科技部補助

大專學生研究計畫研究成果報告

計畫名稱：	Role of PIEZO-type ion channels/protein kinase C signaling in Merkel cells in streptozotocin-induced

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執行計畫學生：李語婕
學生計畫編號：MOST 110-2813-C-040-018-B
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指導教授：曾拓榮

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目錄

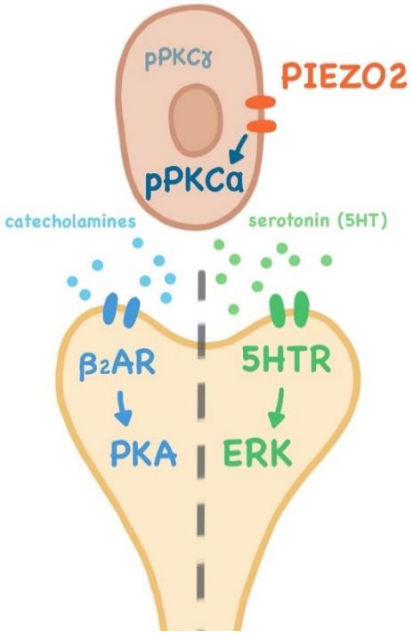
摘要

Diabetic peripheral neuropathy (DPN), one of the chronic complications in human type 1 diabetes mellitus (DM), accompanies an onset of pain symptoms, affecting the quality of life. However, potential mechanisms of peripheral sensitization remain elusive. Merkel cells (MCs) localize in the basal layer of epidermis with their associated primary afferents in dermis to form synaptic-like structures, which is termed as Merkel cell-neurite complex, mediates touch sensation. Our proposal is ongoing investigation regarding whether the interaction between MCs and primary afferents in the skin potentiates a critical molecular cross-talk and, indeed, participates in the maintenance of mechanical hypersensitivity in a rodent model of human type 1 DM**.**

Here, we hypothesize the sequential mechanisms in streptozotocin (STZ)-induced mechanical hypersensitivity, including: (1) MCs activate the PIEZO-type mechanosensitive ion channels/protein kinase C (PKC) signaling for modulating the release of synaptic vesicles; (2) MCs release norepinephrine induces β2 adrenergic receptors (β2ARs)/protein kinase A (PKA) signaling in subepidermal nerve fibers (SENFs); or (3) MCs release tryptophan-derived serotonin (5HT) induces serotonin 1A receptor ($5HT_{1A}R$)/extracellular signal-regulated kinase (ERK) signaling in SENFs to mediate STZ-induced mechanical hypersensitivity.

We established a rodent model of human type 1 DM in Sprague–Dawley rats by a single intraperitoneal injection with STZ (60 mg/kg). About the potential mechanisms of peripheral sensitization in STZ-induced human type 1 DM rats, our results indicate that (1) mechanical hypersensitivity, including mechanical allodynia and mechanical hyperalgesia, were evoked persistently; (2) MCs increase phosphorylated PKC alpha (pPKCα) expressions, which were distinct gathered from the results of phosphorylated PKC gamma (pPKCγ) expression; and (3) progressive dermal denervation was revealed by the reductions of neurofilament 200 (NF200) expression in SENFs. (4) β2AR expressions were not stained in SENFs by the immunohistochemitry. Conversely, phosphorylated PKA (pPKA) expression increased in SENFs. (5) both of 5HTR expression and phosphorylated ERK (pERK) expression were not observed in SENFs.

Even though we could not label PIEZO2 ion channel, β2AR, 5HTR and pERK expressions precisely, we detected intense increases of pPKA expression in SENFs, which means that PIEZO2 ion channel might play a main role in signaling the pain transduction of DPN. Thus, we can target PIEZO2 ion channel/PKCα on the exploration of Merkel cell-neurite complex and investigate the potential mechanisms of peripheral sensitization of DPN.



Keywords: streptozotocin, mechanical hypersensitivity, PIEZO2 ion channel, β2 adrenergic receptor, serotonin 1A receptor, protein kinase A, protein kinase C

前言

Hypersensitivity including hyperalgesia (noxious stimuli), allodynia (innocuous stimuli), and spontaneous pain are caused by progressive peripheral nerve degeneration, especially in human type 1 DM. However, how to set up the clinical strategies for restraining these mechanotransductive pain syndromes to improve the quality of life is very important.

To verify whether the release of synaptic vesicles from MCs activates by PIEZO-type mechanosensitive ion channels/ PKC signaling, which leads to β2ARs/PKA signaling and $5HT_{1A}$R/ERK signaling in SENFs that modulates STZ-induced mechanical hypersensitivity.

研究目的

Our preliminary results showed that an intraperitoneal (i.p.) injection with STZ (60 mg/kg) in rats brought up mechanical allodynia and mechanical hyperalgesia.

We propose experiments in rats injected with STZ to extend these observations by

1) determining whether the potential receptors, PIEZO-type mechanosensitive ion channels (PIEZO2 channels), can be up-regulated for modulating PKC signaling in MCs,

2) determining whether STZ induce the release of monoamines from MCs, which can be measured in dermis,

3) determining whether STZ can affect the integrity of cutaneous SENFs innervation,

4) determining whether β2ARs can be up-regulated in MCs that are expressed similar to PKA activation,

5) determining whether 5HTR can be up-regulated in MCs that are expressed parallel to ERK activation.

These analyses will give us the insight that MCs modulate the release of synaptic vesicles by PIEZO-type mechanosensitive ion channels/PKC signaling. Released norepinephrine activates β2ARs/PKA signaling in SENFs, and released serotonin activates $5HT_{1A}$ R/ERK signaling. The above-mentioned are involved in STZ-induced mechanical hypersensitivity.

文獻探討

Clinically, pancreatic β-cells are destroyed so as to be failure to produce enough insulin in the islets of Langerhans, referred as the human type 1 diabetes mellitus (DM) (Mathis et al., 2001). In type 1 DM patients, the disturbance of glucose metabolism resulted in acute hyperglycemia develops the severe complications, containing the retinopathy, nephropathy, and neuropathy (Tripathi and Srivastava, 2006; Todorovic, 2016). Diabetic peripheral neuropathy (DPN) occurs in the 50 to 70% of type 1 DM patients, which are accompanied nociceptive dysfunctions in a distal symmetrical stocking-and-glove distribution (Todorovic, 2016; Myers et al., 2013). A number of clinical pain symptoms are characterized by the paresthesias, hypersensitivity, and spontaneous pain, have a major influence on the quality of life (Todorovic, 2016). Hypersensitivity is further defined as the decreases of responsive thresholds to noxious stimuli (hyperalgesia) and innocuous stimuli (allodynia), whereas these are setup for assessing the pain symptoms following type 1 DM (Tripathi and Srivastava, 2006; Todorovic, 2016).

Merkel cells (MCs) originated from neural crest cells are found in the epidermis of the skin in vertebrates. Most make contacts with primary afferents in the dermis to form Merkel cell–neurite complexes, which are light-touch receptors (Woo et al., 2014 and 2015; Maksimovic et al., 2014). Piezo2 is known as the Merkel-cell mechano-transduction channel and provide the first line of evidence that Piezo channels have a physiological role in the sense of light touch and pain (Woo et al., 2014; Ranade et al.,

2014). The classic protein kinase C (PKC), including PKC alpha (PKCα), PKC beta (PKCβ), and PKC gamma (PKCγ), play important roles in the regulation of a variety of cellular functions, making those attractive therapeutic targets for a host of clinical diseases (Souza et al., 2002; Zhao et al., 2011). For instance, the phosphorylation of PKCα is involved in the functional expression of extra-synaptic calcium-permeable AMPA receptors in the dorsal horn contribute to maintain complete Freund's adjuvant-induced inflammatory pain (Kopach et al., 2013). Additionally, a persistent increase of PKCγ sustains neuronal plasticity in the dorsal horn following a cellular mechanism of "pain memory" resulting in the activation of NMDA receptors mediates the onset of thermal hyperalgesia and mechanical allodynia (Price and Inyang, 2015; Aira et al. 2013; Zhou et al. 2018).

Primary afferents in the dermis, known as subepidermal nerve fibers (SENFs), has been used to evaluate the integrity of different types of primary afferents. By immunohistochemistry, neurofilament protein 200 (NF200) is expressed in the medium- and large-sized neurons of the dorsal root ganglion, which are restricted to recognize myelinated Aδ and Aβ fibers (Lawson and Waddell, 1991). Interestingly, recent studies demonstrated that norepinephrine is being recognized as a participant in diverse biological events including pain, which is mediated by beta2-adrenergic receptor (β2ARs**) (**Drummond et al., 2020). However, whether MCs or SENFs themselves sense mechanical force is still debated, and the molecular mechanism of mechano-transduction is not completely understood. Intracellular protein kinases mediate the development of chronic neuropathic and inflammatory pain. Interestingly, phosphorylation of protein kinase A (PKA) in the dorsal root ganglion and dorsal horn neurons takes parts in the processing of cancer pain (Zhu et al. 2014; Hang et al. 2012; Hang et al. 2013).

Additionally, it is well known that serotonin is a widely distributed monoamine in both peripheral and central nervous system. It is a participant involved in numerous physiological and behavioral disorders such as pain (Cortes-Altamirano et al., 2018). Studies showed that tactile stimuli trigger vesicular serotonin released from MCs to excite primary afferents in the skin (Chang et al., 2016). In all the 5HT receptors, 5-HT_{1A} receptor is demonstrated to show strong immunoreactions in Merkel cell-nerve endings (Tachibana et al. 2005). 5-HT_{1A} receptors are associated with G proteins, which regulate different kinds of downstream signaling pathways including the extracellular signal-regulated kinase (ERK) signaling pathway (Masson et al., 2012). Notably, findings suggested that nerve injury-induced ERK activation in neurons is essential for the induction of hyperalgesia (Kondo et al. 2020).

研究方法

1. Induction of Human Type 1 Diabetes Mellitus

Adult male Sprague–Dawley rats (200–250 g), were divided into two experimental groups: STZ, which was induced by a single i.p. STZ (pH 4.0, 60 mg/kg, n = 24) injection; and Citrate, i.p. injected with a control citrate solution (pH 4.0, n = 24). All procedures were conducted in accordance with the ethical guidelines set up by the International Association for the Study of Pain (IASP) on the use of laboratory animals in experimental research, and the study protocol was approved by the Animal Committee of Chung Shan Medical University, Faculty of Medicine, Taichung, Taiwan (IASP Committee, 1980).

2. Experimental Design

All rats from the STZ and from the Citrate were monitored the metabolic parameters, containing blood glucose levels and HbA1c levels every 2 weeks. Blood glucose level was tested by a one-touch glucose meter (Johnson & Johnson, US) and HbA1c level was measured using the Test Kit Glyco-Tek Affinity Column Method (Helena Laboratories, Beaumont, TX) (Lin et al., 2008). Behavioral assessments were

evaluated at the following time points: PTW 0 (designated as the pretest baseline data), PTW 2, PTW 4, PTW 8, and PTW 16. Then, all rats were sacrificed periodically at the same time points.

3. Behavioral assessments

A. Mechanical allodynia:

Innocuous stimulation is determined by measuring the withdrawal thresholds to a series of calibrated Senselab aesthesiometer (Somedic Sales AB) according to an up-and-down method. Mechanical threshold is defined as the minimal force (g) initiating a withdrawal response (Ko et al., 2016).

B. Mechanical hyperalgesia:

Mechanical hyperalgesia is evaluated by the noxious pinprick stimulation (A Von Frey–type 0.5 mm filament) with Dynamic plantar aesthesiometer (Code: 37450, Ugo Basile) (Samur et al., 2018). Minimal force (g) is automatically measure as the time elapsed from the onset of rounded tip stimulation to the withdrawal of hindpaw. Each hindpaw is alternatively tested five times with a minimal interval of 5 min between measurements. The values of measurements are used for the analysis and average as a withdraw threshold.

4. Immunohistochemistry

At the end of experiments, rats are anesthetized with isoflurane and sacrificed by an intracardiac perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. Footpad skins are fixed for another 6 h and then changed to 0.1 M PB for storage. After a through rinsing in PB, samples are cryoprotected with 30% sucrose in 0.1 M PB overnight.

The footpad skin perpendicular to the epidermis is sectioned at 30 μm on a sliding microtome (HM440E; Microm), labeled sequentially and stored at -20C. Sections are treated with 0.5% Triton X-100 in 0.5 M Tris buffer (Tris), pH 7.6, for 30 min and processed for immunostaining. Briefly, sections are quenched with 1% H_2O_2 in methanol and blocked with 5% normal goat serum in 0.5% nonfat dry milk/Tris. Sections are incubated with primary antisera against: (1) rabbit PIEZO2 polyclonal antibody (1:200, Invitrogen); (2) rabbit PIEZO2 polyclonal antibody (1:200, Proteintech); (3) rabbit phospho-PKCα (pPKCα, 1:1000, Cell Singnaling); (4) rabbit phospho-PKCγ (pPKCγ, 1:1000, Epitomics); (5) rabbit polyclonal neurofilament 200 (NF200, 1:1000, Sigma Chemicals); (6) rabbit phospho-PKA (pPKA, 1:1000, Cell singnaling); (7) rabbit β2ARs (1:200, Invitrogen); (8) mouse β2ARs (1:50, Santa Cruz); (9) rabbit β2ARs (1:200, Bioss); (10) mouse anti-serotonin receptor 1A (1:50, Millipore); (11) rabbit phosphor-p44/42 MAPK (Erk1/2)(1:200, Cell Signaling) at 4 °C overnight. After rinsing in Tris, sections are incubated with biotinylated goat anti-mouse IgG/biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h and the avidin–biotin complex horseradish peroxidase reagent (Vector Laboratories) for another hour. Reaction products are demonstrated with 3,3-diaminobenzidine (DAB, Sigma Chemicals).

5. Quantitation

A. SENFs areas

The standard procedure for measuring immunoreactive (ir) SENFs area is carried out according to a protocol published in our previous study (Ko et al., 2016).

B. Merkel cells

Standard procedure is following a protocol modified from a published method previously. Briefly, we first photographed the high-definition monochrome images under an Olympus microscope (BH2; Olympus) with a digital camera at a magnification of 100×. All the ir cells in epidermis are calculated with the Image Pro-Plus software (Media Cybernetics) and plotted as the histogram of the cell diameter.

6. Statistical analysis

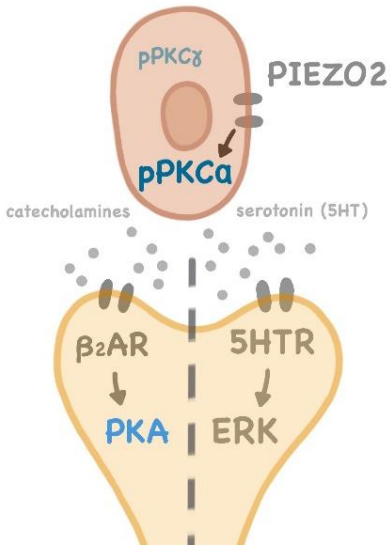
The examiners are blinded to the grouping information when performing the procedures of measurement and quantification. Data following the Gaussian distribution are expressed as mean ± standard derivation of the mean and analyzed with parametric tests. Data not following the Gaussian distribution are analyzed with the nonparametric Mann–Whitney test. Data are analyzed by GraphPad Prism (GraphPad, San Diego, CA). $p< 0.05$ is considered statistically significant. Data from experiments are also performed by one-way repeated measures ANOVA with the post hoc Tukey–Kramer multiple comparison test between three or more independent groups.

結果與討論

糖尿病周邊神經病變（Diabetic peripheral neuropathy）的病理變化包括急性神經纖維異常，接著是慢性神經纖維損傷、萎縮及神經消失。神經纖維的消失是漸進性的，最主要的病理變化是神經軸突消失合併次發性的髓鞘脫失。神經病變是因神經軸突退化所致，由遠端向近端進行，因此神經的末端受傷最嚴重。神經疼痛是伴隨糖尿病和其他疾病的一種常見病痛，包含：1.身體有疼痛感覺（夾鉗感、灼燒感、撕裂感），病因是負責傳導疼痛、溫度和觸覺信號的感覺神經出現損傷；用藥物抑制這種病痛很難見效。2.遠端皮膚感覺異常及知覺缺乏（麻木感，針刺感）易肌肉無力、萎縮。自發性的疼痛反應，疼痛覺過度敏感反應（有害的刺激）以及機械性造成的疼痛覺敏感反應（無害的刺激）現象，都屬於周邊神經損傷產生病變而引起的疼痛反應症狀，且明顯地改變生活的情緒與降低生活的品質。

動物實驗尚未有完整系統性的評估糖尿病周邊神經病變（Diabetic peripheral neuropathy），說明罹患糖尿病病後動物在神經病變併發症的相關研究。本實驗研究鏈脲佐菌素(Streptozocin, STZ)誘發糖尿病動物模式的可能性，並建立在高血糖狀況下造成神經病變併發症引發之疼痛行為，此動物模式能提供一致性且持續性的疼痛症狀表現，並能模擬在臨床上糖尿病周邊神經病變性引發疼痛反應的許多特徵。這些相關性的探討可以藉由背根神經節的周邊小直徑無髓鞘 C 和有髓鞘 Aδ 神經纖維和有髓鞘 Aβ 神經纖維在皮膚內真皮層內的支配表現，評估疼痛行為功能的變化。也可從梅克爾氏細胞(Merkel cells (MCs))和梅克爾神經末梢(Merkel nerve endings)形成梅克爾氏細胞神經突複合物(Merkel cell-neurite complex)來說明傳遞疼痛感覺訊息的路徑活化。證據表明常規的蛋白激酶 C(Protein kinase C (PKC))含有同種型 α，βI，βII 和 γ 可能在疼痛調節中發揮重要作用。因此，在此研究中說明蛋白激酶 Cα(PKCα)在梅克爾氏細胞(MCs)內的調節與神經病變性疼痛症狀的關係。

先前實驗研究並未能在誘發糖尿病的動物模式，建立相關的疼痛感覺訊息的路徑。本計畫主要貢獻是供在誘發糖尿病的動物模式中，顯著地分析影響疼痛行為在周邊神經系統涵蓋的重要機制。以期未來能對臨床上健康醫學針對藥物治療策略的機制有更深層的理解，進而能藉由藥物控制來摒除糖尿病神經病變帶來的疼痛行為和改善生活的品質。



1. Metabolic Parameters in STZ-induced Diabetic Rats

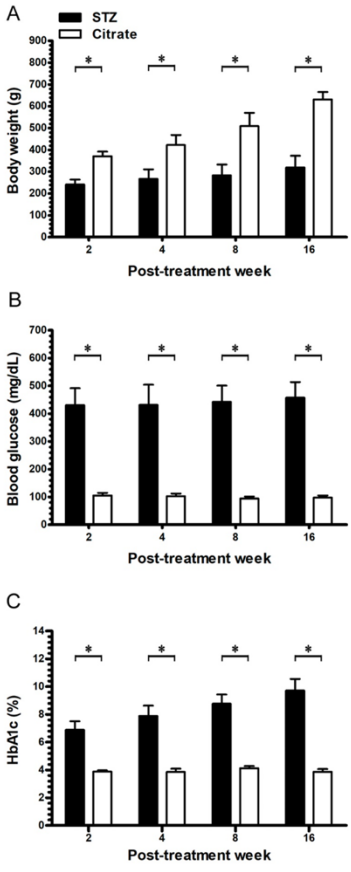


Figure 1. Metabolic parameters in the streptozotocin (STZ)-induced diabetic rats. Graphs showed the temporal changes of (a) blood glucose levels and (b) hemoglobin A1c (HbA1c) levels in the Citrate (open bars) and the STZ (filled bars). All the measurements were expressed as the mean ± standard deviation (SD) (n = 3 in the Citrate and n = 3 in the STZ were sacrificed at each post-induction week (PIW)). * $p < 0.05$ indicated as a significant difference.

2. Mechanical hypersensitivity after STZ induction

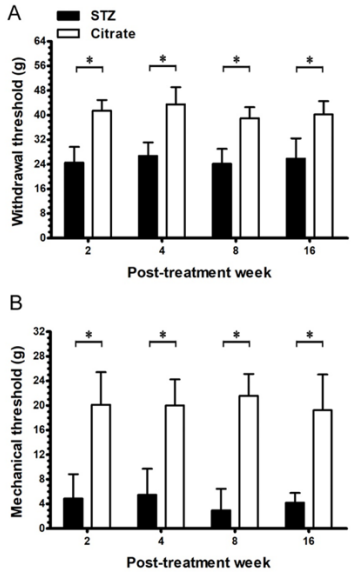


Figure 2. Mechanical hypersensitivity following STZ induction in rats. The temporal changes of mechanical hypersensitivity were shown in (A) hyperalgesia and (B) allodynia. The mechanical threshold of pinprick was demarcated as withdrawal threshold (g). The degree of mechanical allodynia was represented as the mechanical threshold (g) in response to vonFrey monofilaments. * $p < 0.05$ indicated as a significant difference.

3. Expressions of PIEZO2 in the STZ-induced Diabetic Rat Skin

Invitrogen catalog # PA5-2976

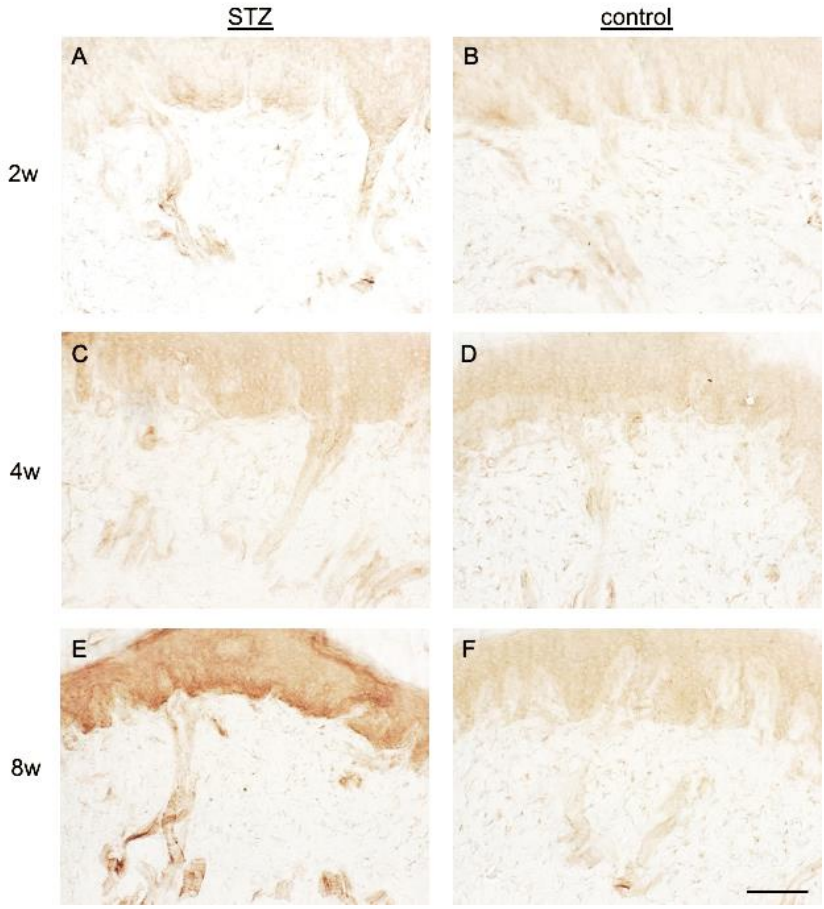


Figure 3-1. Expressions of PIEZO2 in the STZ-induced Diabetic Rat Skin. The sections were immunostained with the antisera against PIEZO2 from Invitrogen at post-treatment week 2. Images showed the (A, B) superficial layer (C, D) , middle layer (E, F) , and deep layer of (A, C, E) STZ group and (B, D, F) control group. Scale bar = 100 μm.

Proteintech catalog # 26205-1-AP

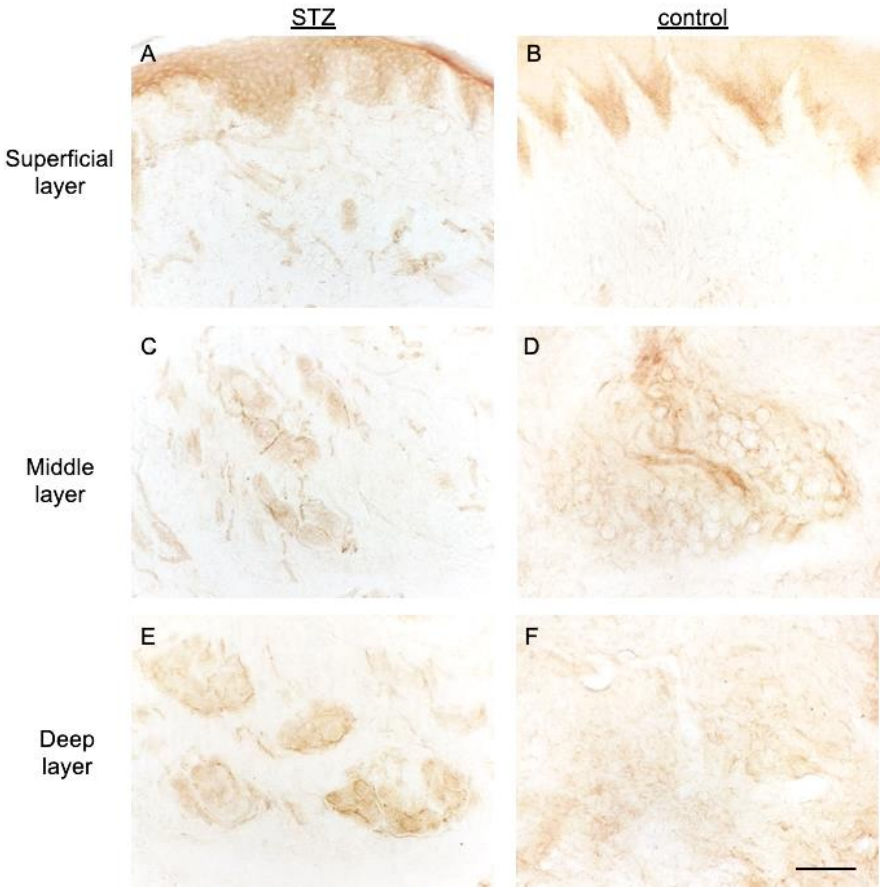


Figure 3-2. Expressions of PIEZO2 in the STZ-induced Diabetic Rat Skin. The sections were immunostained with the antisera against PIEZO2 from Proteintech at post-treatment week 2. Images showed the (A, B) superficial layer (C, D) , middle layer (E, F) , and deep layer of (A, C, E) STZ group and (B, D, F) control group. Scale bar = 100 μm.

4. Expressions of pPKCα and pPKCγ in MCs in the STZ-induced Diabetic Rat Skin

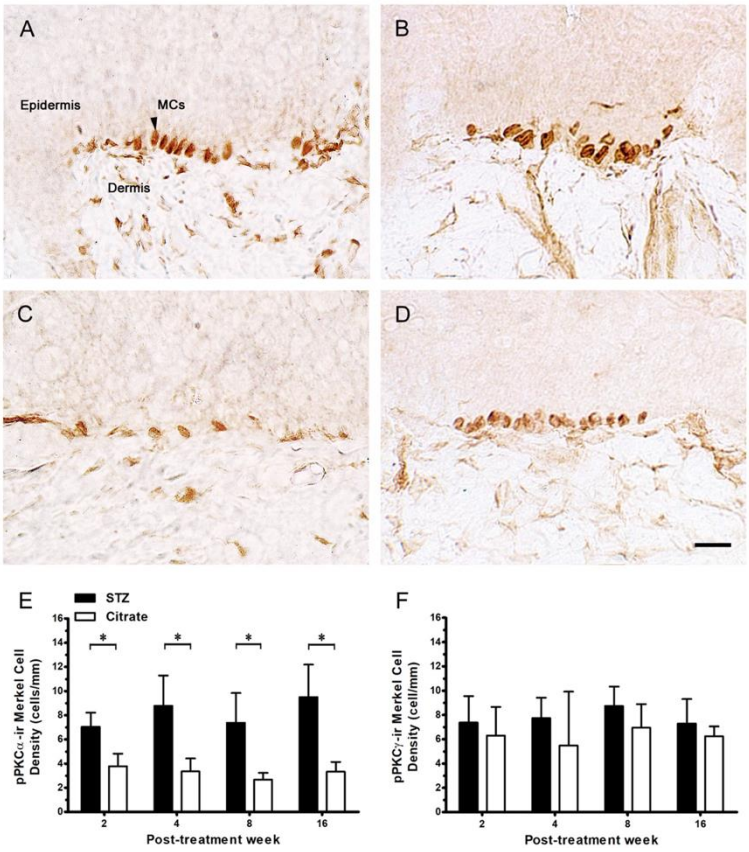


Figure 4. STZ-induced changes of Merkel's cells (MCs) distribution. The sections were immunostained with the antisera against (A, C) phosphorylated protein kinase C alpha (pPKCα) and (B, D) phosphorylated protein kinase C gamma (pPKCγ) at post-treatment week 2. (E, F) Panels showed the temporal changes of quantitative MCs density. * $p < 0.05$ indicated as a significant difference. Scale bar = 25 μm.

5. Distributions of SENFs and PKA in the STZ-induced Diabetic Rat Skin

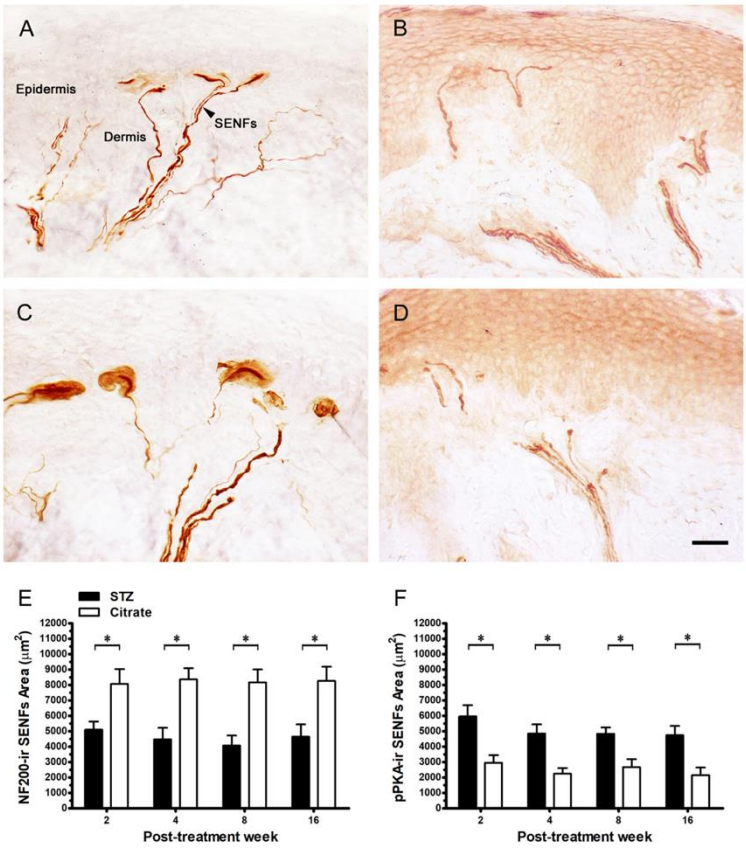


Figure 5. Influences of STZ on subepidermal nerve fibers (SENFs) distribution. The sections were immunostained with the antisera against (A, C) neurofilament 200 (NF200) and (B, D) phosphorylated protein kinase A (pPKA) at post-treatment week 2. (E, F) Panels showed the temporal changes of quantitative immunoreactive (ir) SENFs areas. * $p < 0.05$ indicated as a significant difference. Scale bar = 50 μm.

6. Expressions of β2AR in the STZ-induced Diabetic Rat Skin

Invitrogen catalog # PA5-77283

Santa Cruz sc-271322

Bioss bs-0947R-TR

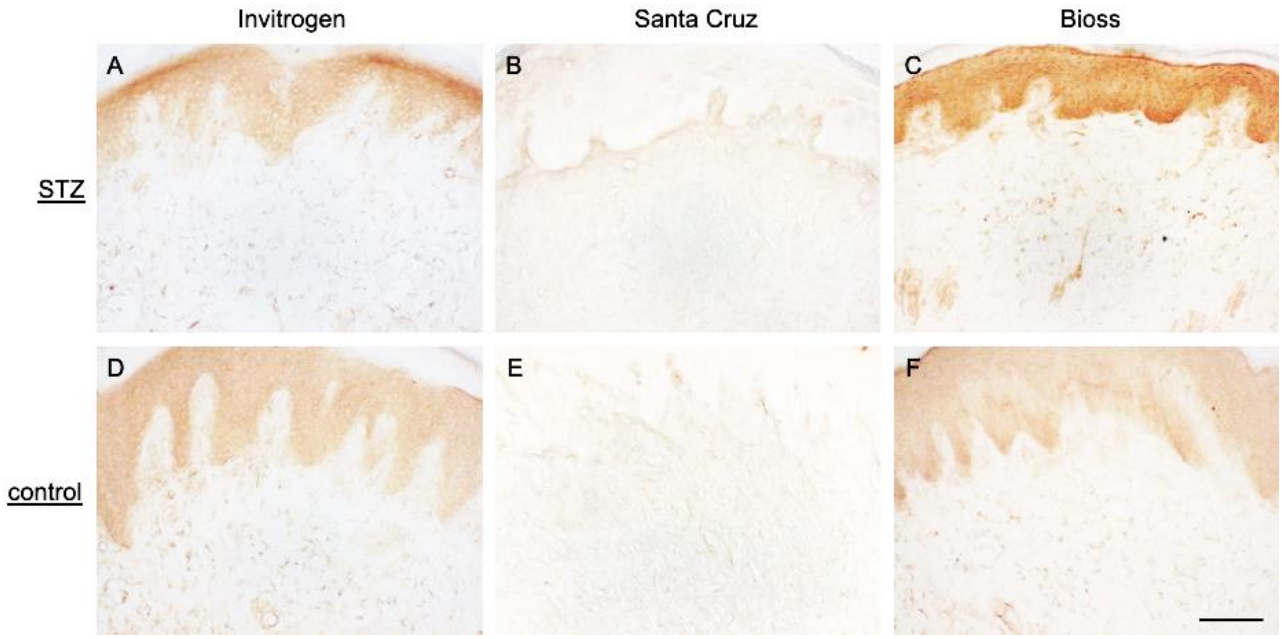


Figure 6. Expressions of β2AR in the STZ-induced Diabetic Rat Skin. The sections were superficial layer of the STZ-induced diabetic rat skin immunostained with the antisera against β2AR respectively from Invitrogen, Santa Cruz and Bioss at post-treatment week 2. Images showed (A, B, C) STZ group and (D, E, F) control group of (A, D) Invitrogen, (B, E) Santa Cruz and (C, F) Bioss. Scale bar = 100 μm.

7. Expressions of $5HT_{1A}R$ and pERK in the STZ-induced Diabetic Rat Skin

MILLIPORE catalog # MAB11041

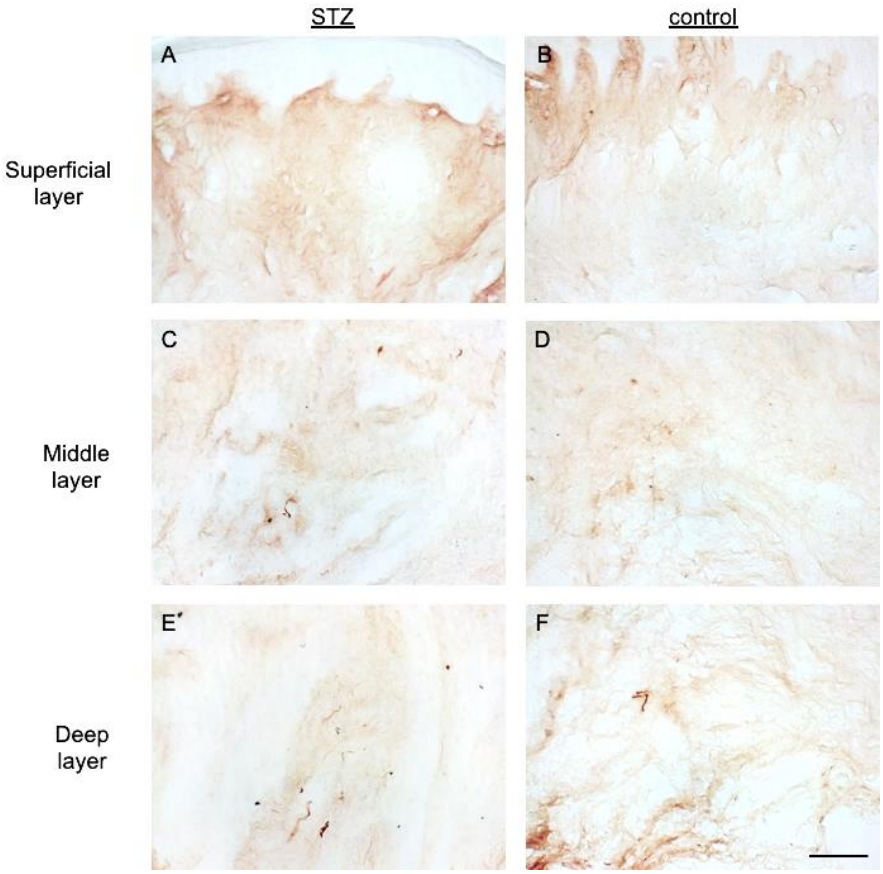


Figure 7-1. Expressions of $5HT_{1A}R$ in the STZ-induced Diabetic Rat Skin. The sections were immunostained with the antisera against 5HT at post-treatment week 2. Images showed the (A, B) superficial layer (C, D) , middle layer (E, F) , and deep layer of (A, C, E) STZ group and (B, D, F) control group. Scale bar = 100 μm.

Cell Signaling #4370

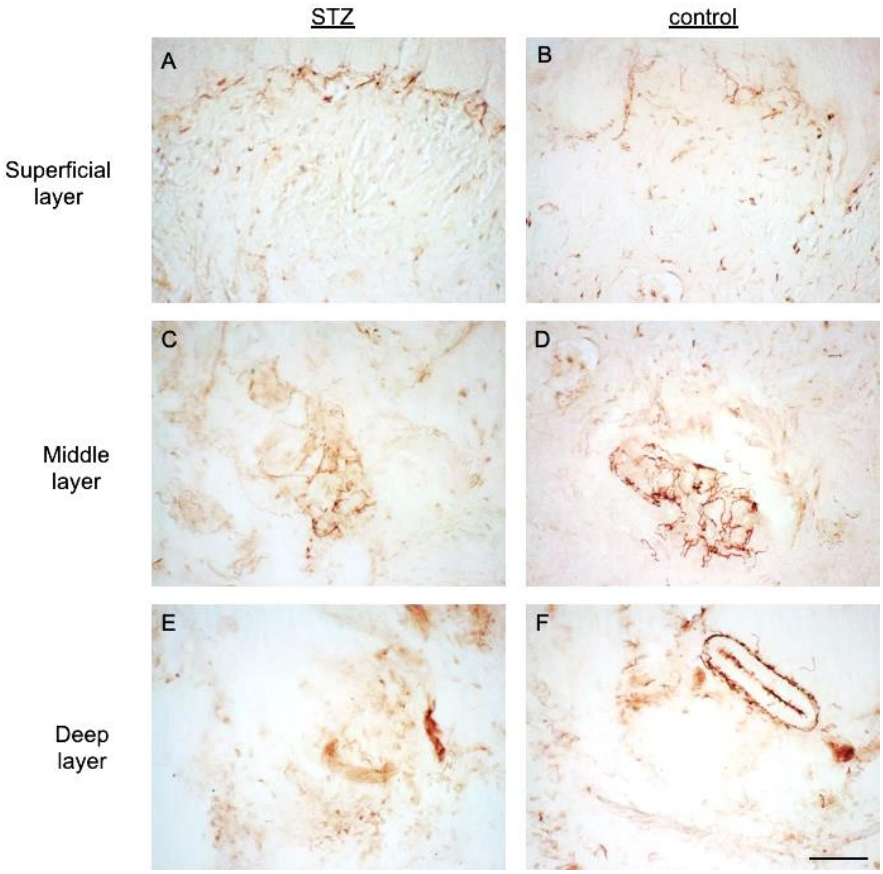


Figure 7-2. Expressions of pERK in the STZ-induced Diabetic Rat Skin. The sections were immunostained with the antisera against pERK at post-treatment week 2. Images showed the (A, B) superficial layer (C, D) , middle layer (E, F) , and deep layer of (A, C, E) STZ group and (B, D, F) control group. Scale bar = 100 μm.

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