Methadone ameliorates multiple-low-dose streptozotocin-induced type 1 diabetes in mice

K. Amirshahrokhi a, A.R. Dehpour a, J. Hadjati b, M. Sotoudeh c, M. Ghazi-Khansari a,⁎

a Department of Pharmacology School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
b Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
c Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

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A B S T R A C T

Type 1 diabetes is an autoimmune disease characterized by inflammation of pancreatic islets and destruction of β cells by the immune system. Opioids have been shown to modulate a number of immune functions, including T helper 1 (Th1) and T helper 2 (Th2) cytokines. The immunosuppressive effect of long-term administration of opioids has been demonstrated both in animal models and humans. The aim of this study was to determine the effect of methadone, a μ-opioid receptor agonist, on type 1 diabetes. Administration of multiple low doses of streptozotocin (STZ) (MLDS) (40mg/kg intraperitoneally for 5 consecutive days) to mice resulted in autoimmune diabetes. Mice were treated with methadone (10mg/kg/day subcutaneously) for 24 days. Blood glucose, insulin and pancreatic cytokine levels were measured. Chronic methadone treatment significantly reduced hyperglycemia and incidence of diabetes, and restored pancreatic insulin secretion in the MLDS model. The protective effect of methadone can be overcome by pretreatment with naltrexone, an opioid receptor antagonist. Also, methadone treatment decreased the proinflammatory Th1 cytokines (interleukin (IL)-1β, tumor necrosis factor-α and interferon-γ) and increased anti-inflammatory Th2 cytokines (IL-4 and IL-10). Histopathological observations indicated that STZ-mediated destruction of β cells was attenuated by methadone treatment. It seems that methadone as an opioid agonist may have a protective effect against destruction of β cells and insulitis in the MLDS model of type 1 diabetes.

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Introduction

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease. Type 1 diabetes results from specific destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans by the immune system (Bach, 1994). It is believed that destruction of pancreatic islet β-cells and consequent IDDM is the result of perturbed immune regulation. Multiple environmental and genetic factors make the immune cells, particularly T lymphocytes to invade islet β-cells and cause pancreatic inflammation (Rabinovitch and Suarez-Pinzon, 1998). This inflammatory response is known as insulinitis (Pukel et al., 1988). Cytotoxicity of T cells towards islet β-cells is generated by cytokines and free radicals. Autoreactive T helper 1 (Th1) cells and proinflammatory cytokines [interferon (IFN)-γ, interleukin (IL)-1 and tumor necrosis factor (TNF)-α] are pathogenic to islet β-cells, whereas T helper 2 (Th2) cells and their cytokines (IL-4 and IL-10) are protective against destruction of β cells (Rabinovitch, 1998). Blocking the function of type 1 cytokines and increasing type 2 cytokines can reduce the development of IDDM in rodent models (Rabinovitch and Suarez-Pinzon, 1998).

Multiple low doses of streptozotocin (STZ) (MLDS) can be used as an animal model for type 1 diabetes. STZ is a potent alkylating agent and damages islet β-cells selectively by two different mechanisms. First, when given in a single high dose, it rapidly destroys islet β-cells by direct cytotoxic action, most probably due to DNA alkylation. Second, when STZ is given in multiple low doses, it induces inflammation of the islets by immune cells, with subsequent destruction of β-cells and progressive hyperglycemia within a few days. This model of diabetes shares many histological and clinical features with human type 1 diabetes, and requires the participation of macrophages and T cells (Like and Rossini, 1976; Rossini et al., 1978; Papaccio et al., 2000). Therefore, the MLDS model has been applied extensively to study the immune pathways (e.g. cytokine signaling, Fas ligand transduction and cytokine-induced β cell apoptosis) involved in destruction of islet β-cells (Kawasaki et al., 2004; Rees and Alcolado, 2005).

It is believed that the endogenous opioid system regulates immune functions. Previous studies have established the presence of opioid receptors (μ, κ and δ) on cells of the immune system (Mehriishi and Mills, 1983). Many animal and human studies have shown that opioids induce immunosuppressive effects, and chronic opioid use has been associated with an increased incidence of infection (Budd, 2006).
These drugs can suppress NK-cell, macrophage and T-lymphocyte functions (Bryant and Roudebush, 1990; McCarthy et al., 2001). It has been shown that chronic morphine treatment inhibits Th1 cytokines and upregulates Th2 cytokines. Moreover chronic morphine treatment in vivo or in vitro decreases IFN-γ, IL-1 and TNF-α production and increases IL-4 and IL-10 protein levels (Roy et al., 1998, 2001, 2004, 2005).

Previous pharmacological studies have suggested that the inhibitory action of opioids on immune responses is mediated by opioid receptors, mostly μ-opioid receptors. Immunosuppressive actions of opioids are absent in μ-opioid receptor-deficient mice and are also blocked by opioid antagonists (Budd 2006; Gaveriaux-Ruff et al., 1998). It has been demonstrated that, like other opioids, methadone has immunosuppressive effects (Li et al., 2002). Methadone is a potent μ-opioid receptor agonist and widely used for the treatment of drug users with opioid dependence. We hypothesized that methadone, due to its immunosuppressive activity, may affect autoimmune diseases such as type 1 diabetes. Therefore, in this study, we decided to investigate whether methadone treatment could prevent the development of type 1 diabetes in mice.

Methods

Animal and chemicals. Male BALB/c mice 6–7 weeks old, weighing 20–25g, were purchased from the Pasteur Institute of Iran. The mice had free access to tap water and ad libitum food, and were housed in a room with a 12-h light/dark cycle. All animal procedures were carried out in accordance with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985). All the chemicals were purchased from Sigma.

Animal treatments. Male BALB/c mice were randomly divided into four groups. The mice in the first and third groups received subcutaneous (sc) injections of methadone (10mg/kg/day). Three days later, the mice in the first and second groups were treated with STZ at 40mg/kg/day intraperitoneally for 5 consecutive days. STZ was administered within 10min of its dissolution (Like and Rossini 1976; Karabatas et al., 2005). The mice in the fourth group received citrate buffer (vehicle) and were used as untreated controls. Blood glucose levels were measured on days 0, 7, 14 and 21 (in relation to the first STZ dose). The blood samples were obtained from the tail vein of non-fasted mice and glucose was measured by a blood glucose meter (Accu-Chech Active). In our study, multiple low doses of STZ did not lead to animal death.

Mice were considered diabetic when non-fasting blood glucose levels were >200 mg/dl for two consecutive days. Mice were anesthetized and pancreata were removed on day 21 for cytokine and histological analysis. Blood was also collected and centrifuged. The plasma was separated and stored at ~70 °C until insulin assay.

Cytokine assay. Pancreas tissue samples were removed from mice and snap frozen in liquid nitrogen. The samples (100mg) were homogenized in 800μl Tris–HCl buffer containing protease inhibitors (Mabley et al., 2002, 2003). All homogenized samples were centrifuged (16,000g, 4 °C) in a refrigerated centrifuge for 30min, and the supernatant was taken and frozen at ~70 °C. Supernatant samples were analyzed for murine cytokine concentrations using specific ELISA kits (Bender MedSystems GmbH). Cytokine levels in the pancreas were expressed as pg cytokine/mg protein, which were determined using the Bradford method (Bradford, 1976).

Plasma insulin determination. Non-fasting blood samples were collected on day 21 in heparinized tubes. Plasma was separated and assayed for insulin concentration. The insulin level of plasma was determined using a commercial Mouse Insulin ELISA kit (Mercodia).

Histological examination. Histological examination of mouse pancreas from each study group was carried out to evaluate the severity of insulitis at day 21. Pancreata were removed and a half of each pancreas was fixed with 10% formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E).

Statistical analysis. Data are expressed as means±SEM. Statistical analysis was performed using one-way ANOVA followed by Student’s t test. P < 0.05 was considered significant.

Results

Effects of methadone on MLDS-induced hyperglycemia and diabetes

In order to determine whether methadone prevents STZ-induced diabetes, blood glucose level was measured in all four groups of mice. When mice were injected with multiple sub-diabetogenic doses of STZ, they presented a progressive rise in blood glucose concentration (Fig. 1A) and the increased incidence of diabetes (Fig. 1B). Administration of methadone (10mg/kg) from 3days before the first STZ injection for 24days reduced incidence of hyperglycemia and diabetes. Control and methadone-treated mice remained normoglycemic throughout the study. Experiments with various doses of methadone showed that the threshold dose for a significant inhibitory effect on blood glucose level

Fig. 1. Effect of methadone treatment on MLDS-induced hyperglycemia (A) and incidence of diabetes (B) in mice. STZ (40 mg/kg/day ip) was injected during five consecutive days. Methadone (10 mg/kg/day sc) was administered from 3 days before the first STZ injection for 24 days. Day 1 was defined as the day of the first injection of STZ. Blood glucose was measured on days 0, 7, 14 and 21. The incidence of diabetes was expressed as an accumulative percentage of mouse blood glucose levels greater than 200 mg/dl. Values are means±SEM for eight mice. ⁎P<0.05; †P<0.01 vs. vehicle-treated mice; ††P<0.05 vs. MLDS-treated mice.
Methadone prevented the MLDS-induced decrease in serum hyperglycemia (Fig. 2). Treatment of mice with naltrexone at a dosage of 10mg/kg/day blocks opioid receptors, which is a weaker antagonist but has a longer half-life. It has been shown that Naltrexone is metabolized to its active metabolite 6-naltrexol, which is Naltrexone is a long-acting opioid antagonist with a half-life of 4h. Determination of serum insulin levels on day 21 showed that in MLDS-treated mice, serum insulin level was dramatically decreased (Fig. 3). Methadone prevented the MLDS-induced decrease in serum insulin. Administration of vehicle and methadone alone had no effect on serum insulin levels.

**Effects of methadone and MLDS on pancreatic cytokines**

To study the mechanism of methadone-induced protection against MLDS-induced diabetes, we measured the levels of Th1 and Th2 cytokines in supernatants of pancreatic tissues from each study group on day 21. As shown in Fig. 4, MLDS injections caused significant elevation in the pancreatic levels of TNF-α, IFN-γ and IL-1β (Th1 cytokines) compared with those in the control group. Treatment of mice with methadone markedly reduced MLDS-induced production of TNF-α, IFN-γ and IL-1β, as compared with that in MLDS-treated mice. Administration of methadone alone did not influence Th1 cytokine levels when compared to mice receiving vehicle. The decreasing effect of MLDS on pancreatic levels of IL-4 and IL-10 was not statistically significant. There was remarkable enhancement of IL-4 cytokine level in mice treated with methadone plus MLDS. There was also a trend toward an increase in IL-10 cytokine level in mice treated with methadone plus MLDS. However, this effect was not statistically significant. These results suggest that methadone protects islets from autoimmune attack.

**Histological studies**

Histological analysis of pancreatic tissue indicated that, in MLDS mice, STZ caused β-cell destruction and severe insulitis (islet leukocyte infiltration). In control mice, islets had intact β cells and insulitis was absent (Fig. 5). In mice treated with methadone plus MLDS, moderate insulitis was obvious, in contrast to control and MLDS alone mice. Prevention of insulitis in methadone-treated mice was significant but not complete. These observations demonstrate that MLDS-induced destruction of β cells and the degree of inflammation were ameliorated by methadone in pancreatic tissue.

**Discussion**

Our study demonstrated that methadone, as an opioid agonist, has protective effect against MLDS-induced type 1 diabetes in BALB/c mice. The ability of methadone to attenuate MLDS-induced diabetes was demonstrated by modulating the activity of the immune system. Diabetes induced by MLDS in mice is considered to be an experimental animal model of autoimmune diabetes. MLDS-induced diabetes exhibits clinical and immunological similarities to type 1 diabetes in humans, which are characterized by infiltration of T cells and macrophages in Langerhans islets, with selective destruction of β cells. It is generally thought that immune cells, cytokines, free radicals and production of nitric oxide have an important role in the pathogenesis of the disease (Kolb, 1994; O’Brien et al., 1996). The Th1 subset of CD4 T cells and proinflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-2, IL-12 and IL-18, have a direct β-cell cytotoxic effect in mice. In contrast, there is inhibition of β-cell damage by Th2 anti-inflammatory cytokines, such as IL-4, IL-5 and IL-10 (Kawasaki et al., 2004; Suarez-Pinzon and Rabinovitch, 2001). The balance between Th1 and Th2 cytokines has been suggested to be the determining factor in diabetes progression.

The cytokines TNF-α, IFN-γ and IL-1β have a central and important regulatory role in β-cell destruction in the pancreas (Lamhamedi-Cherradi et al., 2003). During insulitis, TNF-α is secreted from macrophages and induces production of several other inflammatory cytokines, such as IL-1β and IL-6. It has been reported that inhibitors of TNF-α prevent diabetes in mouse diabetes models (Beales, 1998; Holstad and Sandler, 2001). IFN-γ also mediates β-cell death and anti-IFN-γ mAb prevents insulitis and hyperglycemia in STZ-induced diabetes (Gysemans et al., 2005; Herold et al., 1996). Treatment with neutralizing antibodies against IL-1β has been shown to prevent diabetes in NOD mice (Cailleau et al., 1997).

The role of Th2 cytokines (IL-4 and IL-10) has been extensively investigated in prevention of insulitis and β-cell destruction. Both IL-4 and IL-10 have been shown to suppress insulitis and IDDM in NOD mice.
mice (Cameron et al., 1997; Hancock et al., 1995; Pennline et al., 1994). As mentioned above, it is postulated that any interruption of Th1 cytokines production and enhancement of Th2 cytokines may have protective effects against type 1 diabetes.

There have been several studies on prevention of β-cell damage or type 1 diabetes by anti-inflammatory and immunosuppressive agents (Rydgren et al., 2007; Yang et al., 2003; Maksimovic-Ivanic et al., 2002; Tabatabaie et al., 2000). Several other studies have indicated that opioids interact with the immune system and modulate a variety of immunological parameters (Vallejo et al., 2004; Budd, 2006; McCarthy et al., 2001). In vivo administration of exogenous opioids at pharmacological concentrations has suppressive effects on antibody and cellular immune responses, cytokine expression and graft-versus-host responses (Bryant and Roudebush, 1990). These effects are mediated by opioid receptors, because they are blocked by opioid antagonists (Sacerdote, 2000). Another study has shown that systemic administration of morphine inhibits LPS-induced TNF-α production (Bencsics et al., 1997). Morphine inhibition of phagocytosis and secretion of TNF-α is mediated through μ-opioid receptors (Roy et al., 1998). It is speculated that chronic stimulation of the μ-opioid receptor results in an increase in intracellular cAMP. Opioid-induced increase in intracellular cAMP leads to a decrease in NF-κB activation and suppression of IFN-γ gene expression (Wang et al., 2003). Investigators have reported that morphine treatment in vivo and in vitro decreases both Th1 cytokines (IL-2 and IFN-γ) and increases Th2 cytokines (IL-4, IL-5 and IL-10). They have concluded

![Image](image_url)

**Fig. 4.** Pancreatic levels of Th1 and Th2 cytokines in four study groups on day 21. Methadone (10 mg/kg/day sc) prevented the MLDS-induced increase in TNF-α (A), IL-1β (B) and IFN-γ (C) in pancreata of BALB/c mice, while methadone treatment increased the level of IL-4 (D). Methadone treatment did not significantly increase pancreatic IL-10 levels (E). Results are means ± SEM (n = 4–6). *P < 0.05 compared with vehicle-treated mice; †P < 0.05 compared with MLDS-treated mice.
According to the mechanisms of β-cell destruction leading to diabetes and immunosuppressive activities of methadone, we have investigated the immunological effects of chronic methadone treatment in a murine model of type 1 diabetes. Our study showed that methadone treatment ameliorated MLDS-induced hyperglycemia and incidence of diabetes, and increased pancreas insulin secretion. Methadone has high affinity for μ receptors and lower affinity for δ and κ receptors (Kristensen et al., 1995). The protective effect of methadone against hyperglycemia was reversed by naltrexone, which indicates a contribution from opioid receptors, mostly of the μ type, in the protective effect of methadone against diabetes. Therefore, the protective role of methadone is likely to be mediated by opioid receptor signaling. We showed that chronic methadone treatment had the ability to reduce MLDS-induced Th1 or proinflammatory cytokines, including TNF-α, IFN-γ and IL-1β in the pancreas of mice. This observation is very important, because these mediators play a central role in β-cell damage. In addition, we demonstrated that chronic methadone treatment induced at least one of the Th2 cytokines (IL-4) in the pancreas of MLDS-treated mice. All these actions of methadone may explain how it ameliorates the development of type 1 diabetes. Also, our histological analysis demonstrated the ability of methadone to attenuate β-cell destruction and insulitis development by inflammatory cells.

To the best of our knowledge, the current study is the first to suggest that chronic methadone treatment possesses protective activity against immune-mediated type 1 diabetes in an MLDS mouse model.

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References


According to continuous injection of β-endorphin, as a μ-receptor agonist, induces a shift from Th1 to Th2 cytokine production in mice. While the opioid receptor antagonist naloxone increases Th1 responses (Sacerdote et al., 1998, 2000). Also in vivo and in vitro studies have demonstrated that methadone, as another opioid agonist, has inhibitory effects on function of T lymphocytes and mononuclear phagocytes and natural killer cell cytotoxicity (Li et al., 2002).


