EFFECTS OF NOCICEPTIN ON MARKERS OF OXIDATIVE STRESS AND METABOLIC PROFILES IN DIABETES MELLITUS

PhD Thesis

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Budapest, 2023

INTRODUCTION

Nociceptin (NC) is a small (17-amino acids) endogenous peptide located in the central nervous system [1]. NC, commonly referred to as orphanin FQ (N/OFQ), was discovered in 1995 by two groups of scientists (Meunier et al.; Reinscheid et al.) working independently of each other. NC was originally called „orphanin" because it was deemed to be a peptide of an orphan receptor, which was discovered way before the ligand. This was indeed, a classical example of reverse pharmacology [Hernandez et el, 2021, El Daibani et al., 2022]. NC is a product of a much larger precursor protein, prepronociceptin, which is located on chromosome 8, at p21.1. The nociceptinergic system is well distributed in the nervous system [Neal et al, 1999]. NC and NOP receptors have been detected in the gastrointestinal tract, ductus deferens, and in the cells of the immune system [Tariq et al., 2013, Gyires et al., 2015]. The detection of NC, NOP receptors and their mRNAs in CNS and peripheral nervous systems and other non-neural organs show that the nociceptinergic system is ubiquitous to the human body.

Physiological effects of nociceptinergic system: Although the mechanism by which the nociceptinergic system exerts its function has not been completely elucidated but it has been shown that when NOP receptor is activated it inhibits the activation of adenylyl cyclase and Ca^{2+} channels while stimulating K^{+} channels in a process similar to that observed in the case of opioids. The nociceptinergic system can modulate nociception, musculoskeletal function, inhibition of analgesia induced by pain, lowering of the stimuli of stress, memory consolidation and many others [Tariq et al., 2013].

Diabetes mellitus: Diabetes mellitus (DM) currently affects more than 537 million people worldwide [Diabetes Atlas, 2021]. DM is characterized by hyperglycemia because of insufficient or ineffective insulin molecule to help in the uptake of glucose molecules by target cells such as skeletal muscle, hepatic and fat cells [Calejman et al., 2022]. The inability of the cells to use glucose leads to disruption in the metabolism of carbohydrates, lipids and proteins [Emerald et al., 2022]. Hyperglycemia-induced oxidative stress, coupled with the disruption in the metabolism of carbohydrates, lipids and proteins leads to chronic complications of DM such as diabetic- retinopathy, cardiomyopathy, nephropathy, neuropathy, macro- and micro-angiopathy.

Diabetes mellitus and oxidative stress: Reactive oxygen species (ROS) include radical (superoxide anion, hydroxyl radical, alkoxyl radical, peroxyl radical) and non-radical (hydrogen peroxide, singlet oxygen, hypochlorous acids) species. Reactive nitrogen species (RNS) on the other hand contain nitrogen-centered species. They include nitric oxide nitric and peroxynitrite [Lee et al., 2004]. It has been shown that chronic hyperglycemia (HG) can lead to the release of ROS and RNS through the activation of both enzymatic and non-enzymatic pathways.

Markers of oxidative stress: Markers of oxidative stress can be classified into those that involved in the net antioxidant capacity of the serum lipid peroxidation (isoprostanes, malondialdehyde), oxidative protein modifications (nitrotyrosine, S-glutathionylation), myeloperoxidase, ROS-induced modification in genes (Nrf-2) and endogenous antioxidants [Ho et al., 2013]. In this study we have examined the level of key endogenous antioxidants including catalase (CAT), superoxide dismutase (SOD) and glutathione reductase GRED. CAT is an ubiquitous enzyme capable of converting millions of molecules of harmful hydrogen peroxide into oxygen and water, thereby reducing oxidative stress in cells and tissues [Chelikani et al., 2004]. SOD neutralizes the superoxide radical (a ROS), turning it into oxygen and hydrogen peroxide. The hydrogen peroxide produced in then destroyed by CAT [Hayyan et al., 2016]. GRED, which is conserved in all living organisms contribute to the formation of glutathione, an molecule critical to the reduction of oxidative stress. GRED together with glutathione protect cells from oxidative stress by converting hydrogen peroxide to H_2O_2 and peroxide. GRED is also capable of neutralizing ROS such as hydroxyl radicals and singlet oxygen [Deponte, 2013].

AIMS, HYPOTHESIS AND OBJECTIVES

Hypothesis

We hypothesize that nociceptin (NC) can ameliorate the signs and symptoms of diabetes mellitus in addition to increasing the expression of endogenous antioxidants in several organ systems.

Aims and objectives

Our aim in this project is to investigate the tissue and cellular localization of NC in the pancreas. We also wanted to determine whether nociceptin can influence endocrine release from the islet

of Langerhans of both normal and diabetic rats. Since DM rats suffer from oxidative stress, we wanted to know if NC can increase the expression of endogenous antioxidants.

Objectives of the study

a. To determine tissue and cellular localization of NC
b. To examine the effect of NC on endocrine release in normal and diabetic rats
c. To investigate the effect of NC on the weight and glucose level
d. To determine whether NC can affect the expression of endogenous antioxidants such as catalase, glutathione reductase and superoxide dismutase in the kidney, liver and brain of normal and diabetic rats
e. To determine the effect of NC endogenous antioxidant (catalase, glutathione reductase and superoxide dismutase) expression in pancreatic islet cells

MATERIALS AND METHODS

Experimental animals used in this study: The animals used in this study were Male Wistar rats bred at the Animal House of the College of Medicine & Health Sciences, United Arab Emirates University, Al Ain, UAE. The original strain was bought from Harlan Laboratories, Oxon, England, UK. All experimental animals were placed in large plastic (polypropylene) cages manufactured specifically for murine models. The Animal Facility was fully air-conditioned (23 °C) with 12 h day and 12 h night cycle. Drinking water and rodent laboratory feed obtained from Emirates Feed Factory, Abu Dhabi, United Arab Emirates, were available *ad libitum*.

Diabetes induction: Diabetes mellitus was induced in male 150 - 200 g Wistar rats by a single dose of streptozotocin (STZ) [(60 mg/kg^{-1} body weight; Sigma, Poole, UK, given intraperitoneally, (i.p.)]. STZ was prepared in a buffered citric acid solution. The solution contains, 0.1 M citric acid and 0.1 M sodium citrate. The pH of the buffered solution was adjusted to 4.5. The same volume (0.3 ml) of this vehicle (buffered citric acid) solution was given i.p. to control rats. The confirmation of DM was performed using a One Touch II Glucometer (Life Scan Inc., Johnson & Johnson, Chesterbrook, PA, USA). Rats were considered diabetic if the fasting blood glucose level ≥ 10 mM (180 mg/dl). Experimental rats were

euthanized one month after the induction of DM. Ethical approval for the study was obtained from the CMHS Animal Research Ethics Committee (A5-14).

Treatment of experimental animals with NC: NC was purchased from Abcam (Cat #: ab38198; aa1-17). NC was dissolved in phosphate buffered physiological saline (PBS) and given i.p. to rats at a dose of 10 µg/kg per day for a total of five days to non-diabetic control and diabetic rats. The drug (NC) was administered at 9:00 every morning. Equal amounts of vehicle (PBS) were administered i.p. to another group of 6 non-diabetic rats (normal control) and diabetic rats (diabetic controls) for the same experimental duration as treated. In order to allow the total excretion of STZ from the body of diabetic rats, NC and the vehicle were only given 15 days after the induction of DM.

Immuno-localization of NC in the islet of Langerhans: In the experiment for the localization of NC in the serum and pancreatic islets, blood and tissue samples were collected four weeks post-induction of DM. Rats were euthanized and the pancreas was quickly removed *in toto*. The whole pancreas was portioned into for three separates studies: i). Immunofluorescence (IF) study, ii). Transmission electron microscopy (TEM) and, iii). Insulin secretion.

Effect of NC on markers of oxidative stress: Five days after treatment with NC, blood was collected from the inferior vena cava. In addition, the pancreas, kidney, liver and brain were harvested to study the effect of some selective markers of oxidative stress.

Fixation of pancreatic tissue fragments: Pancreatic tissue fragments were fixed overnight in Zamboni's solution for immunofluorescence study according to a previously reported method [Zamboni & de Martino, 1967]. Small (2 mm^3) fragments from the body of the pancreas were fixed overnight in McDowell solution [McDowell & Trump, 1976] for electron microscopy.

Immunofluorescence study of pancreatic islets of Langerhans: Pancreatic tissue fragments taken from non-diabetic (n = 6) and diabetic (n = 6) rats were fixed in Zamboni's fixative [1967] for 24 h at 4 °C. The pancreatic tissue fragments were then placed in ascending concentrations of ethyl alcohol, embedded in paraffin, and processed for immunofluorescence according to previously described technique [Lotfy et al, 2014].

Immunoelectron microscopy study: Small fragments of pancreatic tissue were cut into small (1mm^3) pieces, fixed in McDowell's solution at 4 °C for 24 h and processed for IEM (immunoelectron microscopy) according to a previous technique [Lotfy et al, 2014].

Stimulation of rat pancreas (normal and diabetic) with NC: Normal and diabetic rat pancreas were expeditiously removed from anesthetized animals, cut into small pieces (1.0 mm^3) before incubation in Krebs buffered (KB) solution (contents in mM: NaCl, 118; KCl, 4.5; KH_2PO_4, 1.4; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 2.8) for 1 h. The pH of the buffered solution was 7.4. Incubation of the tissue in different concentrations of NC (10^{-6}, 10^{-9}, 10^{-12} M) was performed at 37 °C in a water bath and constantly aerated with physiological gas mixture (95% O_2 + 5% CO_2) in accordance to a previous technique [Lotfy et al, 2014].

Insulin RIA: Radioimmunoassay method was used to measure the amount of insulin released into the supernatant after stimulation with different concentrations of NC using a sensitive rat insulin kit (Sigma-Aldrich catalog #: SRI13K). The measurement was performed according to the manual of instruction provided with the kit. All tests including the samples and controls were done as duplicates.

Determination of glucagon concentration: After the stimulation with NC (10^{-6}, 10^{-9}, 10^{-12} M) the concentration of glucagon level in the supernatant was determined using ELISA kit (Catalog #. EK-028-02; Phoenix Pharmaceuticals (Burlingame, CA, USA). The measurement of the concentration of glucagon was conducted based on the instructions given in the instruction manual.

Pancreatic islet isolation and stimulation with NC: Islets of Langerhans were isolated from normal (non-diabetic) and diabetic rats using a previously established technique [Carter et al., 2009].

Measurement of insulin release from the islets of Langerhans: After NC stimulation of pancreatic islet cells, the insulin released was measured in the supernatant using ultrasensitive rat insulin ELISA kit (Catalog #: 10-1251-01; Mercodia Sylveniusgatan 8A, Uppsala, Sweden). Insulin level was done according to the protocol provided by vendor.

Morphometric analysis of endocrine cells: After immunohistochemical staining of the islets of Langerhans of non-diabetic (control) and diabetic rats, the number of nociception-, insulin-, glucagon-, somatostatin- and PP-positive cells was counted with Image J® (NIH, Bethesda, Maryland, USA). The number of either nociceptin-, insulin-, glucagon-, somatostatin-, or PP-immunoreactive cells were tabulated and divided by the islet's total number of cells to determine the percentage of each type of cells in a given islet of Langerhans. A total of 5 islets were counted for each animal per group of six animals (n = 6).

Estimation of the number of NC- and insulin-containing secretory granules: In order to quantify the extent of the distribution of NC and insulin in pancreatic beta cells, the number of secretory granules containing either insulin, NC or both (5 nm, insulin; 10 nm, NC) were estimated on EM images. 5-6 images were counted for each animal per a group of six.

Immuno-expression of endogenous antioxidants in parenchymal organs: As one aspect of our study was to examine on the effect of NC on selected endogenous antioxidants, we used 6 µm thick sections for the immunochemical localization of these bioactive agents. Six µm thick sections mounted on gelatin-coated glass slides were taken through double-labelled immunofluorescence process to identify the pattern of distribution of endogenous antioxidants in the kidney, liver, pancreas and the brain in accordance with a published technique [Lotfy et al, 2014].

Serum catalase activity: The activity of catalase in serum of all animal groups (normal, normal treated, diabetic untreated, and diabetic treated) following the administration of NC was measured using a colorimetric method supplied with commercial kits obtained from Cayman Chemicals (Ann Arbor, MI, USA).

Immunofluorescence images of pancreatic islets: All fluorescence images in both pancreatic islets and other organs (kidney, liver, brain) were obtained with an AxioCam HRc digital camera using AxioVision 3.0 Software (Carl Zeiss, Oberkochen, Germany). Images were processed using Image J 1.8. The fluorescence images were taken without taking into account the intensity (density) of immunofluorescence.

Density of immunofluorescence in kidney, liver and brain: After the normal and diabetic rats were treated with NC, the density of the immunofluorescence staining of CAT, SOD and GRED in the renal cortex, liver, cerebral cortex and C3 region of the hippocampus was determined with Image J software® (NIH, Bethesda, MA, USA). The density of immunofluorescence is directly proportional to the cellular levels of these endogenous antioxidants in the kidney, liver and brain. Since the objective of the study is to examine whether CAT, SOD and GRED co-localize with islet hormones, the density of immunofluorescence in pancreatic sections was not measured. The control (normal) image was taken as 100% . Data were measured as mean ± SEM (n=6 per group).

Chemicals and immunochemical reagents: All chemicals, reagents and immunochemical reagents were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

Statistical Analysis: All experimental data were calculated as mean ± standard error of the mean. Differences between the groups were calculated using One-way ANOVA. Significant differences between mean values of the group, and two different timelines, were calculated with an unpaired t-test. Statistical significance was set at a value of $p < 0.05$.

RESULTS

Total body weight and concentration of blood glucose

The induction of DM caused a significant ($p < 0.002$) loss in body weight when compared to non-diabetic controls 4 weeks into DM. In addition, rats suffering from DM appeared frail with polyuria and polydipsia compared to non-diabetic rats. Some animals developed cataract. Blood glucose concentration was markedly ($p < 0.0001$) higher in diabetic rat, when compared to control.

Pattern of distribution of NC in pancreatic islet cells of non-diabetic and diabetic rats

NC and insulin in islet cells: We wanted to know whether insulin co-localizes with insulin in pancreatic islet cells and to determine whether diabetes alters the pattern of distribution of NC in the islet of Langerhans. Four weeks after the onset of DM, the number of NC-immunoreactive

cells in the islets of Langerhans of diabetic rats was significantly reduced compared to control. Using double labeling immunofluorescence, we observed that NC co-localizes with insulin in pancreatic beta cell both normal and diabetic rats. DM caused a marked ($p < 0.0001$) decrease in the number of insulin-positive cells compared to non-diabetic rats. The extent of co-localization is very high because the percentage of NC- and insulin-immunoreactive cells was similar in both non-diabetic and diabetic rats.

NC and glucagon in pancreatic islets: Since it was not sure whether NC co-localizes with glucagon, we used double labeling immunofluorescence to determine their localization in pancreatic islet cells. Glucagon was observed mainly in the periphery of the islets of Langerhans while NC was mainly located in the central portion of islets. Double labelled IF showed that NC does not co-localize with glucagon in the alpha cells of the islets of Langerhans. It is worth noting that DM alters the pattern of distribution of glucagon-positive cells. In DM, glucagon-positive cells increase markedly ($p < 0.0001$) in number, occupying both the peripheral and central portions of the islets of Langerhans.

NC and somatostatin in islet cells: Somatostatin-immuno-positive cells are located in the peripheral region of pancreatic islet. NC on the other hand is seen in the central portion of islets of Langerhans. Double labelled IF was performed to determine if there is any co-localization between these two hormones in the delta cells of pancreatic islets. Double labelled IF showed that somatostatin and NC do not co-localize within any cells of the islet of Langerhans. However, DM alters the pattern of distribution of somatostatin-immunoreactive cells in pancreatic islets. In DM rats, somatostatin can be observed in the central region of the islets instead of the peripheral location in normal rats. Moreover, the percentage number of somatostatin-positive cells is markedly ($p < 0.0001$) higher in the islet of DM rats.

NC and PP in the islets of Langerhans: Double labeling IF was used to determine whether NC co-localizes with PP in the islet of Langerhans. In normal rat islets, PP-positive cells were observed in the outer part of the islets, while NC-immunoreative cells, occupy the central region of the islet. However, after the onset of DM the number and pattern of distribution of PP-immunopositive cells are altered. The number of PP-immunoreactive cells increased significantly ($p < 0.0001$) in DM rats, while PP-positive cells are now located in the central part of the islet instead of the peripheral location seen in normal rats.

Immunoelectron microscopy of NC in pancreatic islet cells

Double labelled IF showed that NC co-localizes with insulin in pancreatic islet cells. IEM (Immunoelectron microscopy) was used to examine the exact location of NC in pancreatic beta cells. Immunogold particles (5 and 10 nm size) attached to IgG were used to determine the degree of co-localization of NC and insulin in insulin-producing beta cells of the pancreas. TEM of the ultrathin sections processed for immunoelectron microscopy showed NC particles (10 nm) in the secretory granules of pancreatic beta cells with that of anti-insulin 5 nm gold particles. The presence of 5 nm-immunogold particles against insulin and that of 10 nm labelled antibodies against NC on the secretory granules of insulin-producing beta cells showed that NC and insulin are indeed co-localized at the ultrastructural level. After the onset of DM, the number of 5 nm gold particles (directed against insulin) and 10 nm gold particles (conjugated to NC) was significantly ($p < 0.02$) reduced.

Effect of NC on insulin and glucagon release

NC inhibited insulin release from pancreatic tissue fragments of control rat at all concentrations (10^{-12}, 10^{-9}, 10^{-6} M). NC also inhibited insulin secretion from pancreatic tissue fragments of diabetic rats. The inhibitory effect of NC was most notable ($p < 0.01$) when 10^{-9} M was used and least effective after incubation with 10^{-6} M of NC (Fig. 8a.). Incubation of isolated pancreatic islets with NC at 10^{-12}, 10^{-9}, 10^{-6} M caused a large ($p < 0.04$) reduction of insulin secretion in control rats. However, NC (10^{-12} M) induced, marked ($p < 0.02$) elevation in insulin release from islets of DM rats. NC caused marked ($p < 0.001$) glucagon release from the pancreatic tissue fragments in normal but not in DM rats.

Effect of NC on endogenous antioxidants in renal cortex

The immune-expression of three key endogenous antioxidants, catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GRED) was examined in the renal cortex. CAT was observed in the proximal (PCT) and distal (DCT) tubules of non-diabetic and DM rats. The IF staining intensity of CAT was markedly reduced in NC-treated non-diabetic and DM rats compared to untreated control rats (Fig. 9a. - Fig. 9b.). The immune-expression of SOD increased in normal, non-diabetic rats treated with NC. In contrast, the immune-expression of

SOD was significantly reduced in DM rats treated with NC. GRED expression in the renal cortex was markedly increased in non-diabetic, and DM treated with NC.

Effect of NC on endogenous antioxidants in the liver

CAT was observed in liver cells located in the immediate vicinity of the central veins of the liver. CAT immune-expression was significantly lower in the liver of rats treated with NC compared to controls. SOD-immunoreactive hepatocytes located around the central were numerous. These cells contain large quantities of SOD as evidenced from strong SOD IF staining. The tissue level of SOD immune-expression was markedly increased in both normal and diabetic rats treated with NC, when compared to untreated control rats. In addition, treated of normal and DM rats with NC resulted in the increased immune-expression of GRED in liver cells of DM rats compared to untreated controls.

Effect of NC on endogenous antioxidants in the cerebral cortex

Neurons of the cerebral cortex contain CAT. The administration of NC to normal rats lowers the immune-expression of CAT, but caused a significant, increase in CAT immune-expression cerebral cortex of DM rats compared to controls. NC treatment did not significantly alter the immune-expression of SOD in the brain of normal rats. In contrast, treatment with NC significantly increased the SOD concentration in the neurons of the brain in DM rats. The immune-expression of SOD was markedly reduced in the brain of untreated DM rats compared to normal control rats. NC treatment ncreased GRED immune-expression in the cerebral cortex of DM rats compared to untreated DM controls.

Effect of NC on endogenous antioxidants in pancreatic islet cells

IF staining was used to determine whether the administration of NC would alter the immune-expression of CAT, SOD and GRED in the islets of Langerhans.

Effect of NC on CAT immuno-expression pancreatic in islet cells

After NC treatment, double labelled IF of the expression of CAT was performed in conjunction with insulin. The number of CAT-positive endocrine cells in the islet of Langerhans of DM rats increased significantly ($p < 0.05$) after NC treatment. INS does not co-localize with CAT in the

islets of either normal or diabetic rats. NC treatment did not modify the nature of co-localization of these two proteins.

Effect of NC on SOD immuno-expression pancreatic in islet cells

Superoxide dismutase (SOD) was observed in the central region of pancreatic islets of normal rats. These SOD-containing cells co-localizes with insulin (INS). The number of INS and SOD-immunoreactive cells decreased significantly after the onset of DM. However, the percentage number of SOD increase substantially after treatment with NC.

Effect of NC on GRED immuno-expression pancreatic in islet cells

Glutathione reductase (GRED), another key antioxidant is located in cells located in the central part of the islet of Langerhans. In pancreatic islets of normal and DM rats, INS and GRED co-localize. NC treatment significantly increased the number of INS and GRED-immunoreactive cells in the endocrine pancreas.

DISCUSSION

General features of rats with experimental diabetes mellitus

Body weight: During the period of study that spans over 4 weeks, diabetic Wistar rats showed significant loss of body weight compared to non-diabetic normal control rats. The loss of weight is probably due to the destruction of pancreatic beta cells leading to low plasma insulin level. Since insulin is required for the uptake of glucose into hepatic, fat and skeletal muscle cells, the amount of glucose uptake into these cells will be very low in diabetic rats. The low glucose content in these cells would not be enough to drive the metabolism that is required by these energy-consuming cells [Shetty et al., 2009].

Blood glucose: The blood glucose level of diabetic rats increased significantly right from the onset of diabetes to the end of the experiment. This hyperglycemia is a hallmark of diabetes mellitus. The reason behind diabetes-induced hyperglycemia is the destruction of insulin-producing beta cells in the endocrine pancreas. The loss of insulin leads to impaired glucose uptake from blood circulation to peripheral cells such as skeletal muscle, hepatic as well as liver cells from the blood, leaving excess glucose in the blood. The mechanisms by which STZ destroys the beta cells of the pancreas has been previously described. STZ binds to GLUT2

transporter before entering beta cells. This signaling process is then followed by the destruction of pancreatic beta cell [Schnedl et al., 1994].

NC in the cells of the islet of Langerhans

NC and insulin-positive cells: Double labelling immunofluorescence study confirmed that NC co-localizes with insulin in the beta cells of the endocrine pancreas. It was noteworthy to observe that the number of NC-immunoreactive cells was significantly reduced after the onset of DM. This is a feature that is akin to beta cell. The fact that NC is found in the central region of the islet of Langerhans, diminished in number after the induction of DM, and the merging together of NC and insulin, shows that NC and insulin are co-localized and intimately located in pancreatic beta cells. Image analysis confirm that the number of NC-positive islet cells was significantly reduced in diabetic rats compared to controls. This observation corroborates the findings of Tariq et al. [2015].

NC and glucagon-positive cells: We showed that NC co-localized with insulin in the most of the pancreatic beta cells of the endocrine pancreas. Our other objective was to determine whether, this co-localization is also true for glucagon. Using, double-labeling IF technique, no co-localization was observed between NC and glucagon in pancreatic islet cells of Langerhans of either normal or diabetic Wistar rats. Most of the NC-positive endocrine cells were discerned in the core of the islets while glucagon-containing endocrine cells were located in the periphery of the islets. All of these observations indicates that NC do not physically reside with glucagon in a single cell [Al-Shamsi et al., 2006].

NC and somatostatin-positive cells*:* We also wanted to know whether NC co-localized with somatostatin, another major pancreatic hormone. Double-labelling IF showed that somatostatin-positive cells are located in the peripheral region of the pancreatic islets whole NC-positive cells were seen in the central portion of the endocrine pancreas of normal rat. Similarly to glucagon, this findings indicate that NC and somatostatin are not likely to reside in one and the same endocrine cell. We showed that NC does not co-localize with somatostatin in the islets of Langerhans, indicating that NC may not directly regulate the metabolism of somatostatin.

NC and PP-positive cells: In a similar trend with glucagon and somatostatin, double labeling IF showed that the location of NC- and PP-positive cells are different within the endocrine cells of

the islet of Langerhans. Our study clearly showed that NC does not co-localize with PP in the pancreatic endocrine cells of either normal or diabetic rats. This suggests that NC may not directly influence PP metabolism by „contact" within the same islet cell.

Transmission electron microscopy of NC in the endocrine pancreas

Since we have shown by IF that NC is present in pancreatic beta cells of both normal, non-diabetic rats, we were curious to determine the exact location of NC in these cells. Using immunoelectron microscopy we showed that NC is located on the secretory granules of beta cells of pancreatic islets. This observation regarding the presence of NC in secretory granules of pancreatic beta cells of the pancreas concurs with those of Tariq et al. [Tariq et al., 2015]. This observation suggests that NC and insulin are both processed by pancreatic beta cells and could possibly be released simultaneously from secretory granules into blood circulation. It is worth noting that NC is present in the plasma. This close relation between NC and insulin both structurally and ultrastructurally suggests that NC may likely be implicated in the regulation of insulin metabolism.

The role of NC on insulin and glucagon release

NC and insulin release: We showed that NC (10^{-6} -10^{-12} M) induced significant reduction in insulin release from pancreatic tissue fragment of DM rats. This reduction was most potent at 10^{-9} M of NC. It has been shown that some peptides, including galanin can inhibit insulin release rather than stimulating insulin secretion [Adeghate & Ponery, 2001]. To the best of our knowledge, this is the first reported study on the effect of NC on insulin secretion. However, there are reports on the effect of opioid receptors insulin release. Tudurí et al. [2016] reported that stimulation of opioid receptors inhibits insulin release.

NC and glucagon release: In contrast to inhibiting insulin release from pancreatic tissue fragments of non-diabetic rats, NC at different concentrations (10^{-6} -10^{-12} M) stimulated glucagon release from the pancreas of non-diabetic rats. NC did not cause significant increase or decrease in glucagon release from pancreatic tissue fragments of DM rats. These differences in the manner in which NC affects the release of insulin and glucagon may point to a compensatory mechanism within the endocrine pancreas for the maintenance of metabolic equilibrium.

Effect of NC on endogenous antioxidants in the renal cortex

NC and CAT in kidney*:* Our study showed that CAT is discerned in the renal cortex within the epithelium lining the proximal and distal, convoluted tubules in normal, non- diabetic rats. Our findings are in agreement with those of Johkura et al. [1998]. Johkura and co-workers showed, using immunohistochemistry, that developing kidneys contains CAT. We showed that the proximal (PCT) and distal (DCT) convoluted tubules of the kidney of normal and diabetic rats contain CAT. However, the degree of immune-expression of CAT was reduced in untreated diabetic kidney compared to untreated controls. Moreover, the intensity of the immune-expression of CAT was markedly reduced in NC-treated normal rat kidney cortex when compared to untreated normal controls. More studies are needed to be able to decipher the significance of these findings.

NC and SOD in kidney: Treatment with NC caused large and significant elevation of the immune-expression of SOD in the PCT and DCT of renal cortex. However, the immune-expression of SOD was markedly reduced in the renal cortex of DM rats compared to those treated with NC. The reason why the cortical content of SOD is reduced is not clear. The renal content SOD may have been depleted during the course of DM. It is however, well known that chronic DM leads to kidney failure, where most of the renal structures including the PCT and DCT have structural lesions [Singh et al., 2018].

NC and GRED in kidney: NC when given i.p. markedly increased the immune-expression of GRED in the renal cortices of normal and DM rats. This observation suggests that NC can indeed increase the tissue level of GRED both normal, non-diabetic and DM rats. It is difficult to compare our result to that of the literature since, to the best of our knowledge, this is the first reported NC-induced increase immune-expression of GRED in the renal cortex of normal and DM rats.

NC and endogenous antioxidants in the liver

NC and CAT in liver: CAT immune-expression was markedly reduced in the liver after the onset of DM. The plasma level of CAT was also lower in DM rats compared to control. Our results corroborate those reported in the literature, which indicate that DM causes reduction in the plasma level of CAT compared to control [Sindhu et al., 2004]. The administration of NC increased CAT immune-expression in the liver of both normal and DM rats. The author do not

have knowledge of other studies reported on the effect of NC on CAT immune- expression in hepatocytes.

NC and SOD in liver: The results demonstrated that immune-expression of SOD is significantly increased in liver cells of normal, non-diabetic and DM rats after NC treatment. The SOD-containing hepatocytes were observed around the central veins of the liver. We do not know why SOD-positive hepatocytes congregate around the central veins of the liver. It is probable that blood around these parts of the liver contain more free radicals because they actually receive more blood circulation compared to areas distant to them.

NC and GRED in liver: The results demonstrated the presence of GRED in hepatocytes residing around the central veins of the liver. Treatment with NC significantly increased the immune-expression of GRED in hepatocytes located in the immediate vicinity of hepatic central veins. This observation was most visible in DM rats. All of these findings indicate that endogenous antioxidants, such as SOD, CAT and GRED are abundant in parenchymal cells residing around hepatic central veins. This observation suggests that endogenous antioxidants are required in this part of the liver.

NC and endogenous antioxidants in the cerebral cortex

NC and CAT in cerebral cortex: Treatment of normal and DM rats with NC caused significant increase in immuno- expression of CAT in cortical neurons of the brain when compared to untreated controls. The NC-induced increase in the immune-expression of NC in the neurons of the cerebral cortex is most pronounced in DM rats. The reason for these findings is not clear, however, the brain level of CAT in DM rats when compared with of normal non-diabetic controls, leading to an urge for the brain to compensate for this abnormally low tissue level of CAT. Our results corroborate that of a recent report in which a herbal medicine was used to stimulate the level of CAT in the cerebral cortex of DM rats [Liu et al.,2016].

NC and SOD in cerebral cortex: The presence of SOD in cerebral neurons is a logical response to the prominent occurrence of oxidative stress in the neurons of the cerebral cortex. SOD is needed to neutralize toxic effects of reactive oxygen species generated during cellular metabolism in neurons. DM caused a significant reduction in the immune-expression of SOD compared to non-diabetic control. However, NC treatment caused large and significant increases

in the immune-expression of SOD in the cerebral neurons of DM rats, compared to untreated DM control. This indicates that NC can indeed stimulate increases in tissue level of SOD.

NC and GRED in cerebral cortex: DM caused significant depletion of GRED in cerebral neurons compared to non-diabetic controls. NC treatment caused significant increases in the immune-expression of GRED in cerebral cortical neurons of DM rats compared to untreated diabetic controls. A similar investigation demonstrated that melatonin increased the tissue concentration of GRED in the brain of DM rats [Amer, 2021]. These findings suggest that NC is a potent inducer of endogenous antioxidants.

NC and endogenous antioxidants in the endocrine pancreas

Effect of NC on insulin and CAT in islet cells: NC treatment reduced the number of CAT-positive cells in the islets of normal pancreas. In contrast, treatment of DM rats with NC significantly increased the number of insulin- and CAT-immunoreactive cells in the islets of Langerhans. To the best of our knowledge this is the first reported study on the effect of NC on the number of CAT-positive cells in the endocrine pancreas.

Effect of NC on INS and SOD in islet cells: Insulin- and SOD-positive cells are localized to the central portion of pancreatic islets of normal non-diabetic rats. The number of insulin- and SOD-containing cells diminished significantly after the induction of DM. Although NC treatment did not induce significant increase in the number of SOD-positive cells in the islet of normal rats, however, it caused large and marked increases in the number of INS- and SOD-containing cells in DM rats.

Effect of NC and GRED in islet cells: Insulin- and GRED-imunoreactive cells were observed in the core the islets of normal rats. In a similar manner to that of SOD, the number of INS- and GRED-immunopositive cell was significantly reduced after the onset of DM. NC treatment caused a large and marked increases in the number of INS- and GRED-immuno-positive cells in pancreatic islets of DM rats. All these shows the ability of NC to increase the number of islet cells that contains these important endogenous antioxidants.

CONCLUSIONS

1. The study showed that NC is present in the cells occupying a significant part of the core of the islets of Langerhans of non-diabetic rats.
2. The number of NC-immunoreactive cells is significantly reduced after the onset of DM.
3. Double labelling immunofluorescence showed that NC co-localizes with insulin in the endocrine cells of normal and diabetic rats.
4. NC does not co-localize with either glucagon, somatostatin, or PP cells in pancreatic islets.
5. Using transmission electron microscopy, we showed that NC is present in the secretory granules of insulin-secreting beta cells of the pancreas of normal and DM rats.
6. NC markedly inhibit insulin secretion from pancreatic tissue fragments of normal and DM rats. In contrast, NC stimulates insulin secretion from isolated islets of DM rats.
7. All of these observations show that NC is indeed present in insulin-producing pancreatic beta cell and takes part in insulin secretion.
8. Intraperitoneal treatment of normal and diabetic rats with NC significantly increased the immune-expression of SOD and GRED in the pancreas, kidney, liver, and neurons of the cerebral cortex and hippocampus.
9. This NC-induced induction of endogenous antioxidants was especially prominent in DM rats.
10. Therefore, NC may be exerting its physiological and neuroprotective effects by enhancing the expression of endogenous antioxidants in different cell types including neurons.

A. Publications related to the PhD thesis

Full Length Articles

[1] **Adeghate E**, Saeed Z, D'Souza C, Tariq S, Kalász H, Tekes K, Adeghate EA. Effect of nociceptin on insulin release in normal and diabetic rat pancreas. Cell and Tissue Res. 2018; 374: 517-529. [Impact factor: 3.360]

[2] **Adeghate E**, D'Souza CM, Saeed Z, Al Jaberi S, Tariq S, Kalász H, Tekes K, Adeghate EA. Nociceptin Increases Antioxidant Expression in the Kidney, Liver and Brain of Diabetic Rats. Biology (Basel) 2021; 10:621. [Impact factor: 5.168]

Abstracts

[1] **Adeghate E**, Tekes K. Distribution of nociceptin-immunoreactive nerves in the dorsal root ganglion of GK rats. The FASEB J 2016; 30(Suppl. 1): lb35- lb35

[2] Tekes K, **Adeghate E Jr.,** Tariq S, Gáspár R, Adeghate E, Kalász H. Anatomical evidence on the role of nociceptin in experimental diabetes mellitus. Symposium on Recent Developments in Diabetes Mellitus and its Complications. Page 12, 2016.

B. Other publications

Full Length Articles

[1] Kalász H, Ojha S, Tekes K, Szőke E, Mohanraj R, Fahim M, **Adeghate E** and Adem A. Pharmacognostical sources of popular medicine to treat Alzheimer's disease. The Open Med Chem J 12: 23-35, 2018 [Impact factor: 0]

[2] **Adeghate E**, Mohsin S, Adi F, Ahmed F, Yahya A, Kalász H, Tekes K, Adeghate EA. An update of SGLT1 and SGLT2 inhibitors in early phase diabetes-type 2 clinical trials. Expert Opin Investig Drugs 2019; 28:811-820 [Impact factor: 5.081]

C. Scientific Conferences Attended

International Conferences

[1] 47th Annual Meeting of Hungarian Medical Association of America, Sarasota, Florida, USA, October 24 - 31, 2015. Poster Presentation

[2] Experimental Biology, San Diego, CA, USA, April 1-6, 2016, Poster Presentation