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Original articles

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CHRONIC OREXIN-A (HYPOCRETIN-1) TREATMENT OF TYPE 2 DIABETIC RATS IMPROVES GLUCOSE CONTROL AND BETA-CELL FUNCTIONS

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Orexin regulates food intake and energy expenditure. Here, we test the ability of orexin-A (OXA, hypocretin-1) at improving metabolic control in type 2 diabetic animals and elaborate potential mechanisms of action. Rats with experimentally induced type 2 diabetes by a combination of streptozotocin injection and high-fat diet feeding were chronically infused with OXA. *In vitro* experiments were conducted on isolated pancreatic islets, primary adipocytes and insulin secreting INS-1E cells. OXA improved glucose control, enhanced insulin sensitivity and attenuated pancreatic β-cell loss in type 2 diabetic rats. *Ex vivo*, apoptotic death of pancreatic islets isolated from OXA-treated type 2 diabetic animals as well as the impairment of glucose-stimulated insulin secretion were attenuated, as compared to islets derived from vehicle-treated rats. OXA reduced plasma tumor necrosis factor-α (TNF-α) and non-esterified fatty acids (NEFA) levels in type 2 diabetic rats. OXA decreased palmitate- and TNF-α-induced apoptosis of INS-1E cells. OXA improves glucose control by enhancing insulin sensitivity and protecting β-cells from apoptotic cell death in type 2 diabetic animals.

Key words: *orexin, diabetes, β-cells, proliferation, apoptosis, islets, insulin sentistivity, proinflammatory cytokines, glukagon-like peptide*

INTRODUCTION

Orexin is a circulating neuropeptide and neurotransmitter, originally isolated from hypothalamic extracts (1). *Preproorexin mRNA* as well as OXA peptide were also detected in the peripheral tissues (2). Furthermore, OXA is present in the circulation in humans and rodents (3). Two major isoforms of orexin exist: OXA (hypocretin-1) and orexin-B (OXB) (hypocretin-2) (1). OXA and OXB interact with their receptors termed as OXR_1 and OXR_2 (1). Human and animal studies indicated a role of orexin in controlling food intake, body weight and energy expenditure (1, 4-6). Stimulation of food intake by hypothalamic orexin is partially mediated by its interaction with cannabidoid system (7). Low expression or lack of orexin in the body is found in patients with narcolepsy (8, 9). These patients are frequently obese. Furthermore, BMI-matched narcoleptic patients have a higher incidence of type 2 diabetes (12.5% versus 5%) as compared to non-narcoleptic healthy individuals (9, 10).

OXA interacts with pancreatic α- and β-cells, thereby modulating glucose homeostasis (11-15). In non-diabetic rats, OXA acutely inhibited insulin secretion and stimulated glucagon secretion (14). In contrast, we and others found that OXA increased insulin secretion *in vivo* and *in vitro*, and inhibited glucagon secretion in non-diabetic rodents (11-13, 16, 17). Others reported that OXA acutely reduces glycemia, without affecting insulin secretion (18). In non-diabetic and streptozotocin (STZ)-induced type 1 diabetic mice, OXA acutely lowered blood glucose concentrations without any changes in insulin levels (19). Additionally, orexin appears to regulate insulin sensitivity. Overproduction of orexin from an ectopically expressed transgene (*CAG/orexin* Tg) not only rendered animals resistant to diet-induced obesity, but also improved glucose control and attenuated hyperinsulinemia (6). Concordantly, deletion of *orexin* led to a late-onset of insulin resistance (20). Others reported that OXA-deficient mice with STZ-diabetes had improved glucose control (15), questioning the antihyperglycaemic activity of OXA. Thus, the effects of OXA on glucose control in various animal models are inconsistent. Chronic studies evaluating long-term effects of exogenous OXA in type 2 diabetes are not yet available. Since OXA has a short half-life (30.7 min) (21), we studied the effects of continuously infused OXA for 4 weeks in animals with experimentally-induced type 2 diabetes (22) on glucose control, insulin sensitivity and pancreatic islet morphology, and evaluated potential mechanisms of action.

MATERIALS AND METHODS

Reagents

Cell culture media and supplements were from GIBCO Invitrogen (Karlsruhe, Germany) or Sigma-Aldrich (Deisenhofen, Germany). Cleaved caspase 3, rabbit IgG HRP-linked, mouse IgG

HRP-linked antibodies were from Cell Signalling Technology (Danvers, MA, USA). Insulin and glucagon antibodies were from Dako (Glostrup, Denmark) and the β-actin antibody was from Sigma-Aldrich. Palmitic acid (PA) solution was prepared, as described (23). Adiponectin ELISA kit was purchased from MBL International (Woburn, USA), FGF-21 ELISA kit was from Biovendor Inc. (Brno, Czech Republic), total GLP-1 ELISA kit from DRG (New Jersey, USA), leptin, insulin and glucagon RIA kits from Millipore Corporation (Billerica, USA), OXA RIA kit from Phoenix Pharmaceuticals, Inc., TNF-α ELISA kit from R & D Systems, Inc. (Minneapolis, USA), DNA fragmentation ELISA kit from Roche Diagnostics (Penzberg Germany).

Animal experiments

Adult male Wistar rats (200 ± 30 g, 10 weeks of age) were fed high-fat diet (HFD) (60% kcal from fat, diet No. 2127, Kaiseraugst, Switzerland) for 5 weeks, to induce obesity. Control rats were fed a standard chow diet (Labofeed B; Kcynia, Poland). All animals were held in a 12:12 hour light:dark cycle with free access to food and water. Alzet osmotic pumps (model 2ML4; Durect Co., Cupertino, CA) pre-filled with 0.134 mg OXA in 2 ml 0.9% NaCl (Bachem AG, Bubendorf, Switzerland) or vehicle (0.9% NaCl) were placed subcutaneously under general anesthesia at the end of the fifth week of feeding. On the same day HFD-fed animals were injected either with saline (DIO group) or STZ (35 mg/kg body weight), to induce type 2 diabetes, according to the protocol reported by professor Reaven (22, 24). STZ was dissolved in citric buffer and administrated intraperitoneally. Animals were fed HFD for additional 4 weeks. Control lean groups were treated in the same way, except they received chow, instead of HFD. Physiological plasma concentration of OXA in rats is approximately 50 pg/ml (25) which corresponds to 14 pmol/l. In our study, osmotic pumps were filled with 18.81 μM OXA and OXA was continuously infused intraperitoneally (i.p.) at a rate of 47 pmol/h for 4 weeks. The volume hourly released from pumps was 2.5 μl. We calculated that this should yield 0.75 nmol/l OXA concentration in plasma after one hour, which is a pharmacological dose. Note that the total blood volume accounts for approx. 6% of the total body weight in rats and OXA has a half-life of approx. 30 min. OXA degradation at the end of the study was measured from the remaining OXA solution in the osmotic pumps by rat OXA ELISA assay, and compared to freshly prepared OXA. We estimated that OXA degradation was less than 10% after four weeks, comparing to freshly prepared OXA (data not shown). All animal protocols were approved by the Local Ethical Commission for Investigation on Animals, Poznan University of Life Sciences. Principles of laboratory animal care (NIH publication no. 85 – 23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) were followed, as well as specific national laws.

Detection of hormones and cytokines

Blood was drawn from jugular veins of overnight fasted animals in pre-chilled tubes pre-filled with 1 mg/ml EDTA and 500 kIU/ml aprotinin. Plasma was recovered and stored at –80°C.

Intraperitoneal glucose tolerance test (IPGTT)

Animals were fasted for 6 hours (fasting was started at 7 a.m.) and were injected with glucose (1 g/kg body weight, i.p.). Glucose concentration was measured from blood taken from tail veins using a glucometer AccuCheck Active (Roche Diagnostics, Mannheim, Germany).

Determination of surrogate indexes for insulin sensitivity and resistance

Homeostasis model assessment of insulin resistance (HOMA-IR) in diabetic rats, in particularly those with STZ/HFD-induced diabetes was published by others (26-28) and calculated according to the formula: [fasting glucose level (mmol/l) × fasting insulin level (μU/ml)]/22.4. Insulin sensitivity was measured in rats injected i.p. with human recombinant insulin (1.25 IU/kg Humulin R; Eli Lilly, Indianapolis, IN, USA), as described (29). The rate constant for plasma glucose disappearance (K_{ITT}) (insulin sensitivity index) was calculated using the formula: [0.693/biological half - life ($t_{1/2}$)] × 100. Plasma glucose half-life ($t_{1/2}$) was calculated from the slope of the plasma glucose concentration during 30 minutes after insulin injection, using the least square fit when the glycemia declined linearly (30). Quantitative insulin-sensitivity check index (QUICKI) was determined according to the formula: 1/((log(fasting insulin μU/mL) + log(fasting glucose mg/dL)) (31, 32).

Isolation of rat white adipocytes

Primary adipocytes were isolated from epididymal fat pads of male adult Wistar rats (33). In brief, collected adipose tissue was purified from blood vessels, dried and minced with scissors. Thereafter, tissue was digested in Krebs-Ringer HEPES buffer supplemented in collagenase type II (3 mg/ml), glucose (5 mmol/l) for 45 min at 37°C in a shaking water-bath. After digestion cells were filtered through a nylon mesh (250 μm), washed with KRBH and counted using a Burker-Turk counter chamber.

Incorporation of glucose into triglycerides

To measure *ex vivo* incorporation of [U-^{14}C]-glucose into triglycerides isolated primary adipocytes (10^6 cells/ml) were incubated in KRBH (3 mmol/l glucose, 3% w/vol. NEFA-free BSA) with 100 nmol/l OXA +/– 10 nmol/l insulin or vehicle and with 0.5 μCi [U-^{14}C]-glucose for 2 hours at 37°C, as described (25, 34, 35).

Isolation of pancreatic islets and determination of apoptosis markers and insulin RNA content

Pancreatic islets were isolated (11) from randomly selected rats within each experimental group (n = 3), followed by RNA/protein isolation and Western blotting. Relative expression of insulin mRNA in islets was assayed by RT-PCR.

Caspase activity assay

Isolated rat pancreatic islets were lysed in 1X lysis buffer at a ratio of 50 μl per 25 size-matched islets followed by measurement of the total protein concentration using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Ten μg of each lysate was tested using the Caspase 3 Colorimetric Assay Kit (Sigma-Aldrich), with or without caspase 3 inhibitor, in a total reaction volume of 100 μl. The substrate (Ac-DEVD-pNA) concentration was 200 μmol/l, and the inhibitor (Ac-DEVD-CHO) concentration was 0.05 μmol/l. The assay was performed at pH 7.4 at 37°C for 120 min. The results were calculated using a p-nitroaniline calibration curve and expressed as μmol of pNA released per min per μg of proteins in pancreatic islets lysate.

Static incubation of isolated pancreatic islets

To assess glucose-stimulated insulin secretion (GSIS), approx. 50 islets for each experimental group were purified individually by hand picking under stereomicroscope. After washing in PBS the

islets were seeded in 24-wells plates (5 islets/well) and incubated for 2 hours with basal (6.66 mmol/l) or high (24 mmol/l) glucose. Insulin concentration in medium was measured by ELISA.

Cultivation of insulinoma cells

INS-1E is a clonal rat insulin-secreting cell line, derived from INS-1 cells, in which insulinoma was induced by radiation (36). Merglen *et al*. isolated INS-1E clone (37), which is considered as a stable and valuable β-cell model. INS-1E cells were cultured in RPMI-1640 containing 11 mmol/l glucose, 10% FCS, 10 mmol/l HEPES, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 0.05 mmol/l 2-mercaptoethanol, 100 kU/l penicillin, 100 mg/l streptomycin. Cells were maintained at 37°C in a humidified atmosphere (5% CO_2, 95% air).

Immunohistochemistry

The distal 20% of pancreata were fixed in Bouin solution (Sigma, St. Louis, MO) for 24 hours, embedded in paraffin and sectioned with a microtome into 5 – 6 µm-thick specimens. The sections were deparaffinized and incubated in 3% H_2O_2 for 30 min at RT to block endogenous peroxidase activity. After PBS wash, sections were pre-incubated with normal goat serum for 30 min and then incubated with guinea pig anti-insulin polyclonal antibody. After washing in PBS, sections were incubated with secondary antibody and peroxidase (DAKO LSAB 2 System™ HRP). Peroxidase activity was detected using the 3, 3'-diaminobezidine technique (DAKO Liquid DAB Substrate-Chromogen System). Negative controls were carried out by similarly treating adjacent sections and omitting the primary antibody, as well as using primary antibody pre-absorbed with antigen excess.

Morphometric analyses of insulin-immunoreactive cells were performed using Image-J analysis software and particle size analyzer macro (Scion, Frederick, MD). The area of insulin-immunostaining in three sections of pancreas from 4 animals relative to total sectional area examined was quantified by monochromatic threshold.

Real-time PCR

Total RNA extraction and real-time procedures were performed as described (25). Briefly, for cDNA synthesis 1 µg of total RNA and Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) was used according to manufacturer protocol. PCR consisted of an initial predenaturation step at 95°C for 10 min, followed by 35 – 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 30 s and extension at 72°C for 8 s. Standard curves were established for each pair of primers. Additionally, the products were also visualized on 4% agarose gel. The results are shown as relative expression of target gene relative to *Gapdh*. The following sequences of primers were used:
Gapdh (forward: 5'-atggtgaaggtcggtgtga-3',
reverse: 5'-aatctccactttgccactgc-3' and Universal ProbeLibrary probe: #84),
Tnf-α (forward: 5'-tgaacttcggggtgatcg-3',
reverse: 5'-gggcttgtcactcgagtttt-3' and Universal ProbeLibrary probe: #63),
Insulin (forward: 5'-agaccatcagcaagcaggtc-3',
reverse: 5'- cttgggctcccagaggac-3' and Universal ProbeLibrary probe: #73).

Protein isolation and Western blot

Cells were cultured in 6-well plates and serum-deprived for 24 hours. Protein isolation and Western blots were performed as described (25).

Determination of pancreatic insulin content

Whole pancreata were dissected in 0.1 mol/l PBS and weighed. Thereafter, 1/4 of each pancreas (the same duodenal part of the gland) was homogenized using a hand-held homogenizer in 0.14 N HCl/95% ethanol. Aliquots were used for determination of insulin and total protein content (BCA protein assay, Pierce Biotechnology, Rockford, IL).

Cell death assay

INS-1E cells were cultured in a 24-well plate (1×10^5 cells/well) in a RPMI1640 + 10% FCS for 24 hours. Cells were switched to a serum-free medium for additional 24 hours incubation. Thereafter, cells were treated with 0.2 mmol/l palmitic acid (PA) +/– 100 nmol/l OXA or 40 ng/ml TNF-α +/– 100 nmol/l OXA for 24 hours. The choice of OXA dose (100 nmol/l) was based upon previous publications, showing that this dose can effectively affect the functions of adipocytes or pancreatic islets functions *in vitro* (12, 25). In a parallel study, isolated rat pancreatic islets (20 islets/well) were cultured in 6-well plates in RPMI medium with 0.4 mmol/l PA +/– 100 nmol/l OXA for 24 hours. The cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were quantified using Cell Death Detection ELISA.

Statistical analysis

Results are presented as mean ± S.E.M. The numbers of animals and the number of experiments are given in each figure. All collected samples were measured in duplicates. Statistical analysis was performed using Two Way ANOVA followed by the Bonferroni post hoc test. Statistical significance was considered when $P < 0.05$. The trapezoidal rule was used to determine the area under the curve (AUC).

RESULTS

Orexin-A improves glucose control in type 2 diabetic animals

Type 2 diabetic rats had increased fasting plasma insulin, glucagon, and blood glucose levels, as compared to non-diabetic lean animals (*Fig. 1A-1C*). OXA reduced hyperinsulinaemia, hyperglucagonaemia and restored normoglycaemia in type 2 diabetic rats after 4 weeks of treatment (*Fig. 1A-1C*). OXA improved glucose tolerance in type 2 diabetic animals as detected by IPGTT (*Fig. 1D-1G*).

OXA did not affect cumulative daily food intake (data not shown). Type 2 diabetic rats treated with OXA had slightly lower body weights as compared to vehicle treatment ($P < 0.05$) (*Fig. 1H*).

Orexin-A increases insulin sensitivity

HOMA-IR increased from 1.55 (lean controls) to 6.6 in HFD/STZ-diabetic rats, whereas OXA attenuated HOMA-IR in type 2 diabetic rats (1.71) (*Fig. 2A*). Insulin sensitivity index (K_{ITT}) measured by the short insulin tolerance test markedly decreased in HFD/STZ-diabetic rats, as compared to controls ($P < 0.001$) (*Fig. 2B*). OXA increased K_{ITT} in HFD/STZ-diabetic rats. Concordantly, another insulin sensitivity index QUICKI decreased in animals with HFD/STZ-diabetes, whereas OXA potently increased QUICKI in diabetic rats (*Fig. 2C*).

The impact of OXA on insulin sensitivity was measured by insulin-stimulated incorporation of [U-^{14}C]-glucose into triglycerides in isolated adipocytes, *ex vivo* (*Fig. 2D*). Insulin-

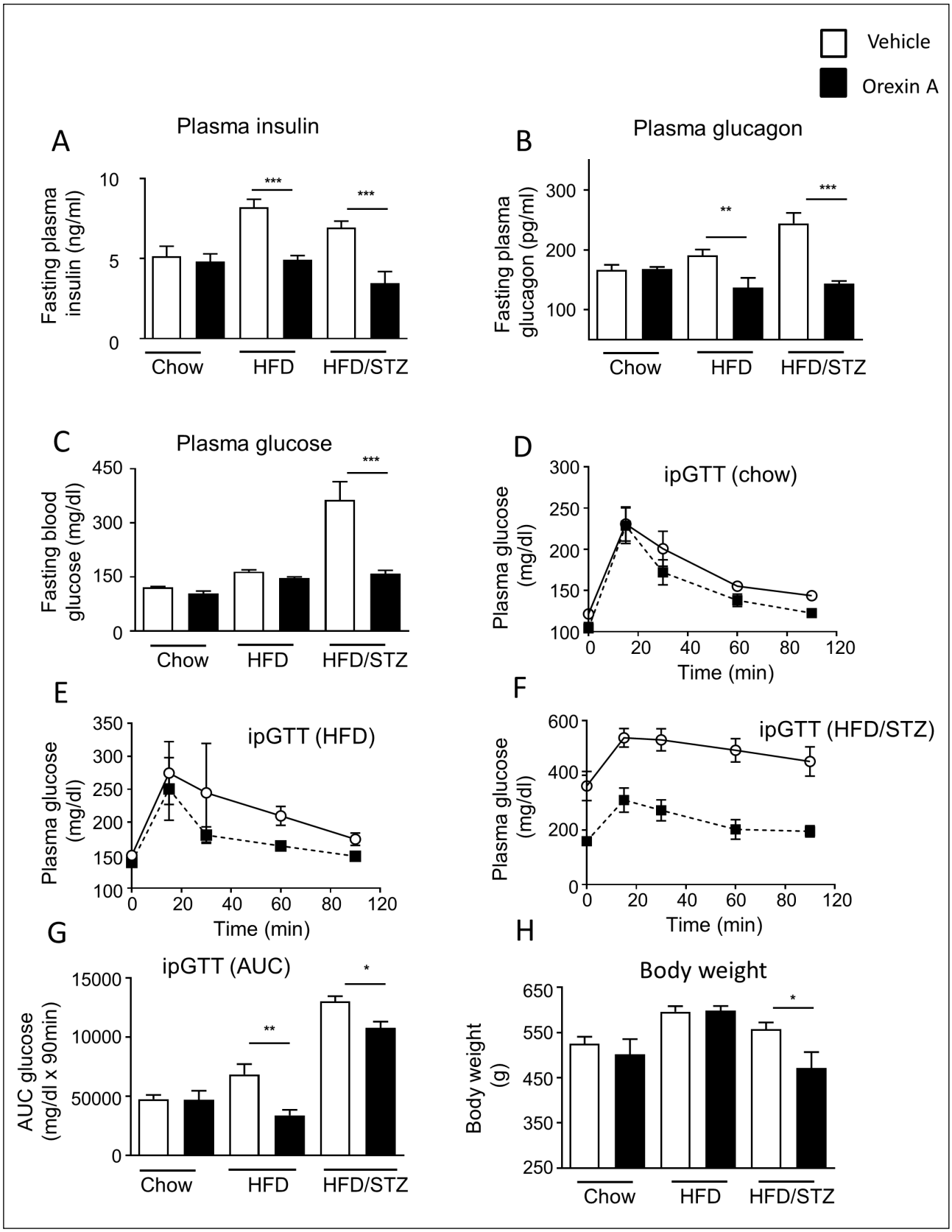


Fig. 1. Effects OXA on parameters of glucose homeostasis. Fasting plasma insulin (*A*), glucagon (*B*) and blood glucose (*C*) levels in non-diabetic chow-fed lean rats and in rats with DIO or HFD/STZ-induced type 2 diabetes after 4 weeks of treatment with vehicle (white bars) or OXA (black bars). Glucose excursions in IPGTT performed in non-diabetic controls (*D*), rats with DIO (*E*) and in type 2 diabetic rats (*F*) after 4 weeks of treatment with vehicle (open circles) or OXA (filled squares). Calculated glucose AUC-90 in all three animal groups (*G*). Determination food body weight at the end of the study (*H*). * denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in comparison with the indicated controls using two-way ANOVA followed by Bonferroni post hoc test. Results are shown as mean ± S.E.M. (n = 7).

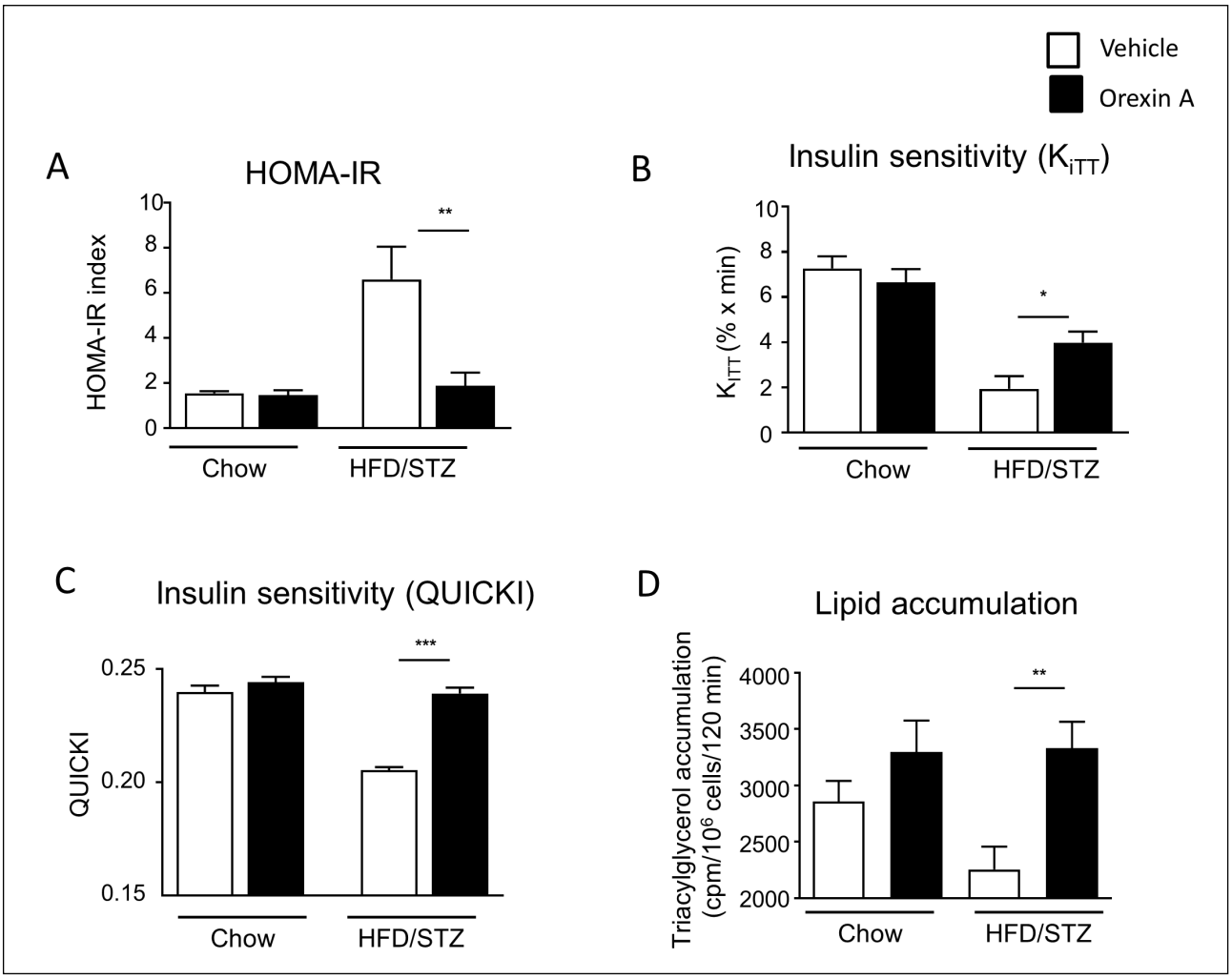


Fig. 2. Effects of OXA on insulin sensitivity index and insulin-stimulated glucose uptake in isolated adipocytes. HOMA-IR index in control in chow-fed, non-diabetic and HFD/STZ-(type 2) diabetic rats after 4 weeks of treatment either with vehicle or OXA (*A*). Insulin sensitivity index (K_{ITT}) determined on the basis of short insulin-tolerance test (ITT) at the end of the study (*B*). Determination of the quantitative insulin-sensitivity check index (QUICKI), calculated as the inverse log sum of fasting insulin (μU/ml) and fasting glucose mgl/dl (*C*). Insulin-stimulated incorporation of [U-^{14}C] glucose into triglycerides detected in isolated primary adipocytes derived from non-diabetic control rats and HFD/STZ-diabetic animals treated for 4 weeks either with vehicle or OXA (*D*). White bars: vehicle treatment, black bars: OXA treatment. * denotes $P < 0.05$; ** $P < 0.01$; ***$P < 0.001$ versus HFD/STZ animals or versus non-diabetic lean control chow-fed rats using two-way ANOVA followed by Bonferroni post hoc test. Results are shown as mean ± S.E.M. (n = 6).

stimulated incorporation of [U-^{14}C]-glucose into triglycerides in adipocytes derived from STZ/HFD-diabetic animals was reduced, as compared to adipocytes derived from chow-fed animals. Insulin potently enhanced [U-^{14}C]-glucose incorporation into triglycerides in adipocytes isolated from OXA-treated type 2 diabetic rats, as compared to adipocytes derived from vehicle-treated diabetic animals.

It is known that pro- and antiinflammatory cytokines and hormones can modulate insulin sensitivity. OXA-treated diabetic rats had lower plasma levels of visfatin, resistin, and leptin, whereas adiponectin, FGF-21 and GLP-1 were higher as compared to vehicle-treated diabetic rats (*Fig. 3A-3F*).

Orexin-A attenuates the loss of pancreatic β-cell mass in type 2 diabetes and improves glucose-stimulated insulin secretion

Type 2 diabetic rats experienced β-cells loss (*Fig. 4A-4D*) and had decreased pancreatic insulin content (*Fig. 4E*), as well as insulin mRNA expression (*Fig. 4F*). OXA treatment partially attenuated β-cell loss and attenuated the decreases of pancreatic insulin content, and insulin mRNA in type 2 diabetic rats. Islets isolated from type 2 diabetic rats had increased apoptosis, as detected by the high abundance of activated (cleaved) CSP3 (*Fig. 4G*), as well as increased CSP3 activity (*Fig. 4H*), in comparison to islets derived from non-diabetic rats. The activation of CSP3 was attenuated in diabetic rats treated with OXA. Pancreatic islets isolated from vehicle-treated type 2 diabetic animals showed impaired insulin release at both basal (6.66 mmol/l) (*Fig. 4I*) as well as high (24 mmol/l) glucose *(Fig. 4J)*. In contrast, chronic OXA treatment of type 2 diabetic rats improved insulin secretion at both glucose concentrations. These findings suggest that OXA attenuates β-cell loss and improves GSIS in type 2 diabetic animals.

Furthermore, we found that type 2 diabetic rats had increased α-cell area and glucagon content which was attenuated by OXA treatment *(Fig. 5A-5E).* OXA protects pancreatic β-cells from

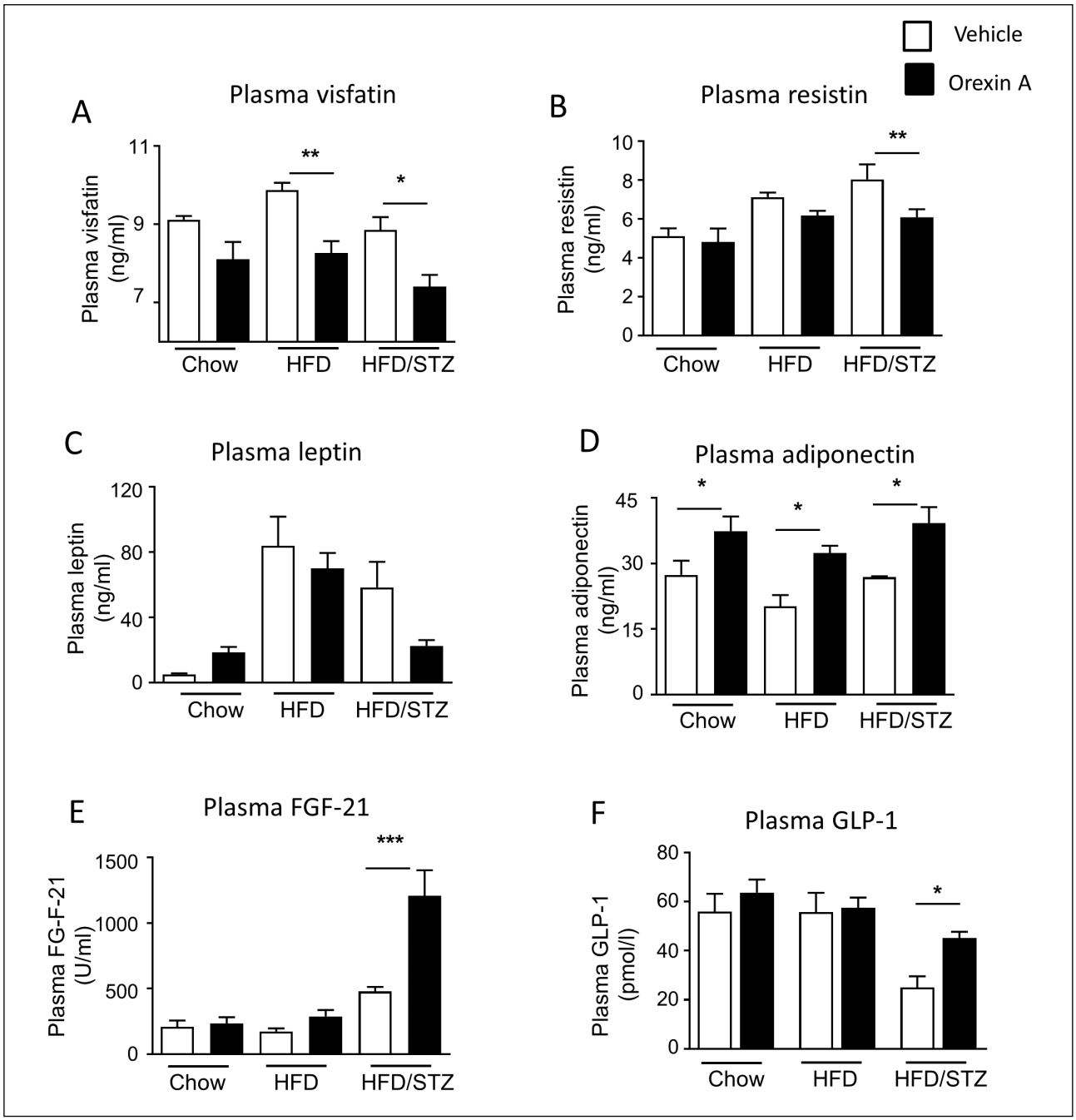
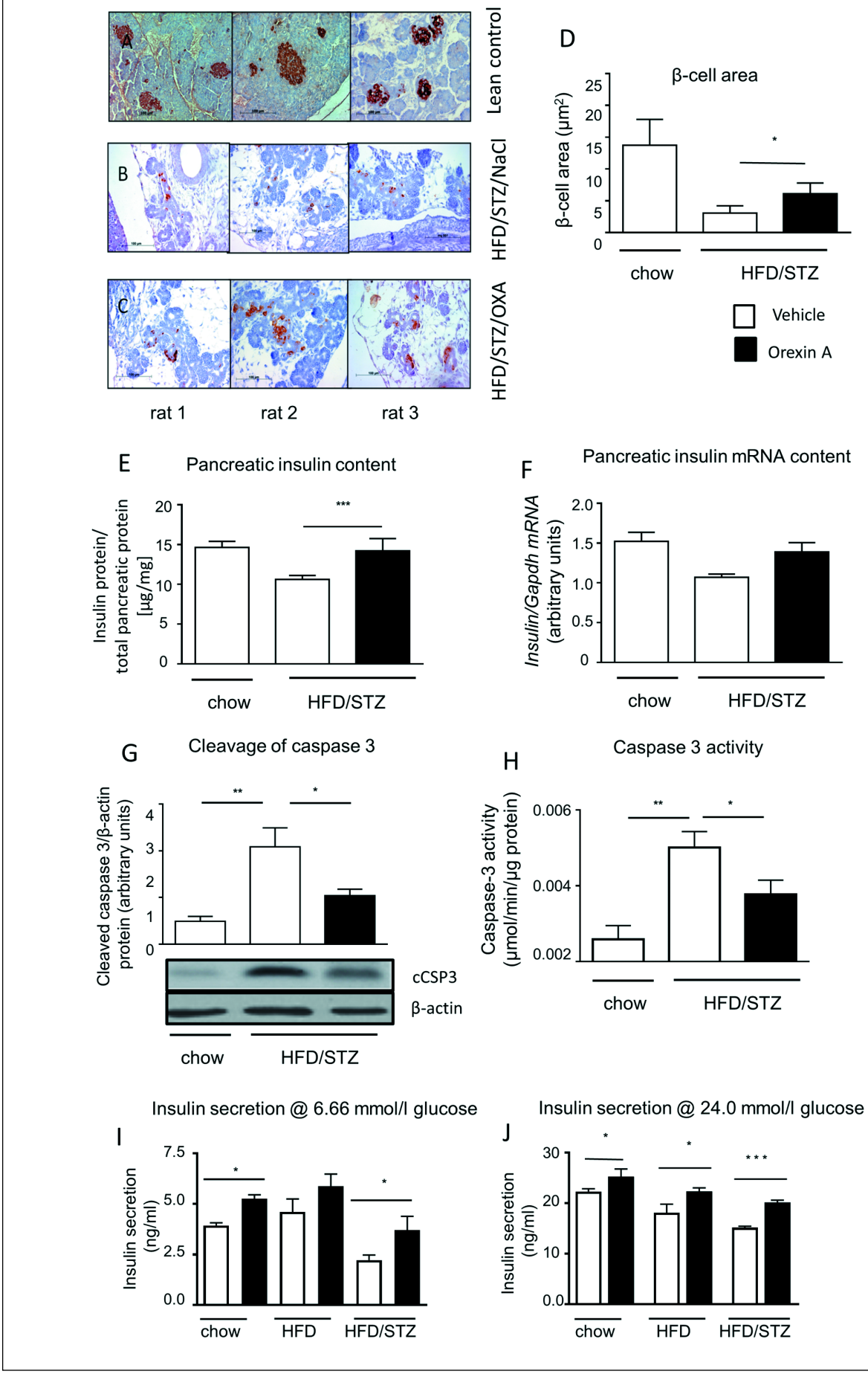


Fig. 3. Changes of plasma levels of pro- and antiinflammatory cytokines in OXA-treated type 2 diabetic, control rats (chow) and in rats with DIO (HFD). Plasma concentrations visfatin (*A*), resistin (*B*), leptin (*C*), adiponectin (*D*), FGF-21 (*E*) and GLP-1 (*F*) detected in animals without or with DIO or T2DM treated for 4 weeks either with OXA or NaCl. The results are derived from n = 6 animals per group. * denotes $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, †$P < 0.05$ versus healthy controls, using two-way ANOVA.

Fig. 4. Alterations of the endocrine pancreatic β-cells morphology and function in rats with experimentally-induced type 2 diabetes chronically treated with OXA. Morphometric analysis for insulin-immunoreactivity of pancreatic sections in lean control rats (upper panel) and rats with HFD/STZ-diabetes, treated continuously for 4 weeks either with vehicle (middle panel) or OXA (lower panel) derived from n = 3 different rats (*A*-*C*). Quantification of the area of insulin-immunoreactivity (β-cell area) in three representative pancreatic sections (*D*). Results are derived from n = 5 animals per group and are expressed as β-cell area relative to the total sectional area (quantification by monochromatic threshold). Insulin content in pancreata isolated from chow-fed or STZ/HFD-diabetic rats treated either with vehicle (white bar) or OXA (black bar) (*E*). Analysis of insulin mRNA content in pancreatic islets isolated from rats with STZ/HFD-diabetes treated either with vehicle (white bars) or OXA (black bars) in comparison to chow-fed control animals (*F*). Determination of caspase 3 (CSP3) cleavage (*G*) and CSP3 activity (*H*) in pancreatic islets isolated from non-diabetic chow-fed rats and from rats with STZ/HFD-diabetes treated either with vehicle (white bars) or OXA (filled bars). *Ex vivo* determination of GSIS at low (6.0 mmol/l) (*I*) or high (24 mmol/l) glucose (*J*) in pancreatic islets isolated from rats with STZ/HFD-diabetes, DIO or non-diabetic lean control rats treated chronically either with vehicle (white bars) or OXA (black bars). * denotes $P < 0.05$, **$P < 0.01$, ***$P < 0.001$ in comparison with the indicated controls using two-way ANOVA followed by Bonferroni test. Results are shown as mean ± S.E.M. (n = 6).


A
B
C
Lean control
HFD/STZ/NaCl
HFD/STZ/OXA
rat 1
rat 2
rat 3
D
β-cell area
β-cell area (μm²)
25
20
15
10
5
0
*
chow
HFD/STZ
Vehicle
Orexin A
E
Pancreatic insulin content
Insulin protein/
total pancreatic protein
[μg/mg]
20
15
10
5
0
***
chow
HFD/STZ
F
Pancreatic insulin mRNA content
Insulin/Gapdh mRNA
(arbitrary units)
2.0
1.5
1.0
0.5
0
chow
HFD/STZ
G
Cleavage of caspase 3
Cleaved caspase 3/β-actin
protein (arbitrary units)
4
3
2
1
0
**
*
cCSP3
β-actin
chow
HFD/STZ
H
Caspase 3 activity
Caspase-3 activity
(μmol/min/μg protein)
0.006
0.004
0.002
**
*
chow
HFD/STZ
Insulin secretion @ 6.66 mmol/l glucose
I
Insulin secretion
(ng/ml)
7.5
5.0
2.5
0.0
*
*
chow
HFD
HFD/STZ
Insulin secretion @ 24.0 mmol/l glucose
J
Insulin secretion
(ng/ml)
30
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10
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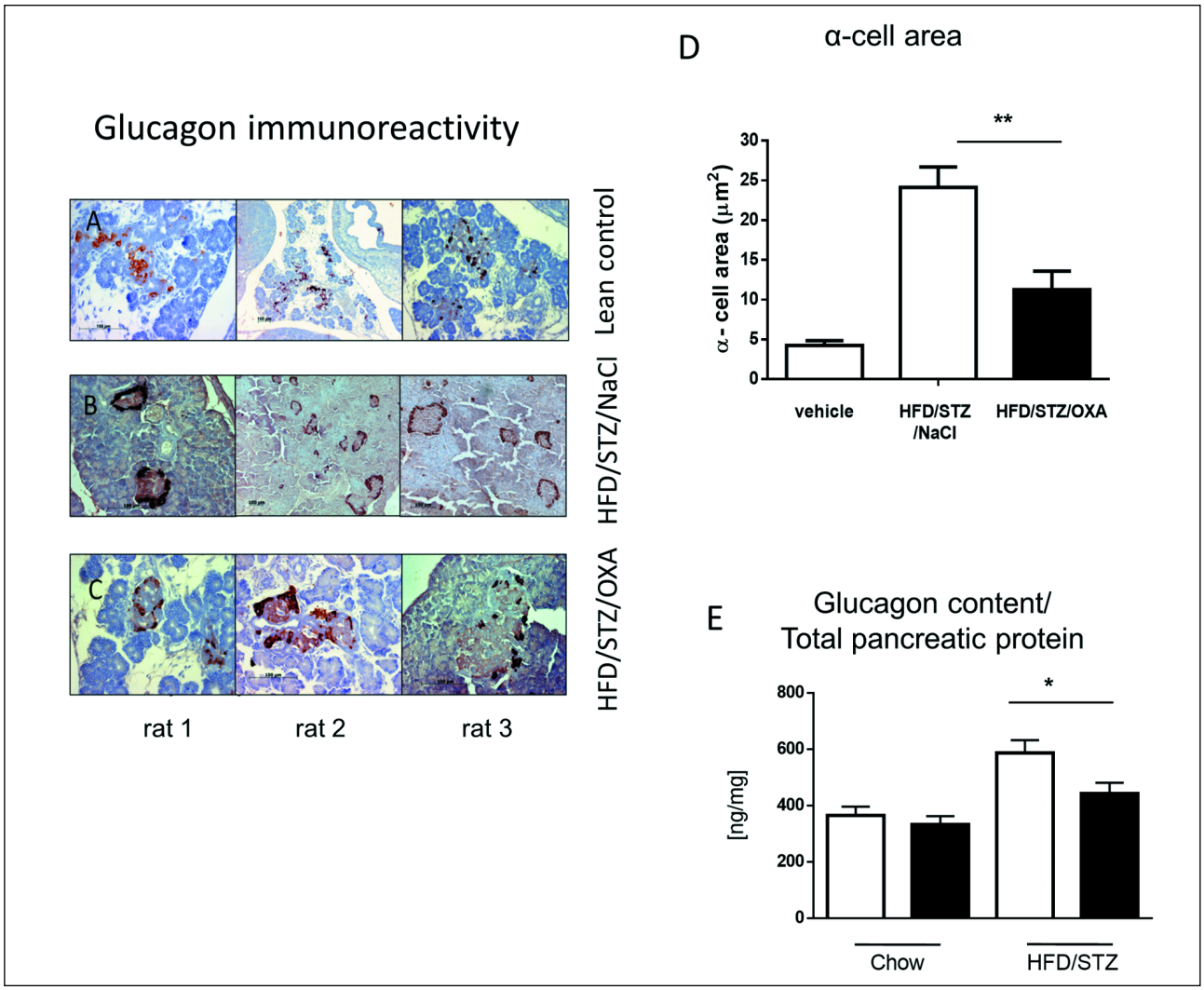

Fig. 5. Pancreatic glucagon-immunoreactivity and glucagon area in lean control, HFD/STZ/NaCl and HFD/STZ/OXA rats. Morphometric analysis for glucagon-immunoreactivity of pancreatic sections in lean control rats (upper panel) and rats with HFD/STZ-diabetes, treated continuously for 4 weeks either with vehicle (middle panel) or OXA (lower panel) derived from n = 3 different rats (*A-C*). Quantification of the area of glucagon-immunoreactivity (α-cell area) in three representative pancreatic sections (*D*). Glucagon content in lean control, HFD/STZ/NaCl and HFD/STZ/OXA rats (*E*). Results represent the mean obtained from n = 3 animals. * denotes P < 0.05, **P < 0.01 versus lean or HFD/STZ/OXA-treated rats using two-way ANOVA.

apoptotic death and enhances β-cell survival *in vitro*. OXA lowered plasma levels of NEFA and TNF-α in type 2 diabetic animals (*Fig. 6A-6B*). Since OXA attenuated β-cell loss and inhibited the activation of the crucial pro-apoptotic protease - CSP3 - in pancreatic islets in type 2 diabetic rats, we further evaluated the effects of OXA on apoptosis *in vitro*. Palmitate induced apoptotic death in pancreatic islets and in INS-1E cells (*Fig. 4C-4D*). OXA attenuated palmitate-induced apoptosis in isolated rat pancreatic islets (*Fig. 6C*) and INS-1E-cells (*Fig. 6D*).

To further elucidate intracellular signaling, through which OXA attenuates palmitate-induced apoptosis, we tested the expression the proapoptotic cytokine TNF-α in isolated rat pancreatic islets. Palmitate stimulated, whereas OXA inhibited the expression of TNF-α in islets (*Fig. 6E*). Next, we tested whether OXA is able to influence TNF-α-stimulated apoptosis of INS-1E-cells. TNF-α stimulated INS-1E cells apoptosis, which was attenuated by OXA (*Fig. 6F*). TNF-α is a well-known activator of CSP3 and therefore we tested the effects of OXA on TNF-α-stimulated activation of CSP3. OXA attenuated TNF-α-stimulated cleavage of CSP3 in INS-1E cells (*Fig. 6G*). Thus, OXA inhibits palmitate-/TNF-α-stimulated apoptosis of pancreatic β-cells.

DISCUSSION

We report here that chronic treatment with OXA improves glycemic control in type 2 diabetes. OXA reduces fasting glucose, circulating insulin and glucagon levels, and improves glucose clearance in type 2 diabetic rats. OXA upregulates the release of anti-inflammatory cytokines from isolated adipocytes and increases plasma levels of these cytokines in type 2 diabetic rats. OXA has opposite effects on pro-inflammatory cytokines. Furthermore, OXA increases insulin sensitivity *in vivo* and *ex vivo* in adipocytes in type 2 diabetes. OXA reduces apoptosis of pancreatic β-cells and attenuates the reduction of pancreatic insulin content in type 2 diabetes. OXA increases GSIS in pancreatic islets isolated from type 2 diabetic rats. Potential

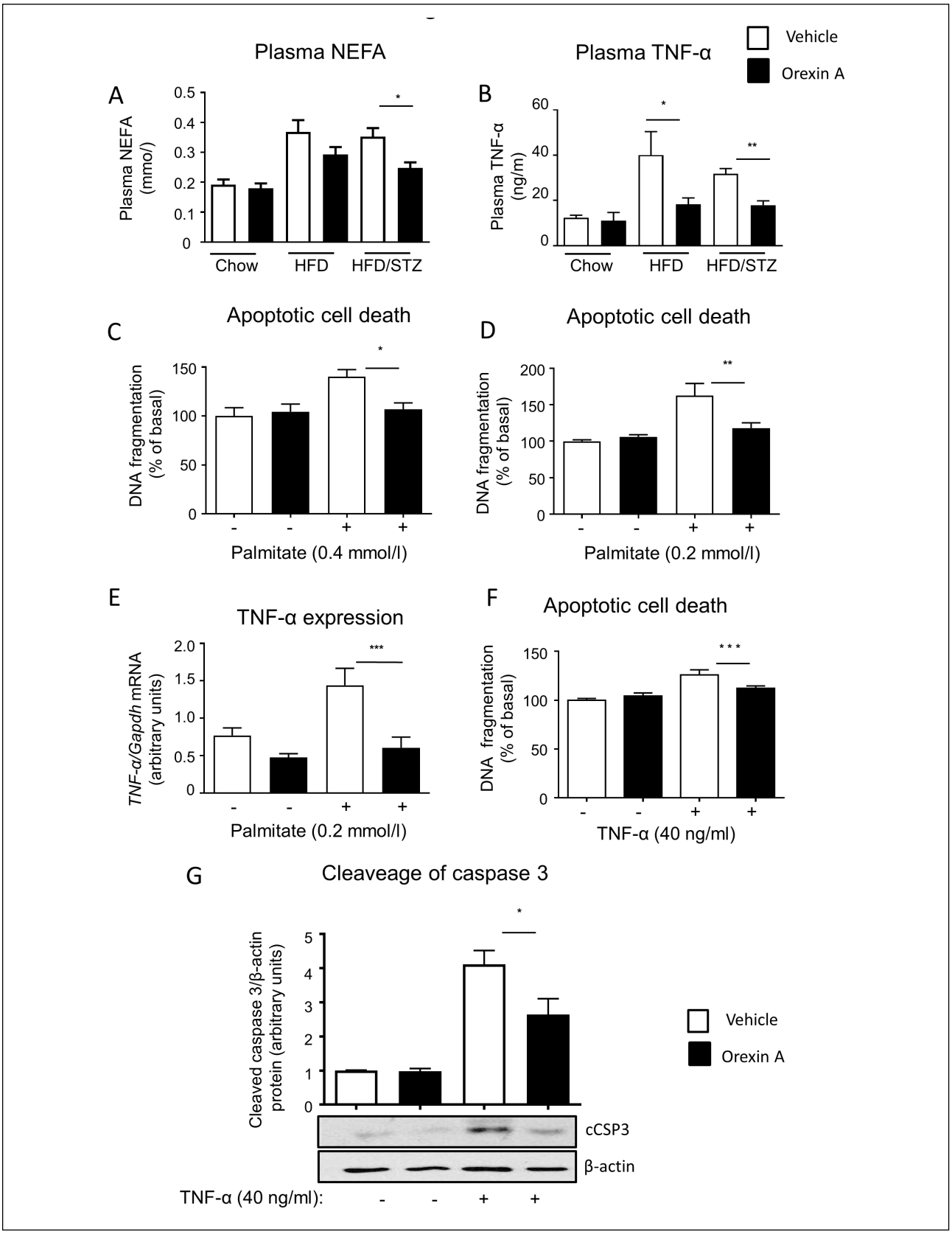

Fig. 6. Effects of OXA on plasma NEFA and TNF-α levels and on apoptosis of pancreatic β-cells and INS-1E cells. Effects of OXA on plasma NEFA (*A*) and TNF-α levels (*B*) in type 2 diabetic animals in comparison to DIO rats and control animals after 4 weeks of treatment. Protective activity of OXA against palmitate-induced apoptosis in isolated rat pancreatic islets (*C*) and INS-1E insulinoma cell line (*D*). Inhibition against basal or palmitate-stimulated *TNF-α* mRNA expression by OXA in isolated rat pancreatic islets (*E*). Inhibition of TNF-α-stimulated apoptosis by OXA in INS-1E cells (*F*). Effects of OXA on TNF-α-stimulated cleavage of CSP3 in INS-1E cells (*G*). Black bars: OXA treatment, white bars: vehicle treatment. * denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus corresponding groups using two-way ANOVA followed by Bonferroni test. Results are shown as mean ± S.E.M. (n = 6 or n = 4 (*Fig. 4G*)).

mechanisms of OXA's action include the inhibition of TNF-α- or palmitate-stimulated apoptosis.

The results of our study are in line with previous reports in animals with DIO or STZ-induced type 1 diabetes or in lean non-diabetic animals (6, 19, 20, 38, 39). However, other studies were performed either in acute settings or in animals with genetic alterations of either orexin or orexin receptors. Furthermore, to date metabolic effects of OXA were not evaluated in animal models of type 2 diabetes, showing alterations in both insulin resistance as well as β-cell dysfunction (22, 24).

In this non-genetic animal model of type 2 diabetes, low fasting insulin and glucose levels in response to OXA-treatment strongly suggest that OXA improves insulin sensitivity. Indeed, we detected concordantly an improvement of insulin sensitivity by OXA in these animals using three independent evaluation tests: ITT, QUICKI, as well as HOMA-IR index. K_{ITT} is considered to be a highly reproducible method, and the values obtained with this index correlate well with the euglycemic hyperinsulinemic clamp test (30). In rats and in humans, QUICKI is considered as a sensitivity index with the strongest correlation with insulin sensitivity, determined by the glucose clamp (26, 40).

To better understand the discrepancies between current findings and the results of acute studies performed earlier by others either using non-diabetic fasted animals or animals with STZ-diabetes (14, 16, 18, 19) it is necessary to emphasize that insulin levels in our animals were much higher. The compensatory increase of circulating insulin results most likely from markedly increased peripheral insulin resistance. However, this finding was surprising because type 2 diabetic animals had reduced β-cell area, as detected qualitatively by immunochemical analysis of representative pancreatic tissue sections. Concordantly, quantitative analysis showed that insulin protein content was reduced by approximately 28%, whereas insulin mRNA was reduced by approximately 33% in HFD/STZ type 2 diabetic animal group as compared to non-diabetic animals fed chow diet. Importantly, a recent work (41) revealed that one of the limitation of the discrepancies between functional and expressional analyses of beta cells may be due to increased insulin degranulation which occurs in diabetic individuals. Thus, it likely that that the loss of β-cells mass detected in type 2 diabetic rats was overestimated. Furthermore, a recent work performed in humans showed that fasting insulin levels do not show any correlation with the degree of pancreatic beta cell loss (42). Rather, a glucose challenge test, as performed in our *in vitro* experiments using isolated pancreatic islets, appears to show a better correlation with the β-cell loss.

OXA chronically infused to rats with HFD/STZ-diabetes improved glucose control not only simply by changing the ratio of insulin to glucagon in plasma, but also by improving peripheral insulin sensitivity and by protecting pancreatic β-cells from apoptotic cell death. These morphological alterations of the endocrine pancreas, accompanied by differential changes of pro- and anti-inflammatory cytokines (e.g. TNF-α) and changes in insulin resistance are strikingly different from the experimental settings described by others. Morphological alterations of pancreatic α- or β-cells in response to OXA investigated here clearly cannot be addressed by performing acute studies. Moreover, the animals studied in the past in the acute settings were fasted for longer periods (up to 18 hours) or have had type 1 diabetes, both of which are characterized by low plasma insulin levels. Therefore it is possible, that the discrepancies between the results of our study compared to earlier publications may result from the inability of OXA to further reduce already very low fasting insulin levels in non-diabetic animals or type 1 diabetic animals with barely detectable, or low insulin levels that were used before.

Orexin receptors are not expressed neither in hepatocytes (43) nor skeletal muscle (44), however, both orexin receptors are expressed in adipocytes (25, 45). We showed that OXA is able to stimulate glucose uptake by fat cells (25, 54). Our *ex vivo* experiments on isolated adipocytes from OXA treated type 2 diabetic animals show that insulin sensitivity in adipocytes was increased. Therefore, we conclude that white adipocytes, at least in part, can contribute to the overall improvement of insulin sensitivity in our type 2 diabetic animals treated chronically with OXA. An open question remains, whether or not OXA contributes to changes of insulin sensitivity in other tissues. An indirect evidence indicating improved hepatic and skeletal muscle sensitivity by exogenous OXA has been shown by others (39). However, a direct measurement of glucose uptake was not performed. Instead, changes of insulin receptor phosphorylation/signaling were reported. However, since orexin receptors are undetectable in skeletal muscle and liver, changes of insulin sensitivity do not result from the direct action of OXA with these tissues. One of the mechanisms that can affect insulin sensitivity of the skeletal muscle in response to OXA could be mediated by β-adrenergic receptors (39). Leptin was also shown to contribute to overall improvement of insulin sensitivity by OXA in animals with DIO (6). In our animal model, pro-inflammatory cytokines, which are known to reduce insulin sensitivity (46, 47) were low, whereas the anti-inflammatory adipokines were increased. Since OXA decreases the pro-inflammatory cytokines in plasma, we suggest that OXA can improve metabolic abnormalities in animals by changing the secretion of proinflammatory cytokines. Which of these cytokines has the major contribution to improved insulin sensitivity, if at all, remains an open question.

Pancreatic β-cells express OXR1 whereas OXR2 protein is absent (11, 13), indicating that all effects of OXA on β-cells, studied so far, are mediated *via* OXR1. The crucial role of OXR1 in regulating various functions of rodent β-cells as well as INS-1 cell was also confirmed using pharmacological blockers of both orexin receptors (13, 48). Despite discrepant reports, it is generally accepted that orexins acutely stimulate insulin secretion *in vivo* and *in vitro* (11, 12, 16, 49-51). In the current study, chronic administration of OXA reduced hyperinsulinaemia and reduced type 2 diabetes-associated loss of pancreatic β-cell mass. Pancreatic insulin content, as well as insulin mRNA were higher in OXA-treated diabetic animals, as compared to vehicle-treated type 2 diabetic rats. Pancreatic islets derived from OXA-treated animals had lower activity of CSP3, which serves as a well-established surrogate biomarker of cellular apoptosis. Furthermore, islets isolated from the OXA group showed a better response to GSIS *ex vivo* at lower as well as higher glucose. Enhancement of GSIS probably results from the higher β-cell mass as well as insulin content of the pancreatic islets. How apoptosis is inhibited by OXA, remains an open issue. While it was found that fasting blood concentration of GLP-1 is not affected in humans who suffer from metabolic syndrome (52) there is evidence that type 2 diabetic patients have lower levels of GLP-1 as compared with normal subjects (53). Furthermore, it is known that GLP-1 can attenuate β-cell loss in type 2 diabetes (54). Since OXA attenuated the reduction of GLP-1 levels in type 2 diabetic rats it is possible that the decreased loss of pancreatic β-cell mass results from a complex interaction OXA with cytokines and GLP-1.

This antiapoptotic activity of OXA on pancreatic β-cells is interesting observation, in view of the fact that both proliferative, as well as anti-proliferative activities were reported for OXA in various cells, a phenomenon appearing to be cell-type specific (55-58). However, we and others showed that OXA stimulates INS-1 and INS-1E cell proliferation (48, 51). Apoptotic death of pancreatic β-cells in type 2 diabetes is triggered by glucolipotoxicity, in addition to the deleterious activity of TNF-α and various interleukins (59). Inhibition of TNF-α production

improves viability and function of isolated islets (60). It is known that TNF-α can stimulate various caspases, thereby activating the pro-apoptotic pathway in pancreatic β-cells (59, 61). The executor protein CSP3 can trigger apoptosis of pancreatic β-cells (61). We report here that type 2 diabetic animals treated with OXA had reduced plasma TNF-α levels, indicating that this cytokine may play a role in OXA-dependent protection against apoptotic β-cell death. Furthermore, increased NEFA in type 2 diabetic rats were lowered by OXA, consistent with the antilipolytic activity of this neuropeptide (25). OXA reduced both basal and palmitate- induced TNF-α expression in freshly isolated islets, and attenuated TNF-α-, as well as palmitate-stimulated β-cell apoptosis. OXA reduced palmitate-stimulated TNF-α expression and attenuated TNF-α-stimulated activation of CSP3 in pancreatic in INS-1E cells. These results gathered *in vivo* as well as *in vitro* (isolated pancreatic islets, INS-1E cells), strongly suggest that OXA reduces β-cell apoptosis by inhibiting TNF-α-/CSP3-dependent pro-apoptotic signaling pathway.

As some of the results were obtained in *in vitro* experiments, the results must be interpreted with caution. As stated before, the final *in vivo* evidence supporting the crucial role of OXA at inhibiting TNF-α induced apoptosis of pancreatic β-cells is missing. The role of numerous cytokines differentially regulated by OXA in the context of type 2 diabetes and β-cell survival remains unclear. Furthermore, we are aware that adipose tissue is not exclusively contributing to the overall improvement of insulin sensitivity. Liver, as well as skeletal muscle are important in this context, however, orexin receptors are not expressed in these tissue and the effects of OXA on insulin sensitivity in both tissues are rather indirectly mediated e.g. *via* sympathoadrenergic system. In addition, we also detected increased α-cell content in pancreatic islets, suggesting that the changes in insulin to glucagon ratio may contribute to the markedly elevated hyperglycaemia in type 2 diabetic animals. Interestingly, OXA was able to decrease α-cell content. It is known, that the loss of pancreatic β-cells in type 2 diabetes is partially due to dedifferentiation into other cell types e.g. α-cells (62). Whether OXA is able to attenuate the dedifferentiation of β-cells to α-cells remains to be investigated. Lastly, it cannot be excluded that peripherally administrated OXA may act directly on the brain. This assumption is supported by data indicating that OXA is able to enter the brain from blood by simple diffusion (21). In brain orexin can promote energy expenditure (63). In our study we observed that OXA reduced body weight in HFD group. Human and animal studies showed that reduction of body weight in obese individuals improves insulin sensitivity (64). Furthermore, it is well-documented that hypothalamic orexin receptor plays a pivotal role in maintaining insulin sensitivity in peripheral tissues including liver and muscles (20, 65). Thus, it is rationale to speculate that OXA mechanism of action may be complex encompassing interaction with peripheral tissues as well as with the central nervous system. Furthermore, ligand induced orexin receptor activation was reported to stimulate the differentiation as well as numerous other biological activities of brown adipocytes (66, 67). It was reported that administration of OXA stimulates UCP1 expression in brown adipose tissue in adult mice, which was associated with improved glucose tolerance and reduced body mass (68). In our *in vitro* experiments OXA increased the expression of *Ucp1*, *Pgc-1α*, *Pparγ* and *Glut4* mRNA in differentiated rat brown adipocytes (data mot shown). Therefore, it is likely that improved metabolic parameters in OXA-treated diabetic rats resulted from enhanced brown adipose tissue activities. Nevertheless, it remains an open issue whether OXA can stimulate brown adipose tissue-mediated thermogenesis and energy expenditure in diabetes. To find an answer to this question a separate study needs to be performed. In addition, the perfect model for the human type 2 diabetes is not available and this is also true for our animals. Therefore studies on other animal models of type 2 diabetes will be valuably, to confirm the crucial findings of our chronic study with OXA.

In conclusion, orexin-A alleviates metabolic abnormalities associated with type 2 diabetes and protects β-cells from apoptotic cell death.

Abbreviations: CSP3, caspase 3; DIO, diet-induced obesity; GLP-1, glucagon-like peptide 1; GSIS, glucose-stimulated insulin secretion; HFD, high fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; IPGTT, intraperitoneal glucose tolerance test; ITT, insulin tolerance test; K_{ITT}, constant rate for blood glucose disappearance during insulin tolerance test; NEFA, non-esterified fatty acids; OXA, orexin-A; OXB, orexin-B; OXR1, orexin receptor type 1 (hypocretin receptor type 1); OXR2, orexin receptor type 2 (hypocretin receptor type 2); PA, palmitic acid; PI, propidium iodide; QUICKI, quantitative insulin-sensitivity check index; STZ, streptozotocin; TG, triglyceride; TNF, tumor necrosis factor.

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