, differentiation and cytokine expression. All these processes may be pharmacologically manipulated, thus representing important targets for therapeutic invention against inflammation-related disorders (Dinarello, 2010). At present, glucocorticoids (GC) still remain the best suited for medicinal applications because of their potent immunosuppressive and anti-inflammatory properties. However, long-term applications are often complicated by severe metabolic adverse effects arising mainly from the transactivation of GC receptors (Kleiman and Tuckermann, 2007).

Thus, a new group of so-called “dissociated” GC receptor ligands are developing, which downmodulate pro-inflammatory gene expression via the transrepression mechanism, without physically binding to DNA (De Bosscher et al., 2010b). Among these novel pharmaceuticals is Compound A (CpdA), or 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethyl-ammonium chloride, a stable analog of the hydroxyl phenyl aziridine precursor found in the Namibian shrub Salsola tuberculatiformis Botsch. (De Bosscher et al., 2005). The therapeutic efficacy of CpdA was demonstrated in several animal models of inflammatory and autoimmune disorders (De Bosscher, 2010a). Since we have recently reported that in vivo administration of CpdA exerted a strong protective effect in type 1 diabetes induced in C57BL/6 mice by multiple low doses of streptozotocin (MLDS) (Saksida et al., 2014), the goal of this study was to investigate in more detail the effects of CpdA on specific players in the pathogenic process, namely multiple immune system components, as well as on target beta cells in direct in vitro exposure.

MATERIALS AND METHODS

Reagents

CpdA (2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethyl-ammonium chloride) was a kind gift from Drs. Kathleen Van Craenenbroeck and Guy Haegeeman (LEGEST, University of Ghent, Ghent, Belgium). It was synthesized and characterized, as described by Louw et al. (1997). CpdA was dissolved in DMSO at 0.01 M concentration for in vitro experiments, and further diluted in medium immediately before use.

Unless otherwise stated all other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA). Cell culture experiments used RPMI-1640 medium (25 mM HEPES, 2 mM L-glutamine) supplemented with 5% fetal calf serum (FCS, PAA Chemicals, Pasching, Austria), 5 µM/ml of β-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin (complete medium).

Immune cell isolation and in vitro treatments

Spleen cells (SC), cervical lymph node cells (LNC) and resident peritoneal cells (PC) were obtained from male C57BL/6 mice, 8-12-weeks-old weighing 20-24 g. All animal procedures complied with the Directive
2010/63/EU on the Protection of Animals used for Experimental and other Scientific Purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research “Siniša Stanković” (application No. 03-01/14). Total PC were harvested by washing the peritoneal cavity with PBS. Solid organs were mechanically disrupted, passed through a 40-μm nylon mesh filter (BD Bioscience, Bedford, MA, USA) and the suspension of SC and LNC was collected by centrifugation. Erythrocytes were lysed using lysis buffer (eBioscience, San Diego, CA, USA). Samples of conditioned medium used for cytokine detection were obtained by seeding PC (1x10⁶ per well), SC (5x10⁶ per well), or LNC (3x10⁶ per well) in 24-well culture plates (Sarstedt, Numbrecht, Germany) at 37°C in a 5% CO₂ incubator. Cells were cultured in complete medium in the presence or absence of CpdA (range 0.1-10 μM) for viability assay, or 10 μM CpdA for cytokine production; SC and LNC were stimulated or not with 1 μg/ml Concanavalin A (ConA) for 48 h, and PC were stimulated or not with 5 μg/ml LPS for 24 h.

Culture and treatment of murine insulinoma cell lines

Rat insulinoma RINm5F cells and rat insulinoma INS-1 cells were cultured in tissue culture flasks (Sarstedt) in complete medium containing 10% FCS, until they reached ~80% confluence, when they were detached by a standard trypsinization procedure. Cells were washed and seeded into 96-well flat-bottomed cell culture plates (6×10⁴ per well) in the presence or absence of a mixture of murine cytokines IFN-γ+TNF-α+IL-1β (R&D, Minneapolis, MN, USA), 10 ng/ml each, with or without CpdA (range 0.1-10 μM). Cell viability and the cytotoxic action of cytokines were evaluated by the MTT colorimetric assay after 48 h of cell cultivation.

Isolation of pancreatic islets and treatments

Pancreata were removed aseptically from C57BL/6 mice, randomly punctured and injected with collagenase V, dissolved in Hanks balanced salt solution (HBSS, 1 mg/ml), and incubated in a water bath at 37°C, with horizontal shaking (300 strokes/min). Pancreatic islets were isolated by hand. Apoptosis of pancreatic islets (groups of 20), cultured for 24 h in 96-well plates with or without cytokine mixture (IFN-γ+TNF-α+IL-1β, 10 ng/ml each), with or without 10 μM CpdA, was measured using Cell Death Detection ELISA (Roche, Basel, Switzerland), an assay based on the presence of internucleosomal histone-associated DNA fragments.

Viability assays

Cell respiration, as an indicator of cell viability and proliferation, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan (Mosmann, 1983). At the end of appropriate treatments, cells were pulsed for 30 min with MTT solution (0.5 mg/ml) and the formazan crystals were dissolved in DMSO. The absorbance was measured in an automated microplate reader (LKB 5060-006, LKB, Vienna, Austria) at 540 nm, and background at 670 nm was subtracted.
DNA-histone complexes present in the cytoplasmic fraction of apoptotic islets were detected using Cell Death Detection ELISA (Roche, Mannheim, Germany) according to the manufacturer's instructions. The absorbance was measured in an automated microplate reader (LKB) at 450 nm, and the background at 670 nm was subtracted.

**Determination of cytokine secretion**

Cytokine concentrations in the cell culture supernatant fractions were determined by sandwich ELISA using MaxiSorp plates (Nunck, Rochild, Denmark) and anti-mouse paired antibodies according to the manufacturer's instructions. Samples were analyzed in duplicate for murine IL-1β, TNF-α, IL-17 (BD Biosciences, Bedford, MA, USA), IL-6, IL-10, IL-2 (eBioscience), IFN-γ and IL-4 (R&D Systems, Minneapolis, MN, USA).

**Statistical analysis**

Results are presented as the mean ± standard deviation (SD) obtained in independent experiments. Each experiment was repeated at least three times. The significance of the changes was evaluated by Student’s t-test. Statistical evaluation of the results was made with Statistica version 6.0 (StatSoft, Tulsa, OK, USA) at the 0.05 significance level.

**RESULTS**

**In vitro effects of CpdA on the survival of immune cells**

Taking into account that CpdA has been shown to possess an inherent potential to generate metabolites with the ability to provoke apoptosis (Wüst et al., 2009), it was of interest to assess whether CpdA may affect immune cell survival. We used unfractionated splenocytes (SC) and lymph node cells (LNC) since these two organs contain several populations of immune cells, including T and B lymphocytes, as well as macrophages. Cells were stimulated with ConA as a model of in vitro T lymphocyte responses, which may mimic the in vivo system to some extent. SC and LNC were incubated in vitro for 48 h with or without ConA in the presence of increasing concentrations of CpdA, and cell viability was analyzed by MTT assay. The results showed that concentrations ranging from 0.1-10 μM CpdA did not show any toxicity against quiescent or mitogen-stimulated SC, nor LNC (Fig. 1).

**Fig. 3.** CpdA altered cytokine profile of T lymphocytes. (A) Splenocytes (5x10^5/well), or (B) lymph node cells (3x10^5/well) isolated from C57BL/6 mice were stimulated with 1 μg/ml ConA for 48 h in the presence or absence of 10 μM CpdA. Thereafter, cell-free culture supernatants were collected for the analysis of cytokine secretion by ELISA. Results are expressed as mean values ± SD obtained from four independent experiments, each done in triplicate. *P < 0.05 compared with their respective group without CpdA.
The effects of CpdA on the production of cytokines by immune cells

Our further goal was to investigate the influence of CpdA on immune cell function by analyzing the production of signature cytokines from the distinct populations of immune cells. Having in mind that macrophages are the first cells that invade the endocrine pancreas during initiation of type 1 diabetes (Jörns et al., 2014), we first examined the production of representative macrophage-derived cytokines induced by lipopolysaccharide (LPS), a classical activator of this cell population. As shown in Fig. 2, treatment of LPS-stimulated PC with CpdA (10 μM) significantly reduced the secretion of IL-1β, TNF, IL-6, and IL-10.

Next, we analyzed the influence of CpdA on T-cell differentiation into distinct T-helper (Th) subpopulations using ConA-stimulated SC and LNC. Treatment of SC (Fig. 3A) and LNC (Fig. 3B) with CpdA (10 μM) significantly reduced the secretion of Th1 and Th17 signature cytokines, IFN-γ and IL-17, while the secretion of IL-2 was not affected. In contrast, the secretion of the anti-inflammatory Th2 cytokine IL-4 was significantly upregulated in the cells of both organs tested (Fig. 3). We therefore suggest that CpdA attenuated Th1, Th17, and M1 macrophage response and facilitated Th2 differentiation.

CpdA does not induce beta cell resistance to cytotoxic cytokines in vitro

We finally assessed the capacity of CpdA to interfere directly with the immune-mediated destruction of beta cells as target cells in type 1 diabetes. To mimic the inflammatory environment within islets attacked by activated mononuclear cells, two murine insulinoma cell lines as beta cell representatives, as well as freshly isolated pancreatic islets, were exposed to a cytokine mixture of IL-1β, TNF and IFN-γ in the presence or absence of 10 μM CpdA. Results from the MTT assay (Fig. 4A) revealed that although CpdA did not affect INS-1 and RINm5F beta cell viability, it was not able to rescue beta insulinoma cells from cytokine-induced apoptotic cells in pancreatic islets was not changed in the presence of CpdA (Fig. 4B).

DISCUSSION

Using a preclinical model of type 1 diabetes in mice, we recently demonstrated the therapeutic effectiveness of CpdA, a plant-derived nonsteroidal dissociated GC receptor modulator, in inhibiting disease progression (Saksida et al., 2014). Based on these results, in the current study we set out to investigate whether...
the compound was able to influence innate and adaptive immune cell differentiation, and/or to preserve pancreatic beta cells from their autoimmune attack.

Classically activated M1 macrophages are the major effector cells responsible for pathological changes in type 1 diabetes (Jörns et al., 2014). Type 1 cytokines of the innate immune system have been proposed as key players in initiating, coordinating and maintaining cell destruction during autoimmune diabetogenesis (Beyan et al., 2003). An interesting immunopharmacological characteristic of CpdA arising from the present data is its impact on the macrophage secretory capacity of the type 1 pro-inflammatory cytokines. In agreement with previous in vivo data (Saksida et al., 2014), in the present study CpdA directly interfered with M1 pro-inflammatory macrophages and their secretion of cytotoxic cytokines. However, in contrast to the in vivo model, CpdA downregulated alternatively activated M2 macrophages, as judged by their IL-10 production, suggesting that in the in vitro conditions CpdA did not induce this regulatory arm of the immune response. This does not yet definitely exclude the indirect influence of CpdA on macrophage polarization, since the optimal anti-inflammatory milieu may be created in the lymph node and spleen micro-environments during animal treatment. Indeed, the increased production of IL-4, seen upon CpdA treatment of SC and LNC, might be involved in the further M2 polarization in vivo (Mosser and Edwards, 2008).

T-helper (Th) cell differentiation is the central process defining the nature of the developing autoimmune responses (Steinman, 2007). Apart from the effect that CpdA exhibits on antigen-presenting cells, it might be involved in Th cell differentiation. Indeed, in this study we demonstrated that the compound exhibited strong immunomodulatory effects on T cells via decreasing signature pro-inflammatory Th1/Th17 versus anti-inflammatory Th2 cytokine ratios. CpdA treatment was not toxic to cells, thus implying that the immunomodulatory features of CpdA on activated T cells were not exhibited by reducing their number or activation. Importantly, CpdA did not reduce the IL-2, an essential factor for the maintenance of regulatory T cells (Treg), which control the reactivity of potentially harmful, self-reactive T cells and prevent autoimmune diseases (Horwitz et al., 2004). In agreement with this, we observed an increase in the Treg number within the pancreas of MLDS-induced mice upon in vivo treatment with CpdA (Saksida et al., 2014). However, we do not have any direct evidence Treg are expanded by the compound.

Beta cell destruction could result from the toxic effect of pro-inflammatory cytokines (IL-1β, TNF and IFN-γ), and other inflammatory products released by activated macrophages and T cells, as well as from beta cells themselves (Rabinovitch and Suarez-Pinzon, 1998). In addition, it has been shown that IL-1β, TNF and IFN-γ may increase vulnerability of pancreatic beta cells to autoimmune destruction by inducing the expression of Fas on the beta cell surface (Wachlin et al., 2003). However, in the current study, in vitro treatment with CpdA was not able to rescue either islets of Langerhans or insulinoma beta cells from cytokine-mediated toxicity. Thus, it seems that the CpdA-mediated preservation of islet beta cells that was observed in vivo (Saksida et al., 2014) is secondary to the reduced synthesis of the cytokine by immune cells rather than to antagonism of their bioactivity.

In conclusion, data from the in vitro model confirmed that CpdA represses inflammatory mediators of activated macrophages and T lymphocytes, and up-regulates IL-4, pointing to a direct regulatory effect of CpdA on both innate and adaptive immune cells. However, the drug could not preserve beta cells from deleterious pro-apoptotic effects of cytokines in an inflammatory milieu.

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Authors’ contributions: MV, TS, IN, IS and SSG performed the experiments and analyzed the data. IS and TS edited the text. SSG conceived and designed the study and wrote the paper.

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