DISTRIBUTION OF NOCICEPTIN IN THE PANCREAS AND UTERUS OF NORMAL AND DIABETIC RATS

PhD Thesis

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INTRODUCTION

Nociceptin/Orphanin FQ (N/OFQ, NC) consists of 17 amino acids. It is the endogenous agonist ligand for nociceptin receptor (NOP). This neuropeptide is expressed mainly in neurons of the central (CNS) and peripheral (PNS) nervous systems. NC and its receptor have also been localized to various organ systems of the body (Houtani et al., 1996 Nothackeret al., 1996). NC has been reported to mediate pain and regulate a large number of physiological functions among others, release of neurotransmitters and hormones (Lü et al., 2010), inhibits uterine contraction (Klukovits et al., 2010) and suppresses oxytocin, vasopressin and GnRH release (Dhandapani and Brann, 2002).

Pancreas: NC has been implicated in pancreatic function, for example, Tekes et al. (2005) showed that long-term diabetes mellitus does not modify the plasma or CSF levels of NC. Prolonged and extended hyperglycaemia reduce pancreatic β-cell responsiveness to stimulants (Poitout and Robertson, 1996). It has also been reported that the NC-NOP receptor system plays an inhibitory role in the regulation of amylase secretion from pancreatic exocrine cells (Linari et al., 2006). Other reports revealed that intracerebroventricular infusion of NC caused increased level of plasma insulin (Matsushita et al., 2009).

Uterus: According to Deák et al., (2013, 2015) NC exerts a relaxant effect in the contraction of human and rat uteri. Additionally, NC like the other opiates seems to function as a neuromodulator of the endocrine component of the reproductive system (Foradori et al., 2007). Bryant et al., (2002) reported that NC stimulated prolactin secretion in male and female rats, with the magnitude of the response being significantly greater in females. It is also speculated that corticotrophin-releasing hormone from the epithelium of the endometrium may be regulated by NC (Gravanis et al., 1999).

Nociceptin in diabetes mellitus: Peripheral neuropathy is a common complication of diabetes mellitus. A common symptom of this disorder is neuropathic pain. It is widely accepted that NC is involved in the pathogenesis of diabetes-induced neuropathic pain (Courteix et al., 2004) at supra-spinal and spinal levels. Intracerebroventricular injection of NC causes hyperalgesia or anti-opioid effects instead of analgesia. However, NC produces allodynia or hyperalgesia when given intrathecally in low doses, but causes anti-nociceptive effects in high doses (Tekes et al., 2005). Liu et al., (2012) reported that concentration of NC is elevated in the CNS and serum of rats with diabetic neuropathy when compared to non-diabetic controls.

AIMS AND OBJECTIVES

Hypotheses

Nociceptin is present in endocrine glands e.g. endocrine pancreas and also in the uterus. The tissue content of NC will change after the onset of diabetes mellitus.

Aims of the study

The aim of the study was to determine the pattern of distribution of NC in the pancreatic islets (classical endocrine) and the uterus (a female reproductive organ) of non-diabetic and diabetic rats.

Objectives of the study

1. Investigate the distribution of nociceptin in the endocrine pancreas of non-diabetic and diabetic rats using immunohistochemical, immunofluorescence, Western blot and immunoelectron microscopy methods.
2. Examine the morphology of the uterus after the onset of diabetes.
3. Examine the ultrastructure of the endometrium and myometrium of non-diabetic and diabetic rats using conventional electron microscopy.
4. Investigate the distribution of NC in the uterus of non-diabetic and diabetic rats using immunofluorescence, Western blot and immunoelectron microscopy methods.

MATERIALS AND METHODS

Experimental animals: Twelve adult male or female Wistar rats, weighing 225-250g, were divided randomly into non-diabetic control group (n = 6) and streptozotocin (STZ)-induced diabetic group (n = 6). Food and water were available *ad libitum*. Female rats were selected on the basis of their oestrous cycle as described by Marcondes et al., (2002). The study was performed with the approval of the CMHS Animal Research Ethics Committee (A5-14).

Induction of experimental diabetes mellitus: Diabetes mellitus (DM) was induced either in male and female rats by a single intraperitoneal injection (i.p.) of STZ (Sigma, St. Louis, MO, USA) at a dose of 60 mg/kg body weight (Adeghate,1999). Rats with blood glucose levels ≥ 280 mg/dl were considered diabetic and used for this study.

Body weight: The body and organ (either pancreas or uterus) weights of non-diabetic and diabetic rats were noted with a 9001 Scale (Sartorius, UK) and expressed as mean ± standard error of the mean (SEM).

Glucose measurement: The blood glucose level was measured in blood samples taken from the tail vein. Data were also expressed as mean ± standard error of the mean (SD).

Glucose tolerance test: Non-diabetic and STZ-induced diabetic rats were subjected to i.p. glucose tolerance test according to the method of Kim et al. (2013).

Tissue collection: A mid-line abdominal incision was made and either the pancreas or uterus was removed after anaesthesia. Representative tissue fragments were collected from the pancreas and uterus and used for immunohistochemical, immunofluorescence, electron microscopy and molecular biology studies.

Light microscopy: Pancreas and uterine tissues from non-diabetic and diabetic female Wistar rats were dissected out, washed in phosphate buffered saline (pH 7.2), blot dried, cut into small pieces and fixed rapidly for 24 hours in freshly prepared Zamboni's fixative (Zamboni and de Martino, 1967). The samples were later embedded in paraffin wax and processed for immunofluorescence and immunohistochemical analysis according to standard procedures (Adeghate et al., 2001).

Single and double-labelled immunohistochemistry/immunofluorescence study:

Deparaffinized and rehydrated tissue sections of the pancreas were processed for imunohistochemistry using the avidin-biotin complex method according to a published protocol (Adeghate et al., 2001). Co-localization of NC with insulin was performed using double-labelled immunofluorescence technique according to a previously described method and NC was localized in the endometrium and myometrium, using established method (Adeghate and Ponery, 2004).

Tissue processing for conventional electron microscopy: Pancreatic and uterine tissues were retrieved from non-diabetic and diabetic rats, washed in 0.1M phosphate buffer (pH 7.2), fixed

in Karnovsky's solution (Karnovsky, 1965) and processed for ultrastructural study according to Adeghate et al (2010).

Single and double-labelled immunoelectron microscopy study: NC was localized to the myometrium using single labelled immunoelectron microscopy technique and co-localization of NC with insulin was carried out at the electron microscopy level according to Lotfy et al., (2014).

Western blotting: The presence of NC in uterine and pancreatic tissues was determined with Western blot technique according to previously described method (Tariq et al, 2015).

Morphometry and Statistical analysis: Morphometric analysis was performed on at least six randomly selected images from each group. Statistical analysis was performed using SPSS software 21.0. Data were expressed as means ± SEM. Mann Whitney *U* test was used to determine the statistical significance. $P \leq 0.05$ were taken as significant.

RESULTS

Pancreas

Body weight and blood glucose level: The average body and pancreas weight of the non-diabetic male rats was significantly ($p < 0.05$) higher compared to that diabetic rats. Moreover, the blood glucose level of diabetic rats was markedly higher when compared to that of non-diabetic controls.

Localization of nociceptin and co-localization with insulin in the pancreas:

Immunohistochemical studies showed that NC-immunoreactive cells are located in the central and peripheral portions of the islet of Langerhans of non-diabetic control rats. The number of NC-positive cells in the pancreatic islet of non-diabetic control rats is higher when compared to those seen in the islets of diabetic rats. Using double labelling immunofluorescence method, we observed that NC co-localizes with insulin in pancreatic β-cells of both non-diabetic control and diabetic rats. However, the extent of co-localization was lower in the islet of diabetic rats when compared to non-diabetic controls.

In order to examine the exact intracellular localization of NC, we performed post-embedding immunoelectron microscopy using gold labelled particles, which showed that NC-positive

particles are indeed located with insulin in secretory granules of pancreatic β-cells. The large and marked reduction in the expression of NC in diabetic rat pancreas was further confirmed with immunoelectron microscopy. The localization of NC in the endocrine pancreas of rat using immunohistochemistry was also confirmed by Western blot technique, which also showed a decreased tissue level of NC after the onset of DM.

Uterus

Body weight and blood glucose level: The average body and uterine weights of the non-diabetic female rats was significantly ($p < 0.05$) higher compared to that diabetic rats. In addition the blood glucose level of diabetic rats was significantly higher compared to that of non-diabetic controls.

Morphology of the uterus: DM was associated with a significant ($p < 0.05$) reduction in the weight of the uterus. This reduction occurs as early as 15 days after the onset of DM. In addition, the number of blood vessels supplying the uteri of diabetic rats appeared to be fewer in number when compared to non-diabetic controls.

Light microscopy study was also carried out on semithin sections of uterus to examine the morphology of the rat uterus after the onset of DM. The endometrium and myometrium layers of the uterus of diabetic control were significantly thinner than those of non-diabetic rats. Moreover, the endometrial glands were less prominent in diabetic rats when compared to non-diabetic control rats. Conventional electron microscopy was carried out to examine the cause of the reduction in size in diabetic rat uterus compared to control. Ultrastructural study showed gross loss of myofibrils, nuclei and degeneration of the epithelial cilia and deposition of lipid droplets in epithelial cells of uterus of diabetic rats.

Localization of nociceptin in the uterus

Immunofluorescence study: Nociceptin is localized to the uterus of both non-diabetic and diabetic rats. Large number of NC-positive cells was observed in the endometrial layer of the uterus. The number of NC-immunoreactive cells was significantly ($p <0.05$) lower in the endometrium of diabetic rats compared to that of non-diabetic controls. In addition, the columnar

epithelium of the endometrium of diabetic rat uterus did not express nociceptin as strongly as that of non-diabetic control.

The number of NC-positive cells seen in the myometrium of diabetic rats was significantly ($p < 0.05$) lower compared to that of non-diabetic control group. Moreover the myometrium of the uterus of both non-diabetic control and diabetic rats contain neuronal cell bodies and nerve fibres. These nerve fibres are varicose in nature. The density of the nociceptinergic innervation of the myometrium of diabetic rats was less pronounced when compared to that of non-diabetic control. Morphometric evaluation of immunofluorescent images showed that the number of NC-positive cells was significantly ($p < 0.05$) reduced after the onset of diabetes. The presence of NC in the uterus was confirmed by Western blot analysis, which also showed that the level of NC in the uterus of diabetic rats is just about half of that of non-diabetic control rats.

Immunoelectron microscopy study: We performed post-embedding immunoelectron microscopy using gold-labelled immuno-particles in order to examine the exact location of NC in the uterus. NC-immunopositive gold particles were observed in the myometrium of both non-diabetic control and diabetic rats. NC was discerned mainly on the myofibrils in longitudinal smooth muscle sections. In circular muscle cells, the NC-labelled colloidal gold particles localization was also noticed. The gold particles were not seen on other cytoplasmic organelles.

In diabetic rat myometrium, NC-labelled colloidal gold particles were observed on the myofibrils of smooth muscle cells. The number of NC-immunoreactive gold particles was much lower in diabetic rat myometrium compared to non-diabetic controls.

Morphometric evaluation of randomly selected sections showed a significant ($p < 0.05$) reduction in the number of NC-labelled immunoparticles in the myometrium of diabetic rats compared to non-diabetic controls.

DISCUSSION

We investigated the presence of nociceptin (NC) in two organ systems, namely, the pancreatic islet of Langerhans (an endocrine organ) and the uterus (a female reproductive organ). The study design included two groups of Wistar rats, non-diabetic controls and streptozotocin (STZ)-induced diabetic rats. Two weeks after the injection of STZ, pancreatic and uterine tissue samples were collected for light microscopy, immunohistochemistry, Western blot and

conventional and immunoelectron microscopy. Animal body weight, pancreas and uterine weights were also taken. Glucose tolerance test was conducted after intraperitoneal injection of glucose.

Metabolic parameters

Body weight: In line with the literature data (Adeghate, 1999, Garris et al., 1986) the body and organ (uterus and pancreas) weights of non-diabetic rats increased steadily over time compared to STZ-induced-diabetic rats.

Blood glucose level and Glucose tolerance test: According to literature data (Liu et al., 2013) the blood glucose level was significantly higher in diabetic rats compared non-diabetic controls. In contrast to non-diabetic controls, the blood glucose level of diabetic rats did not return to normal value even after 120 min post glucose challenge.

Pancreas

Our study is the first publication to quantity the tissue level of NC in the pancreas. The pattern of distribution of NC in islet cells was found to be similar to that of insulin and became deranged after the onset of DM. The reason why the distribution of NC is altered in DM needs further elucidation. Our Western blot study also confirmed our immunohistochemical and immunofluorescence findings on the presence of NC in the pancreas.

Immunofluorescence and immunoelectron microscopy study showed that NC co-localizes with insulin in both non-diabetic and diabetic rat pancreas. Immunoelectron microscopy study confirmed our immunofluorescence result on the presence of NC with insulin in pancreatic β-cells. Furthermore, the degree of co-localization was significantly reduced in DM. Our study is the first description of NC in the endocrine pancreas. NC was found to be localized with insulin in secretory granules of pancreatic β-cells and it was not seen in other cytoplasmic organelles. It is suggested that the main function of NC in the pancreas is to regulate insulin metabolism.

Why is NC co-localized with insulin? In our opinion NC may play a role in the metabolism of insulin either in an autocrine or paracrine manner. It may also inhibit or even stimulate insulin release from pancreatic β-cells. The reduction in the number of NC-containing cells in the islet

cell after the onset of diabetes may be due to reorganization of cells within an organ. The exact role of NC in the regulation of hormones of the pancreas is not clear. Liu et al., (2012) showed that NC level is increased in the CNS of diabetic neuropathy pain model of rats. In contrast, Tekes et al., (2005) observed no difference in the CSF level of NC in diabetic rats. However, it has also been shown that chronic cerebroventricular infusion of NC increased plasma insulin level significantly (Matsushita et al. (2009), supporting the view that NC may have a physiological role in insulin secretion.

Uterus

Gross morphology: We observed a reduction in the size of uterus and density of blood vessels in diabetic rats, which may be due to diabetes-induced lesion in blood vessels. Impaired blood vessels diminish blood flow into the organ and the mass of the organ may also be affected (Tatewaki et al., 1989).

Light microscopy: Our light microscopy study revealed that the width of both the endometrium as well as that of myometrium is reduced after the onset of diabetes. Uterine tissue may therefore undergo severe alterations in the absence of insulin. Insulin is known to promote glucose uptake into cells. The atrophy of the female reproductive tract has been reported in db/db mice **(**Tatewaki et al., 1989, Garris, 2004 ,1986).

Immunofluorescence and immunoelectron microscopy study: Our study showed that NC is present in many cells in the endometrial stroma and in the myometrium as well. The role of NC in uterine function has not been elucidated; however, it has been implicated in labour, which is associated with extreme pain. Studies including that of Gáspár et al. (2015) have indeed implicated NC in uterine pain and labour. Our study is the first to localize NC to uterine tissue and therefore leads a strong support to many physiological as well as pharmacological effects of NC that have been reported in the literature. The localization of NC in the endometrium of uterus further supports the role of NC as a putative modulator of the endocrine function in the endometrium. However, further studies are needed to establish the precise role of NC in endometrial function.

The other observation of our immunofluorescence study is the significant reduction in the uterine tissue level of NC after the onset of DM. This observation corroborates our own findings on the pancreatic tissue level of NC. However, it is impossible to compare our findings with that of the

literature since this is the first study to examine the tissue level of NC by immunofluorescence method in the uterus. Some neuropeptides, such as galanin have also a reduced pancreatic level after the induction of diabetes (Adeghate and Ponery, 2001).Western blotting technique was also performed to confirm the localization of NC in uterine tissue and results were in agreement with those performed with immunofluorescence. In addition to the use of Western blot to confirm the presence of NC in the uterus, we have also used immunoelectron microscopy to determine the exact location of NC in the rat uterus. Our study showed that NC is located on the myofibrils of the smooth muscle cells. This observation supports the role of NC as a regulator of smooth muscle contraction.

Nociceptinergic innervation: Our present study demonstrated, for the first time, the distribution pattern of NC-positive nerves in the uterus of rats. This observation supports the physiological and pharmacological role of NC in the uterus. NC has been reported to induce uterine relaxation (Deák et al., 2013), while some other studies have attributed a contractile role for NC in smooth muscles (Yüce et al., 2007).

Conclusion

- The study showed that nociceptin is present and coexists with insulin in pancreatic β-cells.
- The degree of co-localization of nociceptin with insulin in pancreatic β-cells was altered in streptozotocin-induced diabetes.
- The number of nociceptin labelled colloidal gold particles was significantly lower in the β-cells of the islet of Langerhans after the onset of diabetes compared to non-diabetic controls.
- Our study showed a gross atrophy of uteri as early as 15 days post diabetes.
- Our study also revealed the presence of nociceptin in the endometrium and myometrium of the rat uterus.
- The expression of nociceptin was found to be significantly lower in diabetic rat uteri compared to those of non-diabetic controls.
- The myometrium of both non-diabetic and diabetic rats contained NC-positive nerves with varicosities.
- The expression of NC in the nerves of the myometrium of non-diabetic controls was higher compared to that of diabetic rats.

- Ultrastructural study showed that NC is localized specifically to myofibrils of the smooth muscles of non-diabetic controls and diabetic rat uteri.
- The degree of expression of NC in myofibrils of the smooth muscles of diabetic rat uteri is significantly lower when compared to non-diabetic controls.
- The localization of NC in uterine wall suggests that NC may have a specific physiological role in the modulation of uterine function.
- The atrophy of the uterus in diabetic rats should send alarm signals to clinicians and researchers on how to prevent the uterus from degeneration after the onset of diabetes.
- The degeneration of uterine tissue and loss of NC implicate that the regulation of pain appears to be a "double edge sword" for female diabetics looking forward to getting pregnant.

Publications

Publications related to the PhD dissertation

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