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Glucose concentration in culture medium affects mRNA expression of TRPV1 and CB1 receptors and changes capsaicin toxicity in PC12 cells

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ABSTRACT

Objective (s): Hyperglycemia is widely recognized as the underlying cause for some debilitating conditions in diabetic patients. The role of cannabinoid CB1 and vanilloid TRPV1 receptors and their endogenous agonists, endovanilloids, in diabetic neuropathy is shown in many studies. Here we have used PC12 cell line to investigate the possible influence of glucose concentration in culture medium on cytoprotective or toxic effects of a CB1 [WIN55 212-2 (WIN)], or TRPV1 [Capsaicin (CAS)] agonist.

Materials and Methods: Cell viability was tested using the MTT assay. We have also measured TRPV1 and CB1 transcripts by real time reverse transcription-polymerase chain reaction while cells were grown in low (5.5 mM) and high (50 mM) glucose concentrations.

Results: Real time PCR results indicated that high glucose medium increased (P<0.01) TRPV1 mRNA and decreased (P <0.001) that of CB1. Cell culture tests show that hyperglycemic cells are more vulnerable (Dose × Medium, F (3,63)=41.5, P<0.001) to the toxic effects of capsaicin compared to those grown in low glucose medium.

Conclusion: These findings propose that hyperglycemic conditions may result in neuronal cell death because of inducing a counterbalance between cytotoxic TRPV1 and cytoprotective CB1 receptors.

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Introduction

Diabetic neuropathy is a common complication of diabetes which affects around 50 percent of patients. It has various expressions and is persistent to treatment (1). There is much debate about the pathogenesis of this condition but a lot of studies consider hyperglycemia as the main initiating factor (2-5). Hyperglycemia may lead to neuropathy as a consequence of increasing oxidative stress (6-8), formation of glycation end products (9, 10), accumulation of polyols (11, 12), nerve ischemia due to vasculopathies (13-15) ,etc. Peripheral diabetic neuropathy (PDN) may alter pain sensation (hyper-, hypoalgesia, allodynia) or leave it unchanged (normalgesia)(16).

It is shown that intraneural Ca^{2+} concentration is an important underlying cause that may participate in PND by changing intracellular calcium signaling (17-20). Intracellular calcium concentration is a variable which is, depending on the specific cell type, affected by the integration of many signaling pathways.

Elevated Ca^{2+} may lead to neuronal excitability (sensitization) (21) or death (22), both responsible for diabetic neuropathies (16).

TRPV1 is a nonselective cation channel that belongs to the transient receptor potential (TRP) family of ion channels. It is activated by capsaicin and is responsible for the painful sensation of red peppers. This channel is distributed in many tissues and organs of the body. It senses a vast range of stimuli (temperature, low extracellular pH and some lipid derivatives) and plays role in many physiologic and pathophysiologic conditions. many studies show that it has a crucial role in regulating calcium signaling in many cultured cell lines (23-27). CB1 is a G protein-coupled receptor (GPCR) that is activated by the natural compound (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC) and the endogenous endocannabinoids. The role of CB1 receptors in a variety of conditions such as anxiety, memory, immune function, depression, schizophrenia, Parkinson's disease, etc. is widely investigated.

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Anandamide is the endogenous ligand for both CB1 and TRPV1 receptors (28).

Many studies show that TRPV1 and CB1 receptors, expressed in a single cell, have opposing effects (29-33). There is some evidence to support the idea that changes in CB1/TRPV1 receptor signaling alters the cell response to anandamide or other exogenous more specific ligands (34, 35).

In our study, we have hypothesized that hyperglycemic medium for PC12 cells would cause an imbalance in CB1/TRPV1 signaling in these cells. We test this hypothesis by determining TRPV1 and CB1 mRNA expression in cells grown in normal and hyperglycemic conditions. We also measured toxic effects of capsaicin and protective effects of WIN55 212-2 in different glycemic conditions. The usefulness of PC12 cell line for studying functions and/or expression of TRPV1 and CB1 is discussed elsewhere (34, 36).

Materials and Methods

Cell culture

Rat adrenal pheochromocytoma PC12 cells were grown in RPMI 1640 medium with sodium bicarbonate and L-Glutamine (Sigma-Aldrich) with 10% (v/v) fetal calf serum, penicillin/streptomycin (100 U/ml and 100 µg/ml, Biochrom AG). Maintenance was at 37˚C and 5% CO_2. D-glucose (Sigma-Aldrich) solutions were added to the medium to achieve final glucose concentrations of 5.5 (low glucose) and 50 mM (high glucose), simulating low-glycemic and hyper-glycemic conditions, respectively. D-mannitol (50 mM) was added to some 5.5 mM glucose medium samples to compensate for the higher osmolality. Cells were cultured for 5 days in 6 well dishes and medium was replaced every 2 days. Cells were then transferred to 96 well plates (5000 per well) and incubated for 24 hrs. After settlement of the cells the medium was aspirated and new low or high glucose media enriched with the proper concentration of the drugs were added to the cells.

Drugs

Capsaicin, capsazepine, WIN55 212-2 and AM251 were obtained from Alexis Biochemicals. All drugs were dissolved in dimethyl sulfoxide (DMSO) and added to the media with DMSO concentration not more than 1%. Drug enriched media were added to the cells 24 hr before the MTT assay. To find the minimum toxic concentration of Capsaicin the drug was used at 10, 100, and 200 µM in low and high glucose medium. Cytoprotective effects of WIN 55,212 (at 1, 5, 20 µM) were determined on the minimum toxic concentration of capsaicin (10 µM) in low and high glucose conditions. AM251 (10 µM), capsazepine (10 µM), capsaicin (10 µM) and WIN55 212-2 (10 µM) were coadministered to see the possible interaction of drugs with receptors.

Cell viability (MTT) assay

Cell viability was measured using the colorimetric assay in which live cells can reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan crystal. 24 hr after the treatments cells were incubated with MTT (0.5 mg/ml, Sigma-Aldrich) for 4.5 hr at 37°C and then the wells were aspirated and the blue formazan crystals were dissolved in DMSO for 5 min. The plate was read in a ELx808 microplate reader (Biotek) at 550 nm. Data were collected from 9 independent experiments.

RNA extraction

Total RNA was extracted (n=6) using a High Pure RNA isolation kit (Roche Applied Science, Indianapolis, IN, USA) according to kit instructions.

Reverse transcription

A total volume of 20 µl containing: total RNA 10 µl (0.1 µg/µl), dNTPs (10 mM) 2 µl, expand reverse transcriptase (Roche) 1 µl, oligo (dT) 15 (20 pmol) 1 µl, dithiothreitol (DTT) 2 µl and buffer 4 µl was used for the reverse transcription procedure. The temperature was 42˚C and the reaction ran for 60 min.

Real time polymerase chain reaction

cDNA amplification was done in StepOnePlus real time PCR System (Applied Biosystems,CA, USA).

The internal standard was Beta-actin and the specific primers for CB1 and TRPV1 were as follows: CB1 forward primer 5'-CgT-CgT-TCA- Agg-AgA-ATg-Agg-3', CB1 reverse primer 5'-TgC-CgA-TgA-AgT-ggT-Agg-AAg-3', TRPV1 forward primer 5'-gCg-AgT-TCA-AAg-ACC-CAg-A-3' and TRPV1 reverse primer 5'-ATT-CTC-CAC-CAA-gAg-ggT-CA-3'(37). The reaction contained the following compounds: 6 µl of Power SYBR Green PCR Master Mix 2X (Applied Biosystems, CA, USA), 20 pmol of the primers (0.24 µl), 1.2 µl of template, 4.56 µl RNase free water. The total volume of the reaction was 12 µl. The amplification profile for the genes was as follows: The holding stage (95˚C for 15 min), the cycling stage (denaturation 15 Sec in 95˚C, combined annealing/extension 60 Sec in 60˚C). The number of cycles was 40.

Data analysis

The influence of the medium glucose concentration on capsaicin toxicity was analyzed using two-way analysis of variance (ANOVA). Comparisons versus control group was done using Holm-Sidak post comparisons. Student t-test was used to compare the effect of individual drugs with each other where appropriate. Data are shown as means± SEM. Data were considered significant when

$P< 0.05$. The results of real time PCR were analyzed by the software REST 2008 (Corbett research Pty, V 2.0.7). This software uses the equation $2^{-\Delta\Delta CT}$ (relative quantification method) to calculate the fold change expression ratio of genes. The principles of this method are discussed elsewhere (38).

Results

Toxic effects of capsaicin on PC12 cells in low and high glucose medium

PC12 cells were incubated for 24 hr with doses of 0, 10, 100, 200 μM of capsaicin which were grown in a low (5.5 mM) or high (50 mM) glucose medium for 5 days. The results of capsaicin effects on viability are shown in Figure 1. Two way ANOVA results showed that viability was decreased in high glucose compared to low glucose medium (Glucose, F (1,63)=136.4, $P<0.001$). Viability was decreased at higher doses of the drug both in low and high glucose medium (Dose, F (3,63)=134.3, $P<0.001$) and decrement of viability was greater in the medium with higher glucose concentration (Dose × Medium, F (3,63)=41.5, $P<0.001$).

Cytoprotective effects of WIN55 212-2 on toxic effects of capsaicin

To find out if a CB1 agonist has protective effects on toxicity induced by capsaicin, WIN55 212-2 (0, 1, 5, 20 μM) and capsaicin (10 μM) were added to low and high glucose media 24 hr before MTT assay (Figure 2).

The results show that in the low glucose medium the viability of cells is statistically different ($P< 0.05$) when WIN55 212-2 concentrations are 1, 5 μM compared to when they are grown in WIN55 212-2-free medium. In high glucose conditions viability is only improved ($P<0.05$) when the concentration of WIN55 212-2 is 20 μM.

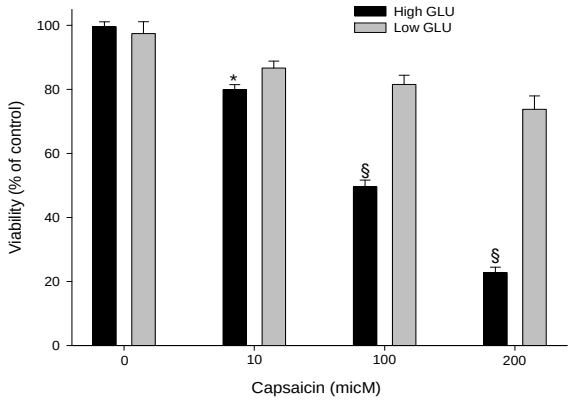

Figure 1. Effect of glucose and capsaicin concentrations in the medium on viability of PC12 cells. MTT was used to measure the viability of cells 24 hr after they were exposed to different (0, 10, 100, 200 μM) capsaicin concentrations. * P =0.02 compared to low glucose medium.§ $P< 0.001$ compared to low glucose medium. The results are shown as mean±SEM

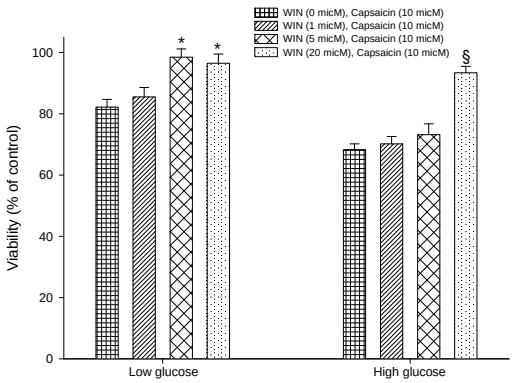

Figure 2. Cytoprotective effects of WIN55 212-2 on toxicity induced by capsaicin. WIN55 212-2 (0, 1, 5, 20 μM) and capsaicin (10 μM) were co-administered on PC12 cells cultured in low (5.5 mM) and high (50 mM) glucose media. MTT was used to measure viability. *$P<0.05$ compared to WIN (0 μM) in low glucose medium. § $P<0.001$ compared to WIN (0 μM) in high glucose medium. The results are shown as mean±SEM. WIN; WIN55 212-2

Effect of TRPV1 and CB1 antagonist on toxic and protective effects of TRPV1 and CB1 agonists

To see the effects of drug antagonism capsazepine (10 μM), as a TRPV1 antagonist was co-administered with capsaicin (10 μM) 24 hr before MTT assay. In another experiment, AM251 (10 μM), as a CB1 antagonist, was co-administered with capsaicin (10 μM) and WIN55 212-2 (10 μM). Tests were done in cells which were grown in low (5.5 mM) and high (50 mM) glucose media for 5 days (Figure 3). T-test results show that in both media co-administration of capsazepine can improve ($P<0.05$) viability of cells compared to capsaicin alone. The results also show that when a CB1 antagonist, AM251 (5 μM), is added to capsaicin and WIN55 212-2 the improving effects of WIN55 212-2 is ablated and the viability of cells is not statistically significant ($P>0.05$) with capsaicin.

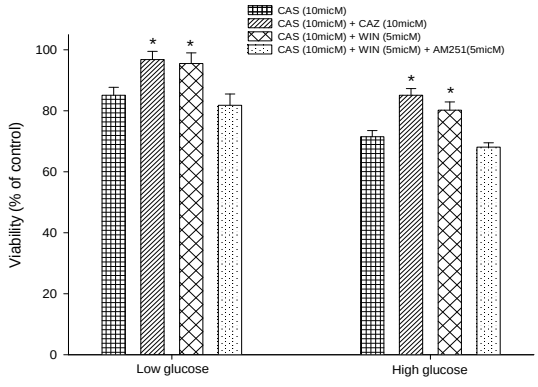

Figure 3. Effects of TRPV1 and CB1 antagonism on PC12 cells exposed to capsaicin. Capsazepine (10 μM) was co-administered with capsaicin (10 μM) and AM251 (5 μM) was co-administered with capsaicin (10 μM) and WIN55 212-2 (5 μM) to see if the effects of agonists were blocked by antagonists. All drugs were administered 24 hrs before the MTT assay. *$P<0.05$ compared to CAS (10 μM). The results are shown as mean±SEM. CAS; capsaicin, CAZ; capsazepine, WIN; WIN55 212-2

Table 1. Effects of medium glucose concentration on CB1 and TRPV1 expression in PC12 cells

	CB1 expression ratio	TRPV1 expression ratio
Low glucose	1	1
High glucose	0.04±1.25	8.7±2.3
P value	<0.001	<0.01
Result on mRNA expression	down-regulation	up-regulation

TRPV1 is up-regulated in high glucose medium in comparison to low glucose medium by an expression ratio of 8.7±2.3 and CB1 receptor is down-regulated in high glucose medium by an expression ratio of 0.04±1.25

Effects of low and high glucose concentration on mRNA expression of CB1 and TRPV1

Data analysis indicates that mRNA for CB1 is down-regulated in high glucose compared to low glucose medium. The fold change in expression ratio of CB1 is 0.04 (P<0.001). TRPV1 mRNA is up-regulated and the fold change in expression ratio is 8.7 (P<0.01) (Table 1).

Discussion

This study provides evidence that hyperglycemia diminishes expression of CB1 and enhances that of TRPV1 receptors in PC12 cell line and shows that this change has negative effects on cell viability in the face of a TRPV1 agonist.

The increasing number of diabetic patients in the world and the related economic and human losses has made diabetes as one of the most important research fields in medicine. CB1 and TRPV1 are two important endovanilliod receptors that recently are the subject of many research articles. Many ongoing studies are conducted to understand the changing pattern of the endovanilloid system in the course of diabetes and there are hopes that modification of its components would open new ways to encounter long term complications of the disease. Neuropathies are some late complications of diabetes that are caused by the increased excitability or death of peripheral or central neurons (16). Many ongoing studies are trying to understand the mechanism of these changes.

Our results show that the high glucose medium has decreased mRNA expression of CB1 receptors (Table 1). Effects of glucose in down-regulation of CB1 receptor in PC12 cells and DRG neurons (isolated from streptozotocin-induced diabetic rats) is reported in previous studies (34). A number of *in vivo* or cell culture based studies show that CB1 is a cytoprotective receptor but the mechanism of this protection is less known (39-42).

In spite of CB1 receptors, hyperglycemia has increased RNA transcripts for TRPV1 gene (Table 1). TRPV1 is a nonspecific cation channel with high permeability for calcium ions (27). Impairment of calcium homeostasis in sensory neurons is an important factor in the development of diabetic neuropathies (19, 20, 43, 44). TRPV1 channels play a major role in neuronal sensitization and chronic pain sensation (45). Prolong activation of TRPV1 channels may even lead to neuronal cell apoptosis (23, 46). In our experiments TRPV1 receptor is up regulated (by the fold change expression ratio of 8.7) in cells grown in hyperglycemic media which is supportive for the higher toxicity of capsaicin in this condition (Figure 1). In low glucose medium WIN55 212-2 has reverted capsaicin (10 μM) toxicity in the minimum concentration of 5 μM while in high glucose medium a minimum concentration of 20 μM is needed (Figure 2). Reduced protective effects of CB1 agonist may be explained by reduced expression of CB1 (by the fold change expression ratio of 0.04) and enhanced expression of TRPV1 genes. These results suggest that one mechanism for cytoprotection of the CB1 receptors is their counteraction with TRPV1 receptors. Osmolality plays no role because there were no significant differences between glucose and manitol osmotic controls (data not shown). In both low and high glucose media the effects of capsaicin (10 μM) is reverted by using a TRPV1 antagonist, capsazepine (10μM), showing that toxicity is receptor mediated. Co-administration of a CB1 antagonist, AM251 (5μM), has also prevented the protective effects of WIN55 212-2 (5 μM) indicative of the role of CB1 receptors in the process (Figure 3). Previous studies show that inflammation is an important underlying cause for enhanced signaling of TRPV1 (20, 44, 45, 47). Enhanced signaling is reported to be related to increased oligomerization, phosphorylation, and/or targeted expression of TRPV1 proteins on the cell surface membrane (47), enhanced cooperative functional expression of Cav3.2 T-type calcium and TRPV1 channels (20), increased translation and transport of TRPV1 ion channels without changing its transcription (44) and enhanced TRPV1 mRNA expression (45). The study by Evans *et al* (2007) has shown that there is a crosstalk between these two receptors and CB1 differently affects TRPV1 signaling at low and high concentrations of nerve growth factor (NGF)(48) so modification of the interaction between receptors may be another reason for increased signaling of TRPV1 channels.

Conclusion

In our study we have demonstrated that higher glucose concentrations are responsible for more toxic effects of TRPV1 activation and less protective

effects of a CB1 agonist. In support of these results Real time PCR assay shows an increase for TRPV1 and a decrease for CB1 receptors. It is also probable that hyperglycemia would pave the way for more toxic effects of TRPV1 agonist by changing the crosstalk between TRPV1 and CB1 receptors. Since anandamide and some other lipid derivatives in the body are agonists for both TRPV1 and CB1 receptors detailed understanding of the changes of these receptors and neural cell response to their endogenous ligands in diabetic patients may help to better understand the pathophysiologic basis of diabetic neuropathy and open ways to introduce new drugs for the prevention and/or treatment of this condition.

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