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Methionine enkephalin (MENK) regulates the immune pathogenesis of type 2 diabetes mellitus via the IL-33/ST2 pathway

Keying Zhang[a], Jing Yang[a], Na Ao[a], Shi Jin[a], Ruiqun Qi[b], Fengping Shan[c], Jian Du[a,*]

[a] Department of Endocrinology, No. 4 Teaching Hospital, China Medical University, Shenyang 110032, China
[b] Department of Dermatology, No. 1 Teaching Hospital, China Medical University, Shenyang 110001, China
[c] Department of Immunology, School of Basic Medical Science, China Medical University, Shenyang 110122, China

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ABSTRACT

The incidence and mortality of type 2 diabetes mellitus (T2DM) rank among the top ten worldwide. Emerging studies indicate pathological roles for the immune system in inflammation, insulin resistance and islet β-cell damage in subjects with T2DM. Methionine enkephalin (MENK) is present in endocrine cells of the pancreas and has been suggested to be an important mediator between the immune and neuroendocrine systems. Therefore, it may play a role in modulating insulin secretion from islet cells. Since little is known about the effect of MENK on T2DM, therefore it was the aim of this study to characterize the role and possible mechanism of action of MENK on plasma glucose and serum insulin levels in T2DM rats and INS-1 cells in vivo and in vitro. MENK significantly decreased the plasma glucose level and increased the serum insulin concentration in T2DM rats. It also increased the serum levels of the cytokines IL-5 and IL-10, while decreased TNF-α and IL-2 levels. We further confirmed that MENK regulated glucose metabolism by upregulating opioid receptor expression and modulating the IL-33/ST2 and MyD88-TRAF6-NF-κB p65 signaling pathways. Based on these results, an intraperitoneal injection of MENK represents a potentially new approach for T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) often leads to disability from cardia-cerebrovascular diseases, blindness, limb amputation and other effects [1] and poses a serious health and economic challenge worldwide. Thus, strategies designed to control the disease development are urgently needed. The bidirectional interaction between the immune system and whole-body metabolism has been well recognized for many years [2,3]. Immunometabolism is a novel therapeutic target to control metabolism in many diseases, including cancer, diabetes and obesity. In addition, an important study elucidated the link between inflammation and glucose metabolism [4]. T2DM results from the generalized activation of the innate immune system, in which chronic cytokine-mediated inflammation exists [5,6]. Therefore, both the immune system and inflammatory mechanisms are involved in the occurrence and development of T2DM.

Based on accumulating evidence, endogenous opioid peptides and opioid binding sites are expressed in the endocrine pancreas of several animal species, including humans [7,8], suggesting that these peptides may also play a role in pancreatic secretion and subsequently influence glucose metabolism. Methionine enkephalin (MENK) is an endogenous opioid peptide composed of Tyr-Ala-Ala-Phe-Met that is derived from proenkephalin. MENK is an important mediator of communication between the immune and neuroendocrine systems by binding to opioid receptors: delta (d-, DOR), kappa (k-, KOR) and mu (m-, MOR) [9]. MENK, an immunoregulatory factor, modulates both the innate and adaptive immune systems, controls the activation and regulation the function of dendritic cells, macrophages, $CD4^+$ T cells, epithelial cells, and mesenchymal cells [10–13]. As shown in our previous studies, MENK regulates the functions of immune cells at a concentration of 10^{-12} mol/L [9,14].

For several years, the development of autoimmune chronic inflammatory diseases has been attributed to the activity of autoreactive $CD4^+$ T helper cells [15,16]. Naïve Th0 cells differentiate into specific Th subsets (Th1, Th2, Th17, Th22, Treg etc.) under the influence of cytokines [17]. Th1 cells, which produce pro-inflammatory cytokines (TNF-α, IL-2, and IFN-γ), support cell-mediated immunity and consequently promote inflammation, cytotoxicity, and delayed-type hypersensitivity. Th2 cells secrete anti-inflammatory cytokines (IL-4, IL-5, IL-10, and IL-13), support humoral immunity and ameliorate the inflammatory actions of Th1 cells [18–20]. The Th1/Th2 balance is very important for maintaining the steady-state immunity in the organism.

* Corresponding author.
E-mail address: dujianbox@126.com (J. Du).

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Group 2 innate lymphoid cells (ILC2s) are considered the first cells to activate the Th2 immune response, serve as a bridge between innate immunity and adaptive immunity, amplify the strength of the body's immune response to damage, and at the same time facilitate the formation of the adaptive immune response [21].

According to a recent study, islet mesenchymal -cell-derived IL-33 orchestrates immunometabolic crosstalk in pancreatic islets that promotes insulin secretion [22]. IL-33 induces T helper type 2 responses by binding to ST2 on resident ILC2s in islets. The IL-33/ST2 pathway prevents an inappropriate Th1-polarized response and induces IL-4 and IL-13 production. Finally, transmembrane ST2 may be a potent negative regulator of Toll-like receptor (TLR) signaling, and its upregulation on immune cells may make them refractory to maturation induced by pro-inflammatory, stimuli [23]. TLRs act via MyD88 and TRAF, triggering NF-κB p65 activation and promoting cytokine secretion and inflammatory responses [24]. However, little is known about the influence of MENK on diabetes to date. Therefore, the aim of this study was to determine the effect of MENK on insulin secretion from the pancreas of T2DM rats and INS-1 cells (a glucose-sensitive rat pancreatic β-cell line) and to characterize the possible mechanism.

2. Materials and methods

2.1. Rats and cells

Four-week-old, SPF-grade, male Sprague-Dawley rats (156 ± 12 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained in specific pathogen-free facilities at the Experimental Animal Center of China Medical University in accordance with the guide for the Care and Use of Laboratory Animals. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the China Medical University. Rat insulinoma INS-1 cells were obtained from the cell resource center of the Beijing Institute of Life Science, Chinese Academy of Science. All experiments were performed in INS-1 cells from passages 25 to 45.

2.2. Reagents

The DOR (cat. no. ab176324), KOR (cat. no. ab183825), IL-33 (cat. no. ab187060), and TRAF6 mAbs (cat. no. ab40675) and the ST2 pAb (cat. no. ab228543) were purchased from Abcam. The MOR (cat. no. NB100-1620) and MyD88 pAbs (cat. no. NB100-56698) were purchased from Novusbio. The NF-κB p65 mAb (cat.no. 3033P) was purchased from Cell Signaling Technology, and the GAPDH mAb (cat. no. CAB932Hu22) was purchased from Cloud-Clone Corp. MENK (≥98% purity) was purchased from Chinese Peptide Company, fetal bovine serum (FBS) was purchased from ExCell Bio, and other frequently used chemicals were all of analytical grade and were obtained from Sigma-Aldrich or TaKaRa.

2.3. Procedures with rats

Rats were acclimatized for 1 week and then weighed and randomly divided into two groups: a T2DM group that received a high-glucose and high-fat diet (31.1% kcal from fat, 20% kcal from carbohydrates and 15.6% kcal from protein) and a normal control group that received a normal diet. After 4 weeks, the T2DM group was administered a single intraperitoneal injection of 35 mg/kg streptozotocin (STZ) dissolved in citrate buffer at a pH of 4.5. Rats were considered to have T2DM if the randomly measured plasma glucose level was ≥16.7 mmol/L after 72 h. The plasma glucose level was measured from a drop of blood collected from the tail vein using a glucose analyzer (Bayer HealthCare LLC). After stabilizing for 10 days, normal control rats were subdivided into three groups: one received 20 mg/kg MENK daily (NC + M), one was administered MENK + naltrexone (NC + M + N), the latter of which was injected 1 h before MENK at 10 mg/kg as described in a previous study [25], and the other group was treated with an equal volume of saline (NC). T2DM rats were subdivided into five groups, three of which were treated with 5, 10 or 20 mg/kg MENK (M5, M10 and M20, respectively), one was administered NTX + MENK (10 mg/kg and 20 mg/kg, T + M + N), and the other group was treated with saline (T). All reagents were administered via an intraperitoneal injection once daily and for 42 consecutive days. Body weight and plasma glucose levels were recorded throughout the study. Rats were fasted overnight and anesthetized with an intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg). Then, blood was collected from the heart and centrifuged at 1000*g* for 20 min. Serum was separated and stored at −20 °C. The fresh pancreas tissues were placed on ice and the adherent fat and connective tissue were removed. Representative fragments were taken from the tail and body of the pancreas and stored at −80 °C until further analysis.

2.3.1. Procedures with cells

Cells were cultured in a 5% CO_2 atmosphere in air at 37 °C in RPMI-1640 cell culture medium containing 11.1 mmol/L glucose and supplemented with 10% (v/v) FBS. Cells were passaged for 4 days when cell confluence reached 80–90% after trypsin-EDTA-mediated detachment. Then, INS-1 cells were cultured in 6-well plates with complete culture medium for 48 h until the cells reached 60% confluence. The medium was removed and cells were washed twice with PBS prior to different treatments. Cells were incubated in RPMI-1640 containing 1% BSA (NC) that was supplemented with MENK (10^{-12} mol/L, NC + M) or with MENK and NTX (10^{-12} mol/L and 50 nmol/L, NC + M + N), and cells were incubated with a final concentration of 25 mmol/L glucose, 0.4 mmol/L palmitic acid in 1% BSA/RPMI-1640 media (HGP) supplemented with or without MENK (HGP + M) or MENK and NTX (HGP + M + N) for 48 h. For the pharmacological inhibition of MENK, cells were treated with 50 nmol/L NTX for 1 h prior to the MENK treatment. The cells and cell culture supernatants were collected for subsequent experiments.

2.3.2. Glucose-stimulated insulin secretion (GSIS)

INS-1 cells (1×10^6 normalized) were incubated with PBS medium for 2 h and then incubated with 2.8 mmol/L glucose in PBS for 1 h (basal samples), followed by an incubation with 16.7 mmol/L glucose in PBS (glucose-stimulated samples) for 1 h. Incubations were performed at 37 °C in an air incubator. The supernatant from each well was collected to separately measure basal and stimulated insulin secretion.

2.4. Enzyme-linked immunosorbent assays (ELISAs) of insulin and cytokine levels

After the experiment, the supernatant and serum were collected and assayed for insulin, TNF-α, IL-4, IL-5 and IL-10 levels using highly sensitive ELISA kits according to the manufacturer's instructions.

2.5. RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from pancreatic tissues and INS-1 cells using a Total RNA Kit (Omega Biotek). The quantity and quality of the extracted RNA were evaluated by measuring the absorption at 260 and 280 nm using a Nano Photometer Pearl spectrophotometer (Implen, Germany). The first strand cDNAs were synthesized using Prime Script RT reagent kits, and RT-qPCR was performed with TB Green Premix Ex Taq II (TaKaRa Bio, Japan). The process was performed with an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.). Primers for gene amplification were synthesized by referencing Primer Bank and are listed in Table 1. The expression of every gene was analyzed in at least 3 biological replicates, and gene expression was quantified and normalized to the β-actin mRNA using the $2^{-\triangle\triangle CT}$ method.

Table 1
PCR primer sequences.

Gene	Primer	Sequence (5′–3′)
Oprd1	Sense	CAACGTGCTCGTCATGTTTGG
	Antisense	CAGGTACTTGGCGCTCTGGAA
Oprm1	Sense	AACTCCACTCGAGTCCGTCAGAA
	Antisense	CTGGATGGTGTGAGACCCAGTTAG
Oprk1	Sense	GCATTTGGCTACTGGCATCATC
	Antisense	AGGAGCATTCAATGACATCCACA
Il33	Sense	AAAGTGCGACAGCACATCAGG
	Antisense	CCTCATGCAGTAGACATGGCAGATA
Il1rl1	Sense	CACACAACTACACAGTGGAAGTGGA
	Antisense	GTTAATCTGCCACAGGACAGCAA
Myd88	Sense	TATACCAACCCTTGCACCAAGTC
	Antisense	TCAGGCTCCAAGTCAGCTCATC
Traf6	Sense	TTTGGCGTCGGAGACACTTG
	Antisense	TCGCTTGAAGACTGGCTGGA
Nfkb1	Sense	TCTTCGACTACGCGGTTACGG
	Antisense	CTCACGAGCTGAGCATGAAGG
Actb	Sense	GGAGATTACTGCCCTGGCTCCTA
	Antisense	GACTCATCGTACTCCTGCTTGCTG

2.6. Western blot analysis

The pancreatic tissues and INS-1 cells were lysed in lysis buffer containing 1 mmol/L PMSF and 1 mmol/L protease inhibitors on ice for 30 min. Supernatants were collected after centrifugation. Protein concentrations were detected using the BCA assay. Thirty micrograms of protein sample were separated on 10% SDS–PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Sigma). Membranes were blocked with 5% BSA in Tris-buffered saline solution (TBS) containing 0.1% Tween-20 and incubated overnight at 4 °C with specific antibodies against MOR (1:500), DOR (1:1000), KOR (1:1000), IL-33 (1:1000), ST2 (1:500), MyD88 (1:500), TRAF6 (1:5000), NF-κB p65 (1:1000), or GAPDH (1:100). Membranes were incubated with a secondary antibody (1:8000) for 1 h at room temperature. Protein signals were visualized using the enhanced chemiluminescence detection system. Protein expression levels were normalized to GAPDH. Experiments were performed in triplicate.

2.7. Immunohistochemistry

The rat pancreas was isolated, fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin, cut into 4 μm-thick sections, and stained with hematoxylin-eosin (HE). Tissue sections were immunostained using streptavidin-biotin horseradish peroxidase. Sections were deparaffinized, rehydrated and then subjected to antigen retrieval in citrate buffer (pH 6.0) at 98 °C for 2 min. Sections were treated with 3% hydrogen peroxide for 10 min, incubated with goat serum for 20 min at room temperature, and then incubated with antibodies against MOR (1:100), DOR (1:100), or KOR (1:50) overnight at 4 °C. Sections were incubated with a secondary antibody, stained with DAB, counterstained with hematoxylin, dehydrated, and transparentized. Stained cells were analyzed by calculating the number of positive pixels per area in 3 locations on each slide using ImageJ 1.48 software.

2.8. Statistical analysis

All statistical analyses were performed with Prism software (GraphPad Software 5.0). Data are presented as means ± SD. Comparisons among multiple groups were performed using one-way ANOVA and comparisons between two groups were compared using unpaired *t*-tests. $p < 0.05$ was considered statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.01$).

3. Results

3.1. Effects of MENK on rats in vivo

3.1.1. MENK reduced glucose levels and increased the body weight of T2DM rats

The plasma glucose level in the T2DM rats gradually increased after the STZ injection (10–11 weeks), while the glucose level in the normal control group was invariable. Beginning at the 15th week (the 14th day after MENK application), the glucose level in the MENK intervention groups began to decrease significantly to different extents, while the plasma glucose level in the T2DM rats treated with saline remained high. This phenomenon was observed almost daily afterwards (Fig. 1A). Based on the comparison of plasma glucose levels in T2DM rats before and after the MENK treatment, all different doses (5, 10 and 20 mg/kg) of MENK significantly reduced glucose levels (Fig. 1B, $p < 0.05$). Then, after comparing the plasma glucose levels in T2DM rats treated with different doses of MENK with the saline control group, 10 mg/kg and 20 mg/kg MENK significantly reduced plasma glucose levels (Fig. 1C–D, $p < 0.05$). The 5 mg/kg MENK group was not significantly different from the control group. No obvious changes were observed after MENK and NTX application compared with those in the normal control group. Based on these results, MENK reduced plasma glucose levels in T2DM rats, and the efficacy varied with the dose.

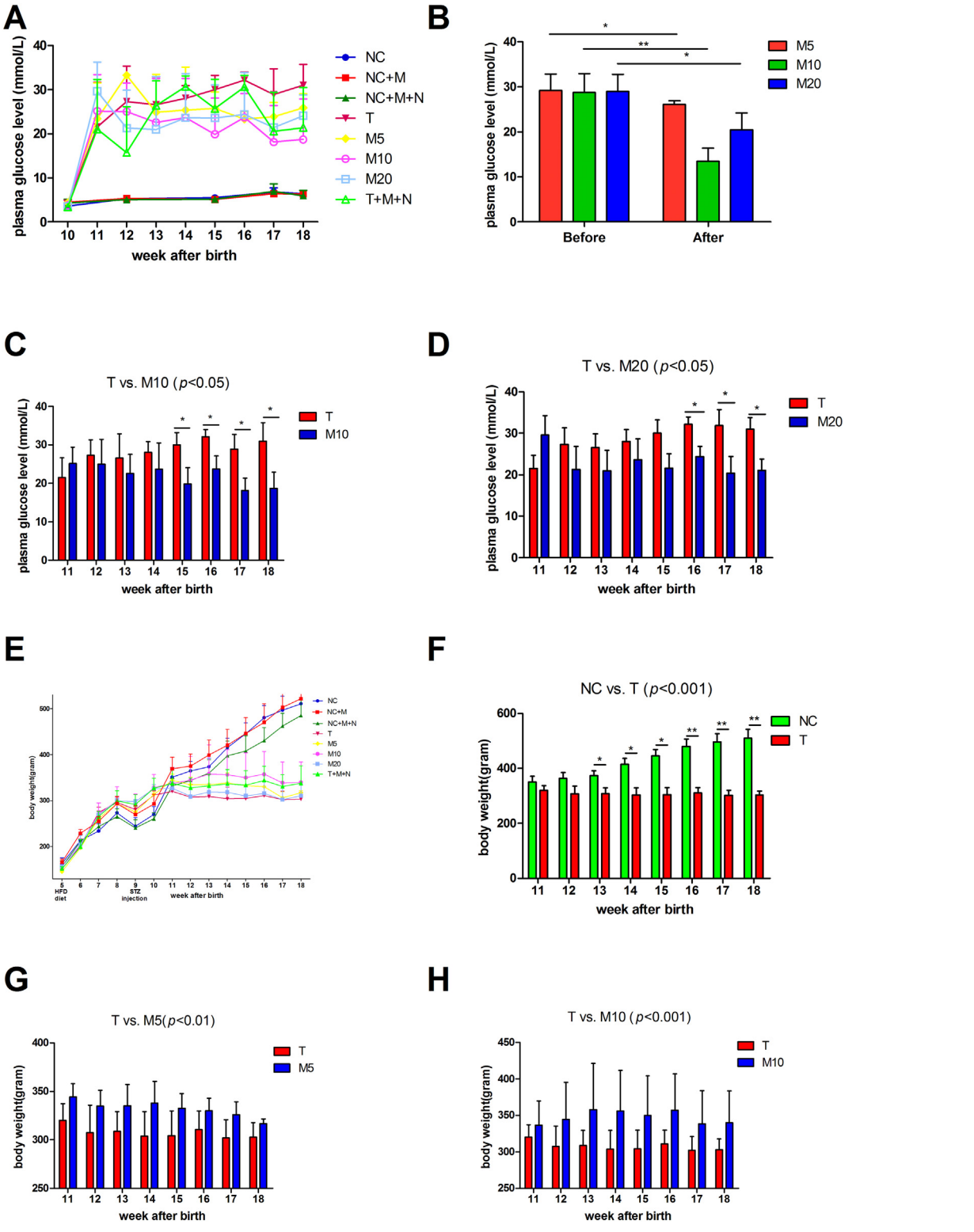
Additionally, after successfully establishing the T2DM rat model, the body weight of the T2DM group no longer continued to increase after the 11th week, while the weight of the normal control group still increased gradually over time. After the MENK intervention, the weight of rats treated with each dose of MENK increased to different degrees compared with those in the T2DM saline control group (Fig. 1E). As weight loss accompanies hyperglycemia, body weight significantly reduced in the T2DM group compared with that in the NC group (Fig. 1F, $p < 0.001$). Moreover, the weight of the rats receiving MENK intervention was significantly improved compared with that of the T2DM rats treated with the saline, and the improvements induced by 5 mg/kg and 10 mg/kg MENK were statistically significant (Fig. 1G–H, $p < 0.01$), while the change induced by 20 mg/kg MENK was not statistically significant. Therefore, the change in body weight was consistent with the change in plasma glucose levels in rats, and we further determined that MENK decreased glucose levels.

3.1.2. MENK increased serum insulin levels in T2DM rats

As shown in Fig. 2, the serum insulin level significantly decreased in the T2DM group ($p < 0.05$), suggesting that the amount of insulin secreted by islet β-cells reduced. After the intervention with different doses of MENK, the serum insulin level significantly increased in T2DM rats, with the maximal effect observed at 10 mg/kg ($p < 0.05$), suggesting that MENK effectively increased insulin secretion from islet β-cells. No differences were observed in the groups of normal control rats.

3.1.3. Effects of MENK on the morphology and number of β-cells in the rat pancreas

Based on the results of HE staining of pancreatic tissues in Fig. 3, islets in the normal group contained spherical endocrine cells with a uniform size that were scattered between the exocrine parts of the pancreas. β-Cells accounted for approximately 70% of the islet cells and were mainly located in the center of the islet. β-Cells were small cells with irregular masses, light staining, clear nuclear chromatin, cytoplasmic boundaries and connective tissue. Compared with those in the normal group, the islet β-cells in the T2DM group were sparse and displayed a disorderly arrangement. Fewer β-cells with a different morphology and unclear cytoplasmic boundaries were observed in these animals. After the MENK intervention, the number of cells significantly increased, the edges were rounder, the nucleoli were clear and the cells were arranged evenly compared with those in the T2DM group. Compared with that in the normal group, the cytoplasm of the



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MENK group was slightly uneven. Thus, the MENK intervention improved the morphology of pancreatic tissue and maintained its normal function.

3.1.4. MENK increased the expression of opioid receptors in the rat pancreas

We detected the levels of the DOR, MOR and KOR mRNAs and proteins in the rat pancreas to elucidate whether opioid receptors may participate in the mechanism regulating the glucose level. The expression of the KOR mRNA was below the limit of detection (the CT value > 35). No significant changes in the expression of the DOR and MOR mRNAs were observed in rats from the NC + M and NC + M + N groups compared to that in the NC group, as shown in Fig. 4A–B. However, a statistically significant decrease in the expression of the

Fig. 1. Changes in plasma glucose levels and body weight in rats. Methionine enkephalin (MENK, M) was intraperitoneally injected with 5, 10 and 20 mg/kg/day (M5, M10, M20) and naltrexone (N) was injected 1 h before MENK at 10 mg/kg into Sprague-Dawley rats for 42 days. Plasma glucose levels were measured in a drop of blood collected from the tail vein. A, the plasma glucose level in the type 2 diabetes mellitus (T2DM, T) rats gradually increased after the streptozotocin (STZ) injection (10–11 weeks). Beginning at the 15th week, the glucose level in the MENK intervention groups began to decrease significantly to different extents, while the plasma glucose level in the T2DM rats treated with saline remained high. This phenomenon was observed almost daily afterwards. B–D, The glucose levels before and after MENK treatment and the differences in glucose levels between groups were compared. Before and after the MENK treatment, all different doses (5, 10 and 20 mg/kg) of MENK significantly reduced glucose levels. MENK significantly reduced plasma glucose levels in the T2DM rats treated with 10 mg/kg and 20 mg/kg MENK with the saline control group. E–H, Body weights were recorded and compared. E, after successfully establishing the T2DM rat model, the body weight of the T2DM group no longer continued to increase after the 11th week, while the weight of the normal control (NC) group still increased gradually over time. After the MENK intervention, the weight of rats treated with each dose of MENK increased to different degrees compared with those in the T2DM saline control group. F, the body weight significantly reduced in the T2DM group compared with that in the NC group. G–H, the weight of the rats receiving 5 mg/kg and 10 mg/kg MENK intervention was significantly improved compared with that of the T2DM rats treated with the saline. Six rats per group were analyzed. Values are presented as means ± SD, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

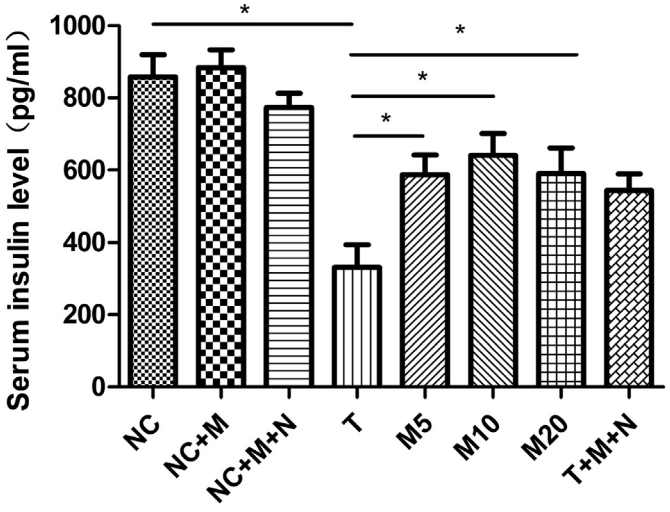


Fig. 2. The serum insulin levels in rats. Methionine enkephalin (MENK, M) was intraperitoneally injected with 5, 10 and 20 mg/kg/day (M5, M10, M20) and naltrexone (N) was injected 1 h before MENK at 10 mg/kg into Sprague-Dawley rats for 42 days. Serum insulin levels were measured using a rat insulin ELISA kit. The serum insulin level significantly decreased in the type 2 diabetes mellitus (T2DM, T) group compared with the normal control (NC) group. After the intervention with 5 mg/kg, 10 mg/kg and 20 mg/kg MENK, the serum insulin level significantly increased in T2DM rats. Six rats per group were analyzed. Values are presented as means ± SD ($n = 6$), $*p < 0.05$.

MOR and DOR mRNAs was observed in rats in the T2DM group compared with that in the NC group ($p < 0.01$). The DOR mRNA was expressed at significantly higher levels in groups that received various doses of MENK (5, 10 and 20 mg/kg) than that in the T2DM group ($p < 0.05$). The MOR mRNA was expressed at significantly higher levels in animals treated with 5 mg/kg and 20 mg/kg MENK, but a significant difference between 10 mg/kg MENK intervention group and the T2DM group was not observed ($p > 0.05$). As shown in Fig. 4C–F, levels of the DOR, MOR and KOR proteins in the pancreas of T2DM rats significantly decreased compared with those in the normal control group ($p < 0.05$). After the MENK intervention, these decreases were reversed, and the improvements in MOR and DOR levels induced by both the 5 and 20 mg/kg doses were statistically significant ($p < 0.05$); the improvement in KOR levels induced by the 10 mg/kg dose was also statistically significant ($p < 0.05$).

The results of immunohistochemical staining were shown in Fig. 4G–I. MOR, DOR and KOR were mainly located in epithelial cells, mesenchymal cells and inflammatory cells of the pancreas in rats. KOR was expressed at very low levels, as shown in Fig. 4I, which was also consistent with the mRNA expression. As shown in Fig. 4G–H, MOR and DOR were expressed at significantly lower levels in the T2DM group than those in the NC group. MENK-treated rats exhibited significantly higher expressions than T2DM rats did. MOR or DOR levels were not changed in the NC + M and NC + M + N groups compared with those in the NC group. Taken together, we concluded that MENK upregulated the expressions of opioid receptors.

3.1.5. MENK downregulated the MyD88-TRAF6-NF-κB p65 signaling pathway and IL-33/ST2 expression in rats

The TLR signaling pathway promotes inflammatory responses. Thus, we measured the expressions of downstream molecules in the TLR pathway, MyD88, TRAF6 and NF-κB p65, in pancreatic tissues to explore the mechanism by which MENK induces hypoglycemia. We also analyzed the expression of intermediates in the IL33/ST2 pathway in pancreatic tissues. As shown in Fig. 5A–E, the MyD88, TRAF6 and NF-κB p65 mRNAs were expressed at significantly higher levels in T2DM rats than those in the control group ($p < 0.05$). After the MENK intervention, the expression of the MyD88, TRAF6 and NF-κB p65 mRNAs was normalized. The decreases in the expression of the MyD88 and TRAF6 mRNAs in all dose groups and NF-κB p65 in the 10 mg/kg and 20 mg/kg dose groups were statistically significant compared with those in the T2DM group ($p < 0.05$). Similarly, the IL-33 and ST2 mRNAs were expressed at significantly higher levels in the pancreas of the T2DM group than in the control group ($p < 0.05$). However, after treatment with various doses of MENK, the expression of the IL-33 mRNA significantly decreased to various degrees ($p < 0.05$). However, the expression of the ST2 mRNA only decreased by 10 mg/kg MENK. MENK did not exert a significant effect on the expression of mRNAs encoding the proteins involved in both pathways in the three normal control groups. We also detected the levels of the corresponding proteins using western blotting, as shown in Fig. 5F–K.

3.1.6. MENK regulates serum Th1/Th2 cytokine levels

The MENK treatment inhibited the secretion of inflammatory cytokines, as shown in Fig. 6A–D. High levels of inflammatory cytokines were detected in T2DM rats, including TNF-α and IL-2. Serum IL-5 concentration significantly decreased in T2DM rats compared with those in normal control rats. The Th1/Th2 ratio exhibited a shift towards the TNF-α and IL-2-producing Th1 phenotype in T2DM rats. After the MENK intervention, TNF-α and IL-2 levels decreased, while IL-5 and IL-10 levels increased and the Th1/Th2 ratio displayed a decrease in Th1 cells and an increase in Th2 cells, suggesting that MENK transformed the response from a Th1 to Th2 response.

3.2. Effects of MENK on rat insulinoma INS-1 cells in vivo

3.2.1. MENK improves GSIS in INS-1 cells

After the cells were incubated with a final concentration of 25 mmol/L glucose and 0.4 mmol/L palmitic acid in culture medium (HGP) for 48 h, the insulin level in the supernatant was lower than in the NC group. In the HGP + M group, MENK (10^{-12} mol/L) evoked large and significant increases in insulin secretion compared with the HGP group, as shown in Fig. 7A ($p < 0.05$). We examined GSIS in INS-1 cells to confirm that MENK improved β-cell function. As shown in Fig. 7B, under low glucose conditions (2.8 mmol/L), significant changes in insulin secretion were not observed in cells incubated with the

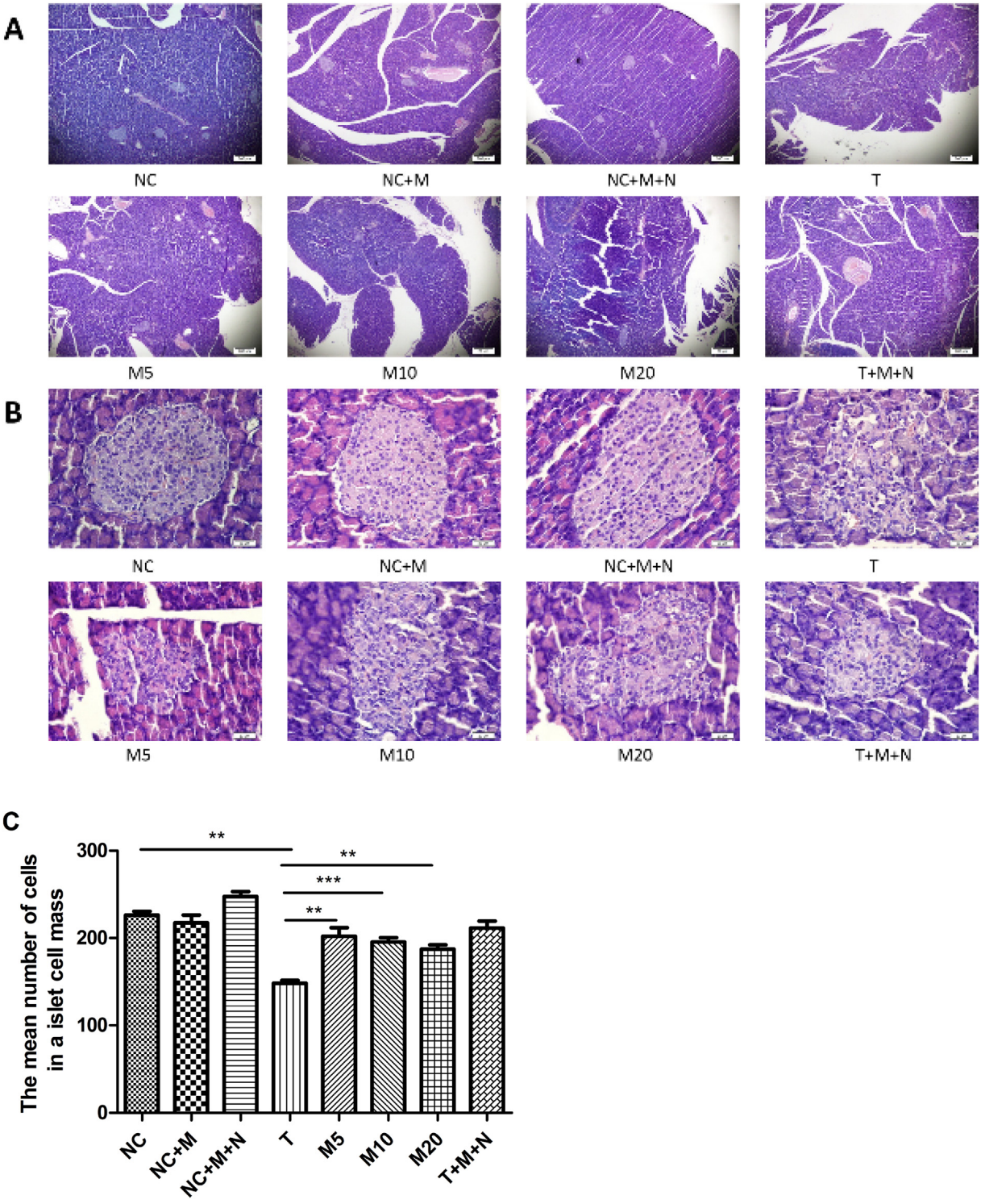


Fig. 3. Changes in the morphology and number of cells in the rat pancreas. Methionine enkephalin (MENK, M) was intraperitoneally injected with 5, 10 and 20 mg/kg/day (M5, M10, M20) and naltrexone (N) was injected 1 h before MENK at 10 mg/kg into Sprague-Dawley rats for 42 days. Images of hematoxylin-eosin (HE) staining of pancreatic tissues from each group are shown. Images in A have a scale of 200 μm, and images in B have a scale of 20 μm. The mean number of cells were analyzed and calculated in 3 islets from each slide using ImageJ 1.48 software and a statistical test. Fewer β-cells were observed in type 2 diabetes mellitus (T2DM, T) animals compared with the normal control (NC) animals. After the 5 mg/kg, 10 mg/kg and 20 mg/kg MENK intervention, the number of cells significantly increased. Six rats per group were analyzed. Values are presented as means ± SD ($n = 6$), $^{**}p < 0.01$ and $^{***}p < 0.001$.

different media. However, in the presence of high glucose (16.7 mmol/L), the GSIS in INS-1 cells treated with HGP reduced, and 10^{-12} mol/L MENK significantly increased insulin secretion in the medium compared with that in the HGP group ($p < 0.05$). Based on these data, the MENK treatment preserved β-cell function in the presence of high-glucose and high-palmitic acid medium.

3.2.2. MENK increased the expressions of opioid receptors on INS-1 cells

We detected the expression of the MOR, DOR and KOR mRNAs and proteins in INS-1 cells to assess the mechanism underlying the effect of

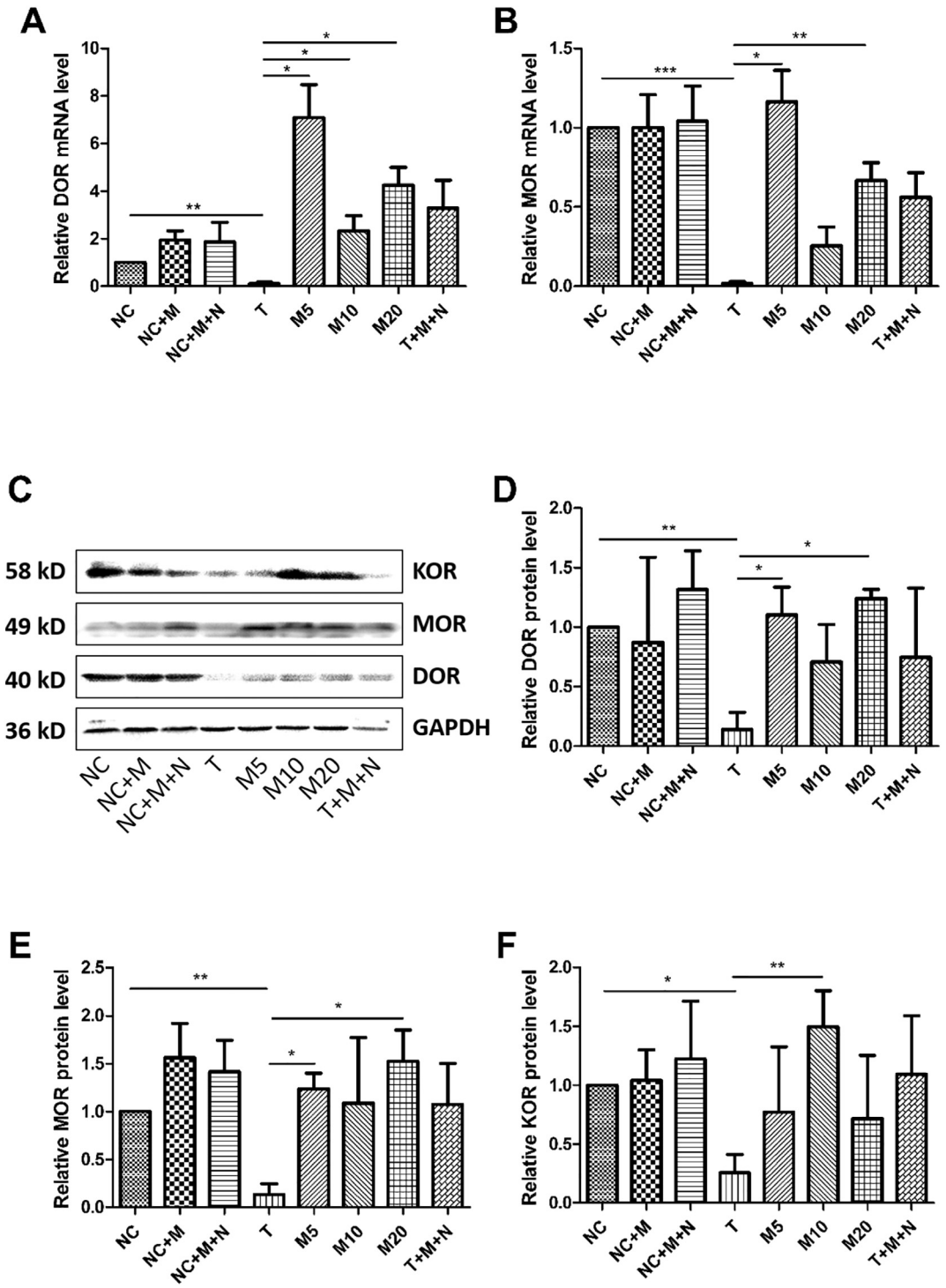
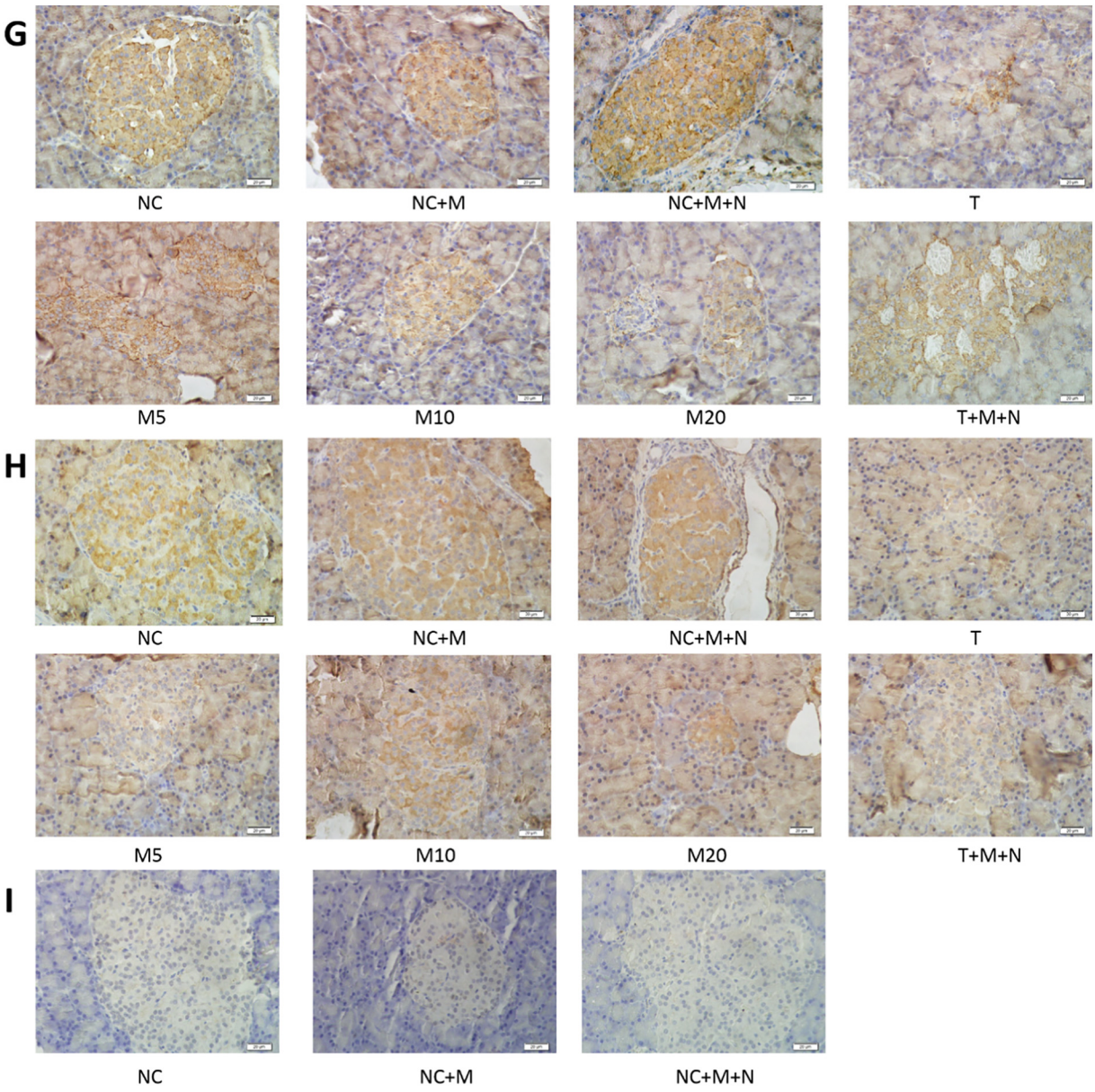


Fig. 4. Levels of opioid receptors (DOR, MOR and KOR) mRNAs and proteins expression and immunohistochemical staining in rats. Methionine enkephalin (MENK, M) was intraperitoneally injected with 5, 10 and 20 mg/kg/day (M5, M10, M20) and naltrexone (N) was injected 1 h before MENK at 10 mg/kg into Sprague-Dawley rats for 42 days. Levels of the delta -, kappa - and mu - opioid receptor (DOR, MOR and KOR) mRNAs (4A–B) and proteins (4C–F) in pancreatic tissues were measured. A statistically significant decrease in the expression of the MOR and DOR mRNAs was observed in rats in the type 2 diabetes mellitus (T2DM, T) group compared with that in the normal control (NC) group. The DOR mRNA was expressed at significantly higher levels in groups that received various doses of MENK (5, 10 and 20 mg/kg) than that in the T2DM group. The MOR mRNA was expressed at significantly higher levels in animals treated with 5 mg/kg and 20 mg/kg MENK. The levels of the DOR, MOR and KOR proteins in the pancreas of T2DM rats significantly decreased compared with those in the NC group. After the MENK intervention, these decreases were reversed, and the improvements in MOR and DOR levels induced by both the 5 and 20 mg/kg doses were statistically significant; the improvement in KOR levels induced by the 10 mg/kg dose was statistically significant. Immunohistochemistry was conducted to observe the localization and expression of DOR (4G), MOR (4H) and KOR (4I) in the pancreas. Stained cells were analyzed by calculating the average number of positive pixels per area in 3 locations on each slide using ImageJ 1.48 software. MOR and DOR were expressed at significantly lower levels in the T2DM group than those in the NC group. MENK-treated rats exhibited significantly higher expressions than T2DM rats did. Six rats per group were analyzed. Values are presented as means ± SD ($n = 6$), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.



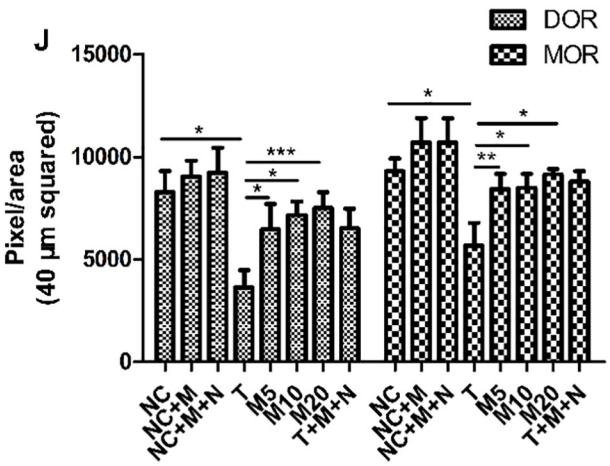


Fig. 4. (*continued*)

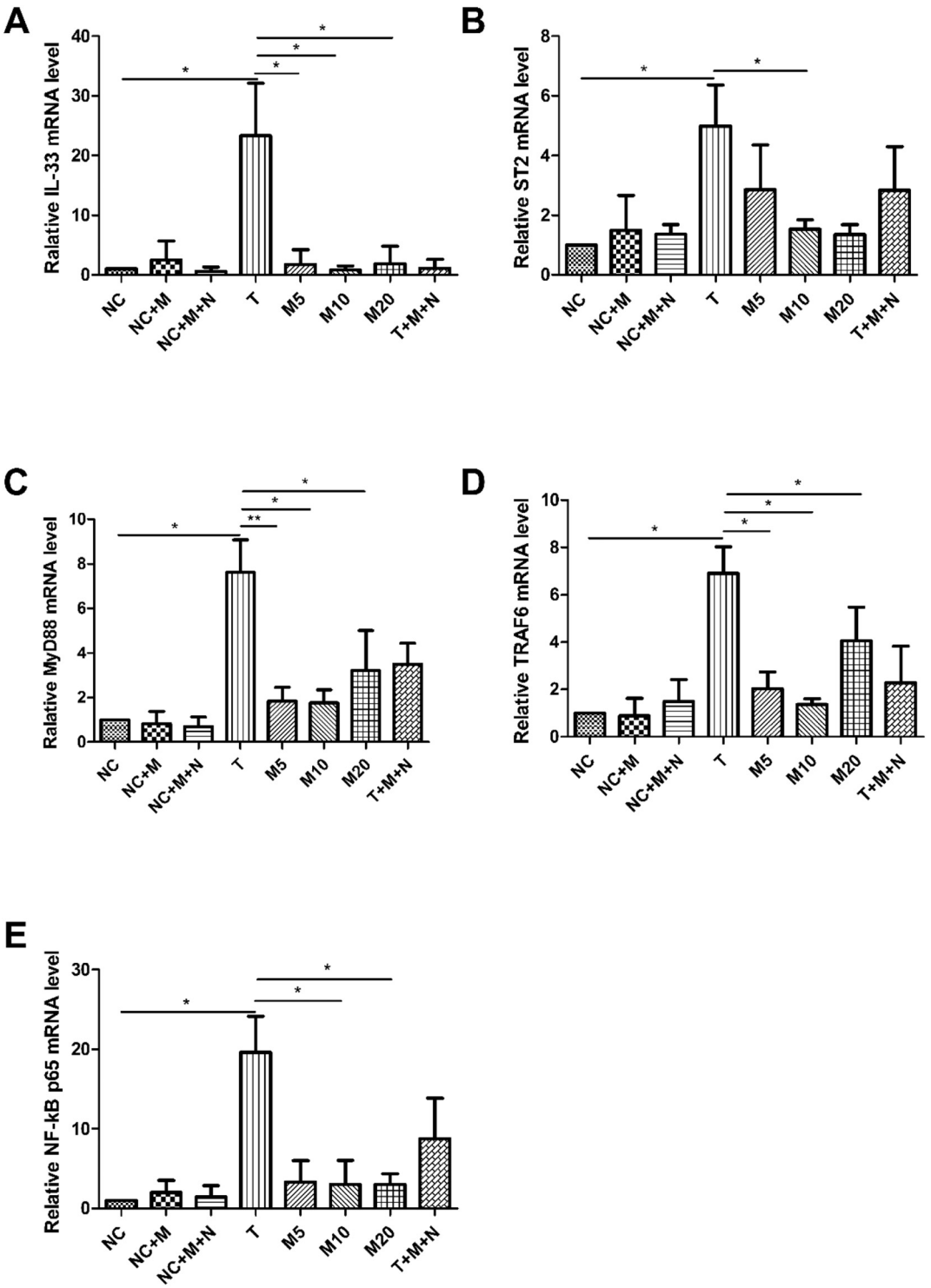


Fig. 5. Expression of mRNAs and proteins involved in the MyD88-TRAF-NF-κB p65 and IL33/ST2 pathways in rat pancreatic tissues. Methionine enkephalin (MENK, M) was intraperitoneally injected with 5, 10 and 20 mg/kg/day (M5, M10, M20) and naltrexone (N) was injected 1 h before MENK at 10 mg/kg into Sprague-Dawley rats for 42 days. The mRNA and protein levels of intermediates in the aforementioned signaling pathways in rat pancreatic tissues were measured. The MyD88, TRAF6 and NF-κB p65 mRNAs were expressed at significantly higher levels in type 2 diabetes mellitus (T2DM, T) rats than those in the normal control (NC) group. After the MENK intervention, the decreases in the expression of the MyD88 and TRAF6 mRNAs in all dose groups and NF-κB p65 in the 10 mg/kg and 20 mg/kg dose groups were statistically significant compared with those in the T2DM group. The IL-33 and ST2 mRNAs were expressed at significantly higher levels in the pancreas of the T2DM group than in the NC group. After treatment with all doses of MENK, the expression of the IL-33 mRNA significantly decreased, the expression of the ST2 mRNA only decreased by 10 mg/kg MENK. We also detected the similar levels of the corresponding proteins using western blotting. Six rats per group were analyzed. Values are presented as means ± SD (n = 6), $*p < 0.05$ and $**p < 0.01$.

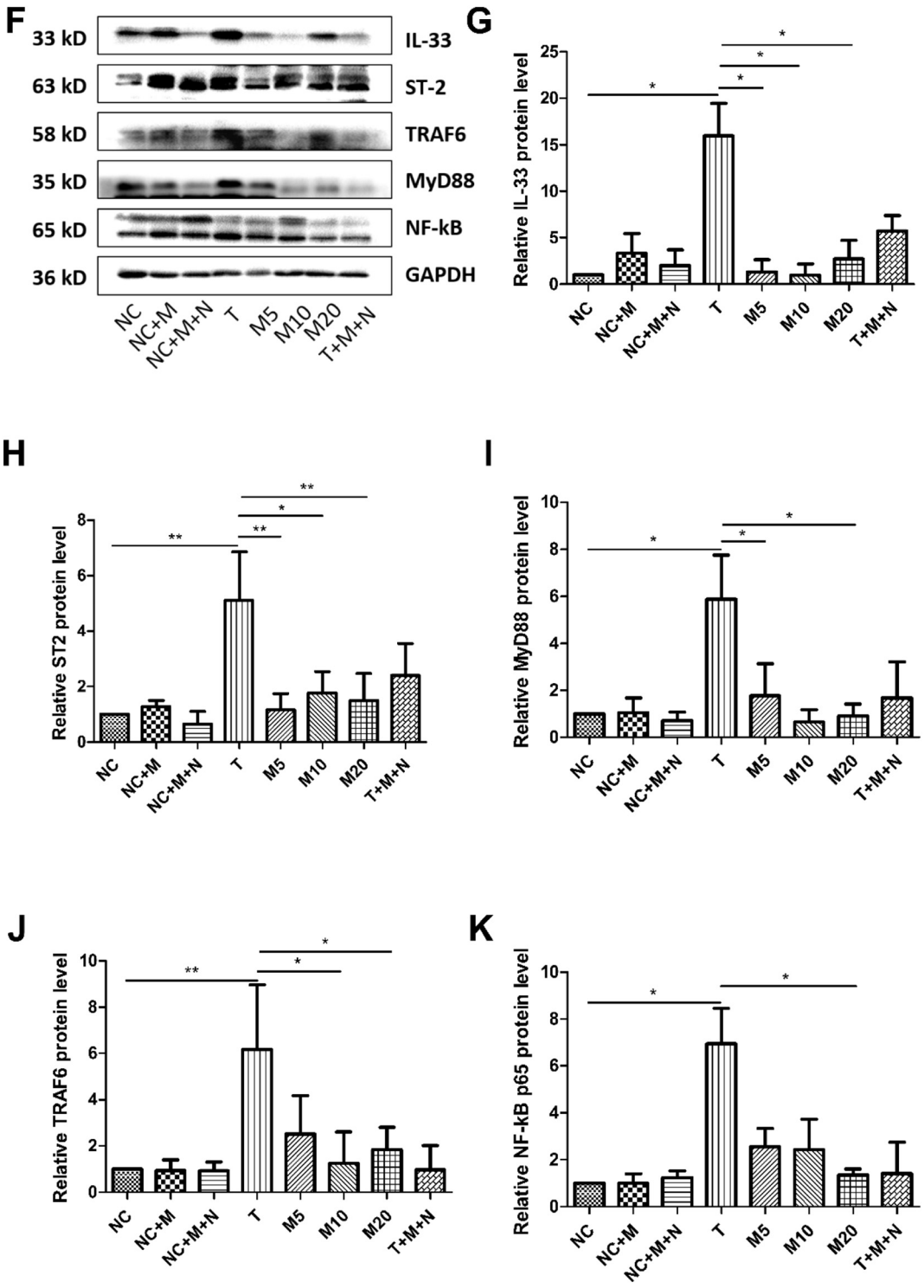


Fig. 5. (*continued*)

MENK on insulin secretion. MOR and KOR were expressed at very low levels in INS-1 cells, the CT values were > 35, and the differences observed after grouping cultures were not significant. As shown in Fig. 8A, the level of the DOR mRNA significantly decreased in the HGP group compared with that in the NC group and significantly increased in the HGP + M group compared with that in the HGP group ($p < 0.05$). However, significant changes were not observed compared with the levels measured in cells from the NC, NC + M and NC + M + N groups. Then, the levels of opioid receptor proteins were detected in INS-1 cells (Fig. 8B–E). Levels of opioid receptor proteins significantly reduced after culture with high-glucose and high-palmitic acid media. After the MENK treatment of injured cells, DOR and MOR levels restored, and the results were statistically significant ($p < 0.05$). Therefore, MENK upregulated the expressions of opioid receptors.

3.2.3. MENK downregulated MyD88-TRAF6-NF-κB p65 signaling pathway and IL-33/ST2 expression in INS-1 cells

As shown in Fig. 9A–E, the expression of the MyD88, TRAF6 and NF-κB p65 mRNAs significantly increased in INS-1 cell cultured with high-glucose and high-palmitic acid media ($p < 0.05$). However, these

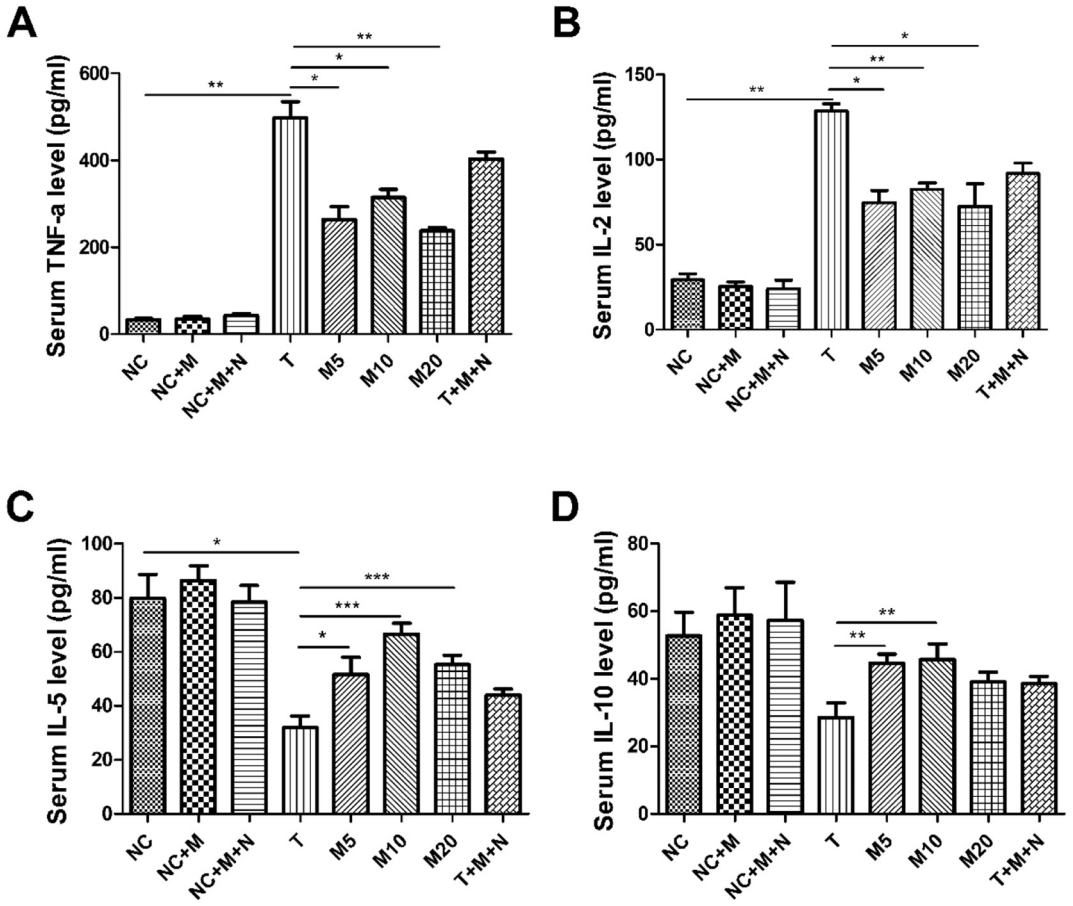


Fig. 6. Serum cytokine levels in rats. Methionine enkephalin (MENK, M) was intraperitoneally injected with 5, 10 and 20 mg/kg/day (M5, M10, M20) and naltrexone (N) was injected 1 h before MENK at 10 mg/kg into Sprague-Dawley rats for 42 days. Serum cytokine levels were measured using rat ELISA kits. Serum TNF-α and IL-2 concentrations were significantly increased and serum IL-5 concentration significantly decreased in type 2 diabetes mellitus (T2DM, T) rats compared with those in normal control (NC) rats. After the MENK intervention, TNF-α and IL-2 levels decreased, while IL-5 and IL-10 levels increased. Six rats per group were analyzed. Values are presented as means ± SD ($n = 6$), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

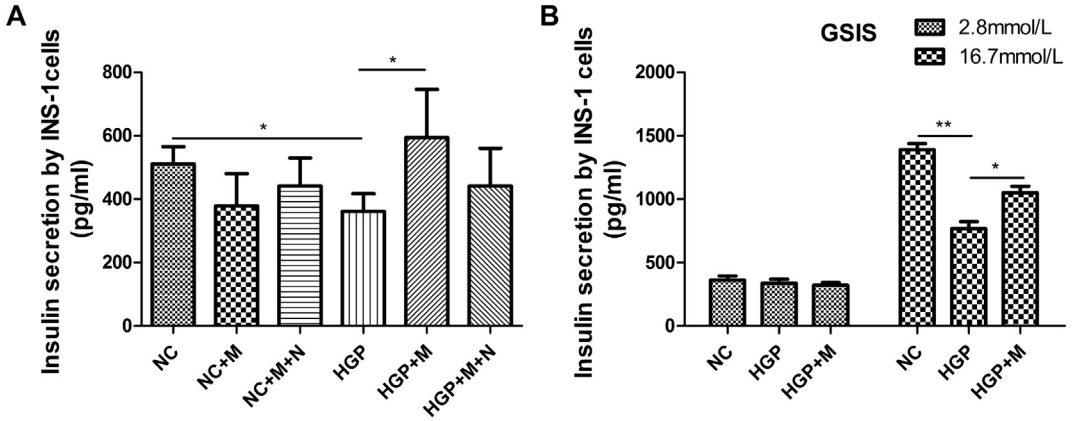


Fig. 7. Insulin secretion by INS-1 cells. INS-1 cells (1×10^6 normalized) were cultured with different culture media (A). Cells were incubated in RPMI-1640 containing 1% BSA (NC) that was supplemented with methionine enkephalin (MENK, 10^{-12} mol/L) or with MENK and naltrexone (NTX, 50 nmol/L), and cells were incubated with a final concentration of 25 mmol/L glucose, 0.4 mmol/L palmitic acid in 1% BSA/RPMI-1640 media (HGP) supplemented with or without MENK or MENK and NTX for 48 h. After the cells were incubated in HGP culture medium, the insulin level in the supernatant was lower than in the NC group. MENK evoked significant increases in insulin secretion compared with the HGP group. INS-1 cells (1×10^6 normalized) were treated with PBS, HGP and HGP + M in low (2.8 mmol/L) or high glucose (16.7 mmol/L) medium (B). In the presence of high glucose (16.7 mmol/L), the GSIS in INS-1 cells treated with HGP reduced, and 10^{-12} mol/L MENK significantly increased insulin secretion in the medium compared with that in the HGP group. The supernatants (2 mL) were collected after INS-1 cells were incubated with different culture media for 48 h. The amount of insulin secreted into the culture medium was measured using a rat insulin ELISA kit according to the manufacturer's instructions. The results are presented as means ± SD, $^{*}p < 0.05$ and $^{**}p < 0.01$.

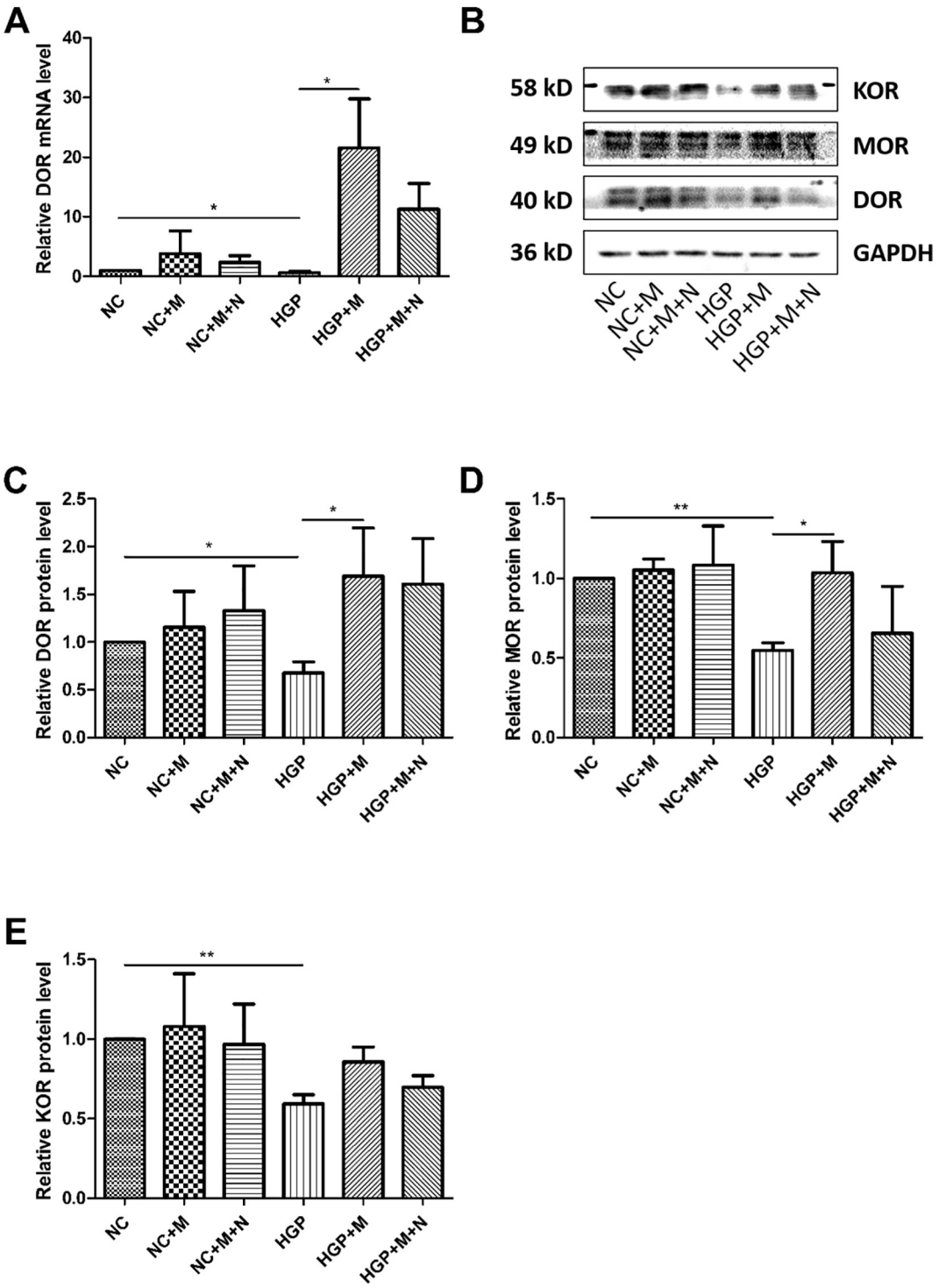


Fig. 8. Levels of opioid receptors (DOR, MOR and KOR) mRNAs and proteins in INS-1 cells. INS-1 cells (1×10^6 normalized) were cultured with different culture media. Cells were incubated in RPMI-1640 containing 1% BSA (NC) that was supplemented with methionine enkephalin (MENK, 10^{-12} mol/L) or with MENK and naltrexone (NTX, 50 nmol/L), and cells were incubated with a final concentration of 25 mmol/L glucose, 0.4 mmol/L palmitic acid in 1% BSA/RPMI-1640 media (HGP) supplemented with or without MENK or MENK and NTX for 48 h. Cells were collected for RT-PCR and western blotting. Levels of the delta -, kappa - and mu - opioid receptor (DOR, MOR and KOR) mRNAs (A) and proteins (B–E) in INS-1 cells were measured. The level of the DOR mRNA significantly decreased in the HGP group compared with that in the NC group and significantly increased in the HGP + M group compared with that in the HGP group. Levels of opioid receptor proteins significantly reduced after culture with HGP media. After the MENK treatment of injured cells, DOR and MOR levels restored. Values are presented as means ± SD, $^*p < 0.05$ and $^{**}p < 0.01$.

increases were inhibited by the MENK treatment ($p < 0.01$). We further confirmed the changes in the protein levels of these molecules using western blotting. As shown in Fig. 9F–K, levels of the MyD88, TRAF6 and NF-κB p65 proteins significantly increased in the HGP group, but the changes significantly reduced by the MENK treatment ($p < 0.05$). At the same time, the levels of the IL-33 and ST2 mRNAs and proteins markedly increased in INS-1 cells cultured with high-glucose and high-palmitic acid media. After treatment with MENK

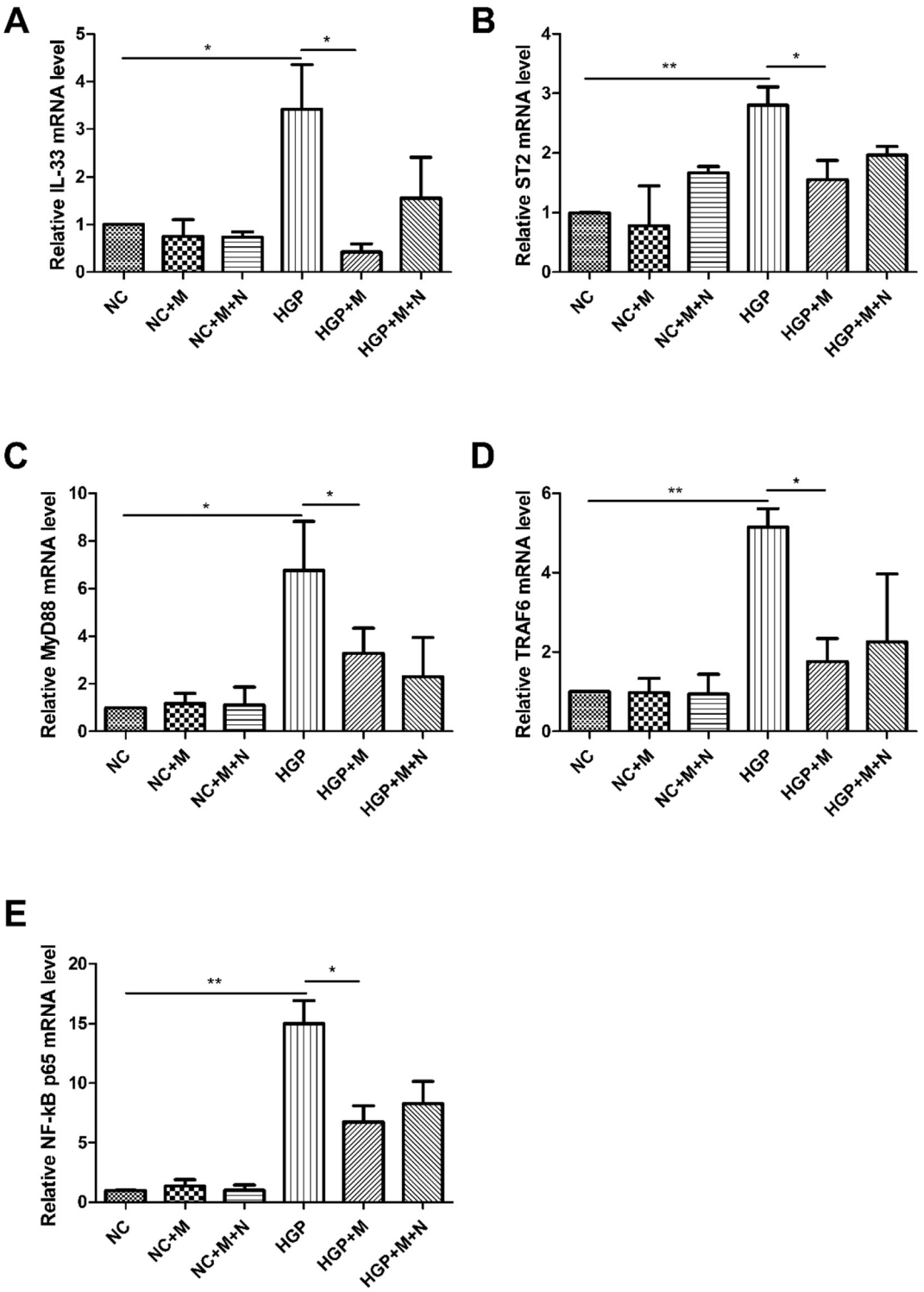


Fig. 9. The mRNA and protein levels of intermediates in the MyD88-TRAF-NF-κB p65 and IL33/ST2 signaling pathways in INS-1 cells. INS-1 cells (1×10^6 normalized) were cultured with different culture media. Cells were incubated in RPMI-1640 containing 1% BSA (NC) that was supplemented with methionine enkephalin (MENK, 10^{-12} mol/L) or with MENK and naltrexone (NTX, 50 nmol/L), and cells were incubated with a final concentration of 25 mmol/L glucose, 0.4 mmol/L palmitic acid in 1% BSA/RPMI-1640 media (HGP) supplemented with or without MENK or MENK and NTX for 48 h. Cells were collected for RT-PCR and western blotting. The mRNA (A–E) and protein (F–K) levels of the corresponding molecules in INS-1 cells were measured. The expression of the MyD88, TRAF6 and NF-κB p65 mRNAs significantly increased in INS-1 cell cultured with HGP media. These increases were inhibited by the MENK treatment. The levels of the MyD88, TRAF6 and NF-κB p65 proteins significantly increased in the HGP group, but the changes significantly reduced by the MENK treatment. The levels of the IL-33 and ST2 mRNAs and proteins markedly increased in INS-1 cells cultured with HGP media. After treatment with MENK (10^{-12} mol/L), the levels of the IL-33 and ST2 mRNAs and proteins restored. Values are presented as means ± SD, $^*p < 0.05$ and $^{**}p < 0.01$.

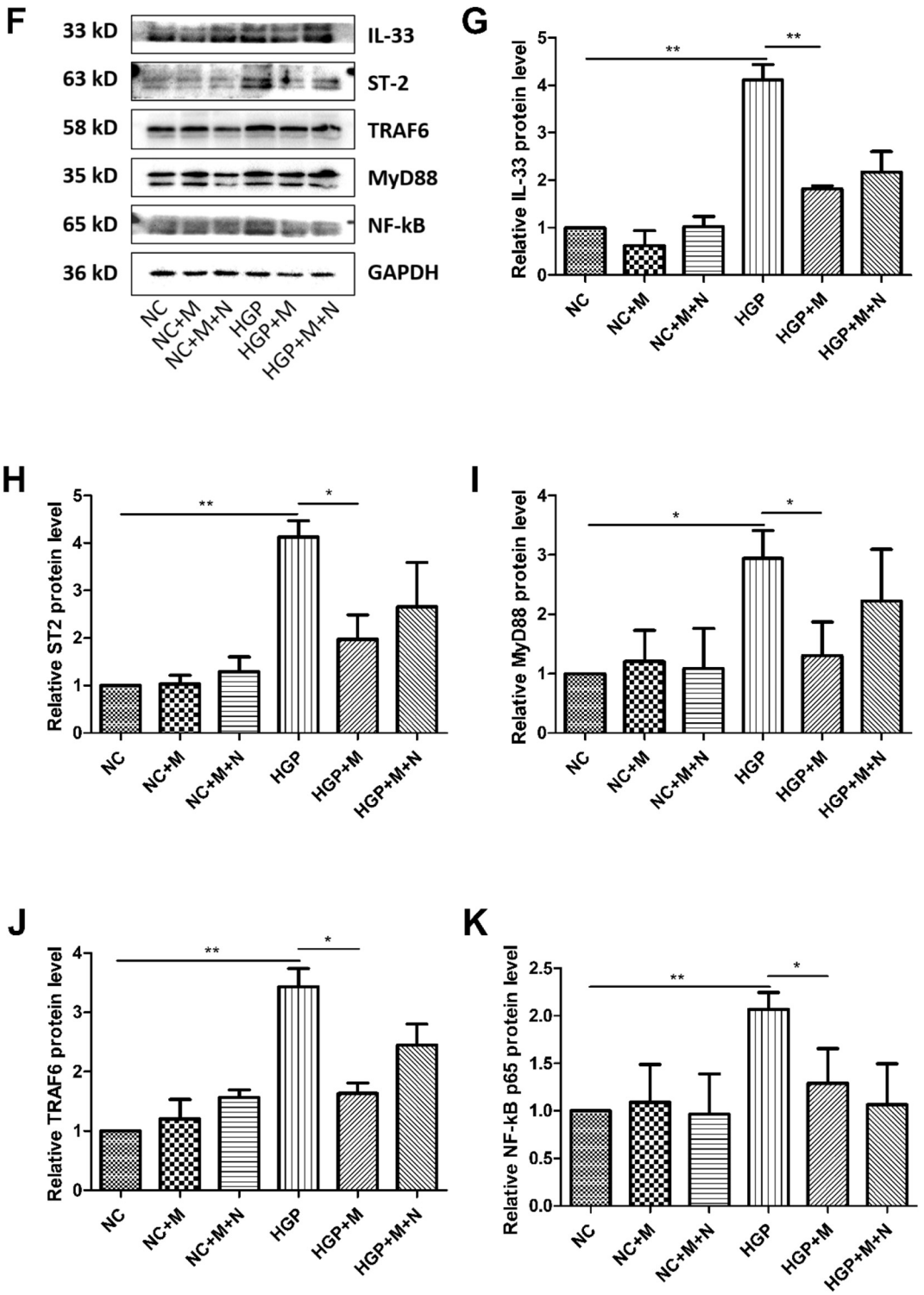


Fig. 9. (*continued*)

(10^{-12} mol/L), the levels of the IL-33 and ST2 mRNAs and proteins restored ($p < 0.05$). The trend towards the inhibition of inflammation was similar to the data obtained from rats in vivo.

3.2.4. MENK regulated the secretion of Th1/Th2 cytokines from INS-1 cells

MENK also inhibited the secretion of inflammatory cytokines in vitro, as shown in Fig. 10. In the HGP group, the inflammatory cytokines TNF-α and IL-2 were secreted at high levels. In contrast, the cytokines IL-5 and IL-10 were secreted at significantly lower levels in the HGP group than that in the NC group. Correspondingly, MENK reduced the levels of the inflammatory cytokine TNF-α compared with that in the HGP group ($p < 0.05$). Significant increases in IL-5 and IL-10 levels were observed in the MENK group ($p < 0.05$) compared to those in the HGP group. The Th1/Th2 ratio shifted towards the Th2 phenotype after the MENK treatment.

4. Discussion

Although multiple treatments have been reported to improve blood glucose control in patients with T2DM, the current medical treatments

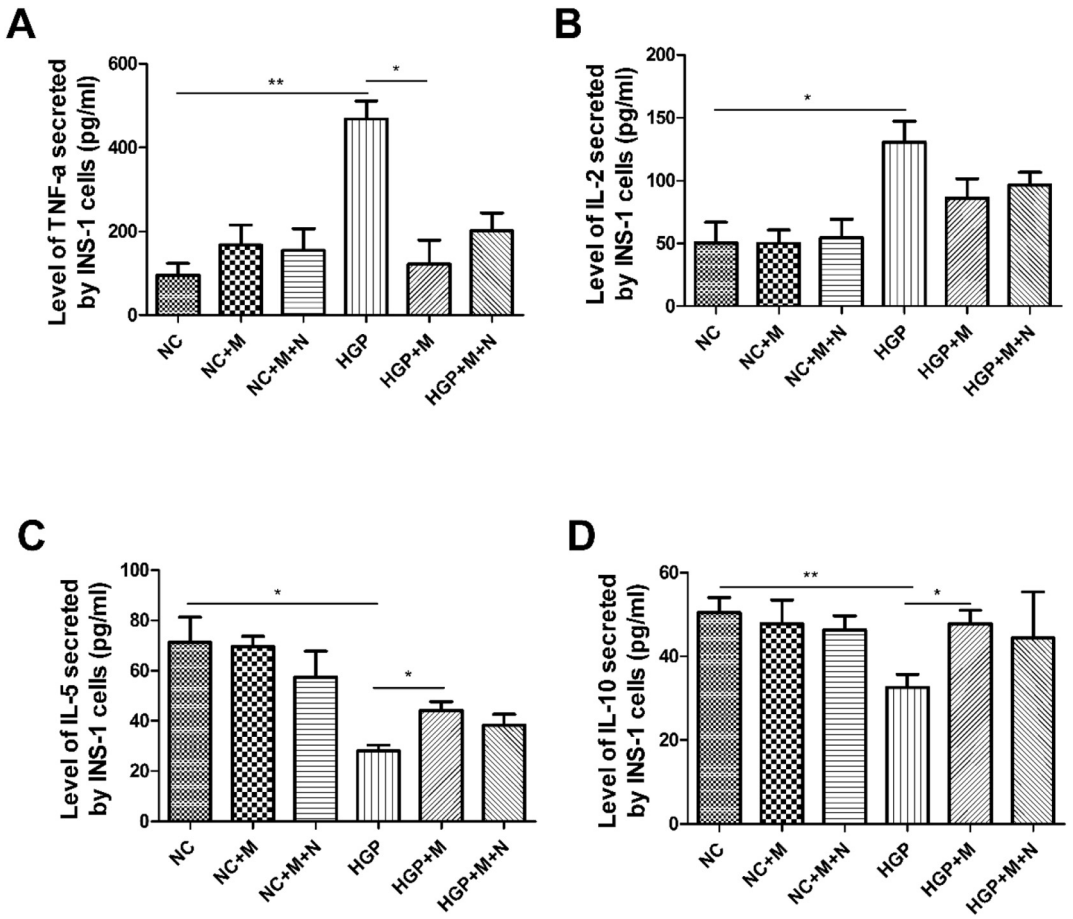


Fig. 10. Levels of cytokines secreted by INS-1 cells. INS-1 cells (1×10^6 normalized) were cultured with different culture media. Cells were incubated in RPMI-1640 containing 1% BSA (NC) that was supplemented with methionine enkephalin (MENK, 10^{-12} mol/L) or with MENK and naltrexone (NTX, 50 nmol/L), and cells were incubated with a final concentration of 25 mmol/L glucose, 0.4 mmol/L palmitic acid in 1% BSA/RPMI-1640 media (HGP) supplemented with or without MENK or MENK and NTX for 48 h. The supernatants (2 mL) were collected to analyze the secretion of TNF-α, IL-2, IL-5 and IL-10 from INS-1 cells cultured with different media for 48 h. The levels of cytokines secreted into the culture media were measured using rat ELISA kits according to the manufacturer's instructions. In the HGP group, the cytokines TNF-α and IL-2 were secreted at higher levels and the cytokines IL-5 and IL-10 were secreted at lower levels than that in the NC group. MENK reduced the levels of the cytokine TNF-α compared with that in the HGP group. Significant increases in IL-5 and IL-10 levels were observed in the MENK group compared to those in the HGP group. The results are presented as means ± SD, $*p < 0.05$ and $**p < 0.01$.

for T2DM are not ideal. Because proper insulin release is crucial to maintain glycemia within a physiological range, enhancing insulin production from β-cells remains an important therapeutic approach [26].

In current study, the plasma glucose levels of T2DM rats increased while the body weights were no longer increasing, and the amount of insulin secreted into serum reduced compared with that in the control group. The number of cells significantly reduced, insulin secretion decreased, and GSIS decreased when INS-1 cells were cultured with high-glucose and palmitic acid medium. Islets are an important site of injury during the occurrence and development of diabetes. Based on HE staining, sections of pancreas generally presented unclear edges and an uneven arrangement of cells, and the number of islet β-cells was significantly reduced in T2DM rats. Elevated glucose and non-esterified fatty acid levels, which are termed glucolipotoxicity, are known to increase islet β-cell death, decrease insulin secretion, induce defects in GSIS and are major contributors to T2DM [27,28].

In current experiment each dose of MENK significantly reduced the plasma glucose level in T2DM rats. Opioid peptides have consistently been shown to influence β-cell secretory function [29–31]. However, these observations are conflicting, with marked distinctions. Now we confirmed that the insulin-secreting function of islet β-cells was significantly improved by the MENK intervention both in vivo and in vitro. The MENK treatment prevented further body weight loss in T2DM rats, with a tendency consistent with improving glycemia. We attempted to resolve the controversy regarding the dose [32], and both low and high MENK concentrations in T2DM rats stimulated insulin secretion in the present study. MENK (10^{-12} mol/L) improved GSIS in INS-1 cells, suggesting that MENK also improved the insulin reserve function of islet cells. Combined with the results of pancreatic HE staining after MENK treatment, we speculated that the increased insulin secretion was partially attributed to the MENK-induced reduction in islet cell damaged and increase in islet cell number. MENK did not exert obvious effect on animals with normal glucose metabolism, indicating that MENK only exerted its therapeutic effect on T2DM rats by increasing insulin levels.

MENK is an opioid peptide that binds to opioid receptors to perform its functions. Three well-known opioid receptors, DOR, MOR and KOR, belong to the seven-transmembrane G coupled-protein receptor family [33]. In the present study, we identified the localization and expression of the three opioid receptors in the pancreatic tissue. The number of opioid receptors in the pancreas of T2DM rats significantly reduced compared with that in the control group, but the expression of opioid receptors significantly increased by the MENK intervention, consistent with the amount of insulin released. However, these levels were not changed in the rats of the three normal control groups. Taken together, we concluded that MENK upregulated the expressions of opioid receptors as part of the mechanism by which it regulates insulin secretion and ameliorates T2DM.

Chronic, low-grade inflammation in islet cells is also thought to play an important role in the development of pathological processes in T2DM. The increased expression of inflammatory factors such as TNF-a and IL-1 activate NF-kB in islet cells and accelerate the cell apoptosis mediated by inflammation [34]. Inflammation also impairs insulin secretion and cell apoptosis after glucose stimulation by damaging the function of the mitochondria, glucose kinase and glucose transporter and by triggering oxidative stress and endoplasmic reticulum stress, among other changes. Glucolipotoxicity initiates inflammatory signaling pathways by activating TLR in β-cells, which induces β-cell apoptosis [4,35]. The TLR family could stimulate the downstream signaling protein MyD88; then, MyD88 binds TRAF and activates the NF-κB signaling pathway to induce the production of pro-inflammatory cytokines, activate inflammatory responses and induce innate and adaptive immunity [36]. Levels of MyD88, TRAF6 and NF-kB p65 increased in the T2DM rats compared with those in the control groups. After the MENK intervention, the expression of MyD88, TRAF6, and NF-κB p65 significantly reduced compared with T2DM rats. Based on this finding, the hypoglycemic activity of MENK is associated with the inhibition of inflammatory responses by downregulating the expression of proteins in the TLR-MyD88-TRAF6-NF-κB p65 signaling pathway. Our findings are similar to published reports that MENK decreases the activity of the TLR7-MyD88-dependent NF-κB p65 signaling pathway to protect against influenza A virus infection [37]. In support of our findings, anti-inflammatory drugs are being developed to treat T2DM [38].

Recently, IL-33 has emerged as a cytokine that plays an important role in several inflammatory diseases [39–41]. In the present study, IL-33 and ST2 expressed at significantly higher levels in the pancreatic tissues of the T2DM group than that in the control group. These results are with published reports that IL-33 is produced by islet mesenchymal cells and its secretion is increased by a diabetes milieu [22]. IL-33 is a member of the IL-1 cytokine family that interacts with a heterodimeric receptor comprising ST2 (encoded by the Il1rl1 gene) and the IL-1 receptor accessory protein (IL-1Racp) [42,43]. When cells are injured or necrotic, intracellular IL-33 is released as an “alarmin” and activates NF-κB, which is required for immune responses and tissue repair [44–47]. As shown in the present study, HE staining of pancreatic tissues from T2DM rats revealed a significant destruction of islet cells. This destruction increased IL-33 expression in T2DM rat islets compared with the normal group. After the MENK intervention, the expression levels of both proteins significantly decreased. We speculated that IL-33/ST2 was down-regulated by the MENK treatment because MENK ameliorated the destruction of islet cells, reduced the release of IL-33 from islet cells, and reduced the inflammatory response of the pancreas.

Inflammation is a critical mechanism leading to β-cell dysfunction and death, in which pro-inflammatory cytokines such as ILs and TNF-α play important roles [48,49], and the levels of multiple cytokines increase in pancreatic islets of subjects with diabetes [50]. Cytokines enable the body to respond rapidly to an immune challenge by coordinating an appropriate immune response [51–53]. In the immune response, antigen-presenting cells secrete a variety of cytokines by stimulating $CD4^+$ T cells to differentiate into Th1 or Th2 cells through a process known as polarization. In this experiment, the serum levels of the pro-inflammatory cytokines TNF-a and IL-2 significantly increased in T2DM rats, while the serum levels of the anti-inflammatory cytokines IL-5 and IL-10 significantly decreased compared with those in the control group, consistent with a previous study [54]. Thus, in T2DM, the Th1/Th2 balance is shifted towards Th1 cells, which secrete pro-inflammatory factors, potentially causing or exacerbating T2DM [55]. Previous study has shown that MENK could inhibit uncontrolled innate immune responses, reduce secretion of inflammatory cytokines, such as TNF-α and IL-1β, through downregulating the TLR7-MyD88-dependent NF-κB p65 signaling pathway [37]. According to our data, MENK significantly reduced the abnormal expression of TNF-α and IL-2 and increased the expression of IL-5 and IL-10 to alleviate the pathological damage to the pancreatic tissue caused by the Th1/Th2 imbalance. Therefore, the increased secretion of Th2 cytokines, decreased secretion of Th1 cytokines and maintenance of the Th1/Th2 balance are essential for controlling T2DM. Thus, MENK regulated the immune response by regulating cytokines production as a treatment for T2DM.

IL-33 plays important roles in inflammatory diseases and autoimmune diseases [56,57]. IL-33 has recently been shown to exert a protective effect on obesity [58–60]. An IL-33 treatment decreases fasting blood glucose levels, and mice lacking the IL-33 receptor ST2 develop hyperglycemia and impaired insulin secretion when fed a high-fat diet [61]. IL-33 polarizes macrophages towards the alternatively activated M2 type and, analogously, T -cells towards Th2 cells, enhances the Th2 response and induces the accumulation of Th2 cytokines in serum [42,62]. Therefore, IL-33 functions as an anti-inflammatory cytokine [47,63]. ST2 is expressed on Th2-associated immune cells such as ILC2s and Th2 cells, which is consistent with the ability of IL-33 to induce the expression of Th2 cytokines [64–66]. In the present study, when IL-33 levels significantly increased in the islets of T2DM rats, IL-33 alternatively activated the M2 type macrophages, induced T -cells to differentiate into Th2 cells and the accumulation of Th2 cytokines, which exerted anti-inflammatory effects and regulated the Th1/Th2 balance to enhance islet cell function. IL-33 has recently been shown to enhance β-cell function through islet-resident ILC2s that produced type 2 immunity-related cytokines, including IL-5 and IL-13, and induce the development of $CD4^+$ Th2 cell-dependent immunity in response to IL-33 [22].

Coincidentally, the production of MENK by ILC2s was confirmed by a flow cytometry analysis of ILC2s purified by cell sorting. Following IL-33 stimulation, ILC2 production of MENK peptides increased in the plasma of patients with diabetes [21,67]. In the present study, when a large amount of IL-33 acted on the ILC2 cells in the pancreatic tissue of T2DM rats, the expression of the ST2 and Th2 cytokines, such as IL-5, IL-10 and MENK, increased on the surface of ILC2 cells. Therefore, we speculate that the increasing IL-33-induced decrease blood glucose levels is related to the effect of increasing MENK concentrations. Therefore, the administration of MENK to T2DM rats significantly reduced blood glucose levels and increase insulin secretion.

Based on these and other previous findings, we concluded that MENK reduced glucose levels and stimulated insulin secretion from the pancreas by regulating immune responses and inhibiting inflammatory responses following its binding to opioid receptors. Thus, MENK might have clinical potential as a therapy and as an adjunct to other hypoglycemic therapies for T2DM.

Conflict of interest

All of the authors formally inform the editorial office that we have no conflicts of interest to declare.

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