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Capsaicin induces cytotoxicity in pancreatic neuroendocrine tumor cells via mitochondrial action

M. Skrzypski [a,b,*], M. Sassek [b], S. Abdelmessih [a], S. Mergler [c], C. Grötzinger [a], D. Metzke [a], T. Wojciechowicz [b], K.W. Nowak [b], M.Z. Strowski [a]

[a] Department of Hepatology and Gastroenterology & the Interdisciplinary Centre of Metabolism: Endocrinology, Diabetes and Metabolism, Charité-University Medicine Berlin, 13353 Berlin, Germany
[b] Department of Animal Physiology and Biochemistry, Poznań University of Life Sciences, 60-637 Poznań, Poland
[c] Department of Ophthalmology, Charité-University Medicine Berlin, 13353 Berlin, Germany

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ABSTRACT

Capsaicin (CAP), the pungent ingredient of chili peppers, inhibits growth of various solid cancers via TRPV1 as well as TRPV1-independent mechanisms. Recently, we showed that TRPV1 regulates intracellular calcium level and chromogranin A secretion in pancreatic neuroendocrine tumor (NET) cells. In the present study, we characterize the role of the TRPV1 agonist – CAP – in controlling proliferation and apoptosis of pancreatic BON and QGP-1 NET cells. We demonstrate that CAP reduces viability and proliferation, and stimulates apoptotic death of NET cells. CAP causes mitochondrial membrane potential loss, inhibits ATP synthesis and reduces mitochondrial Bcl-2 protein production. In addition, CAP increases cytochrome c and cleaved caspase 3 levels in cytoplasm. CAP reduces reactive oxygen species (ROS) generation. The antioxidant N-acetyl-L-cysteine (NAC) acts synergistically with CAP to reduce ROS generation, without affecting CAP-induced toxicity. TRPV1 protein reduction by 75% reduction fails to attenuate CAP-induced cytotoxicity. In summary, these results suggest that CAP induces cytotoxicity by disturbing mitochondrial potential, and inhibits ATP synthesis in NET cells. Stimulation of ROS generation by CAP appears to be a secondary effect, not related to CAP-induced cytotoxicity. These results justify further evaluation of CAP in modulating pancreatic NETs in vivo.

1. Introduction

Capsaicin (CAP) (8-methyl-*N*-vanillyl-6-nonenamide) is an irritant pungent molecule isolated from chili peppers which has the ability to activate heat-activated ion channels such as the transient receptor potential receptor vanilloid 1 (TRPV1) [1,2]. TRPV1 is a non-selective cation channel which is (over)expressed in highly malignant cancers [3]. CAP is able to reduce cell proliferation and to induce apoptotic death, particularly in highly proliferating cancer cells (reviewed in [4]). Several studies showed that genotoxic and proapoptotic activities of CAP are rather restricted to tumor cells, whereas in normal healthy cells CAP fails to induce cytotoxicity [5–8]. Due to inhibition of cancer growth and progression, as well as due to induction of apoptosis, CAP has been proposed to be a novel candidate in cancer therapy [9]. One of the mechanisms of CAP-induced cell death encompasses TRPV1-dependent stimulation of excessive calcium (Ca^{2+}) influx [10,11] which can be suppressed by activation of cannabinoid receptors [12,13]. However, some reports showed that TRPV1 and Ca^{2+} are not entirely responsible for CAP-induced cell death [14,15]. There is evidence suggesting that, in addition to TRPV1 activation, CAP is able to kill cancer cells by causing apoptosis, which depends on the mitochondrial oxidative metabolism regulation. CAP disrupts mitochondrial membrane potential and can cause rapid reactive oxygen species (ROS) overproduction [7,16]. Therefore, the mechanisms of CAP activation appear to be multifaceted and cell type specific.

Recently, it was found that CAP disturbs the mitochondrial membrane potential, triggers ROS generation and induces apoptosis in human pancreatic adenocarcinoma cells AsPC-1 and BxPC-3 [6,7]. Pharmacological blockade of TRPV1 failed to prevent these cells from apoptotic death, induced by CAP [6]. In contrast, reduction of ROS overproduction reversed CAP-induced apoptosis, suggesting that oxidative stress and mitochondrial damage confer proapoptotic effects of CAP in pancreatic AsPC-1 and BxPC-3 cells [6]. Therefore, these data collectively indicate that CAP may be considered as a potential therapy for pancreatic adenocarcinomas.

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CAP, capsaicin; CPZ, capsazepine; DCFDA, 2′,7′-dichlorofluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-L-cysteine; NET, neuroendocrine tumor; ROS, reactive oxygen species; tbHP, tert-butyl Hydrogen Peroxide; TRPV1, transient receptor potential vanilloid channel 1.

* Corresponding author at: Department of Hepatology and Gastroenterology & the Interdisciplinary Centre of Metabolism: Endocrinology, Diabetes and Metabolism, Charité-University Medicine Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1, 13353 Berlin, Germany. Tel.: +49 30 450 553022; fax: +49 30 450 553902.
E-mail address: marek.skrzypski@charite.de (M. Skrzypski).

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In contrast to other solid cancers e.g. pancreatic adenocarcinoma, studies addressing the influence of CAP on growth and apoptosis in pancreatic neuroendocrine tumors (NETs) are not available.

Here, we characterize the effects of CAP on viability, proliferation and apoptosis in pancreatic NET cell lines BON and QGP-1, and describe the cellular mechanisms of action. Our data indicate that CAP reduces viability and proliferation, and induces apoptosis of BON and QGP-1 cells. The proapoptotic activity of CAP depends on its ability to reduce mitochondrial membrane potential, while the regulation of ROS appears not to be related to this cytotoxic activity of CAP.

2. Material and methods

2.1. Reagents

Cell culture media and supplements were purchased from AG Biochrom (Karlsruhe, Germany) and PAA Laboratories, Inc. (Westborough, MA, USA). MTT was from Calbiochem (San Diego, CA, USA). Unless otherwise stated, all reagents were from Sigma-Aldrich (Deisenhofen, Germany). Primary antibodies for Western blot detection of cleaved caspase 3 (Cat. No. 9661), Bcl-2 (Cat. No. 2876) cytochrome c (Cat. No. 4272) and COX IV (Cat. No. 4850) were from Cell Signalling Technology (Danvers, MA, USA).

2.2. Capsaicin solution preparation and administration

Capsaicin was dissolved in DMSO (100 mM stock solution). Unless otherwise indicated, cells were incubated overnight in serum-free medium to synchronize cell cycle and remove the effects of additional factors from serum. After incubation in serum-free medium cells were treated with test agents or DMSO alone at the final concentration of 0.1–0.2% (vol/vol). These concentrations of DMSO were not toxic to cells in our experimental conditions.

2.3. Cell culture

BON cells were cultured in DMEM/Ham's F-12 (1:1) medium and QGP-1 cells in RMPI 1640 medium [17,18]. Media were supplemented with 10% FCS, 2.5 mM L-glutamine, 100 kU/l penicillin and 100 mg/l streptomycin. Cells were maintained at 37 °C in a humidified atmosphere (5% CO_2, 95% O_2).

2.4. Cell proliferation

Cell proliferation was assessed using a Cell Proliferation ELISA BrdU colorimetric kit (Roche Diagnostics, Penzberg, Germany). In brief, BON and QGP-1 cells were seeded in 96-well plates and cultured in growing medium until they reached 80% confluence (approximately 48 h). Cells were then incubated overnight in serum-free medium and then treated with test agents for the indicated time periods. Thereafter, BrdU solution (10 μM) was added and cells were incubated for 2 h. The incorporation of BrdU into the DNA was measured according to the manufacturer's protocol.

2.5. Cell viability

BON and QGP-1 cells were treated with test agents in the same way as described for BrdU assay. Afterwards MTT solution in PBS (0.5 mg/ml) was added to the incubation medium and cells were incubated for additional 30 min. Medium was then removed and the formed formazan crystals were dissolved in 100 μl of DMSO. Optical density was measured by a plate reader (SpectraMax Plus^{384} microplate reader, Molecular Devices, Ismaning, Germany) at 570 nm and 650 nm (reference wave length).

2.6. Apoptosis

BON and QGP-1 cells were cultured in 24-well plates to reach approx. 80% confluency. Thereafter, cells were incubated overnight in serum-free medium. Then, cells were treated with test agents. Apoptosis was measured by quantifying the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) using Cell Death Detection ELISA kit (Roche Diagnostics, Penzberg, Germany).

2.7. ROS production

Reactive oxygen species (ROS) were measured by DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, UK), according to manufacturer's protocol. In brief, BON cells were plated in black 96-well plates or in 4 chamber tissue culture slides (BD Falcon, BD Biosciences, San Jose, CA, USA) for microscopic examination. Then, cells were incubated with 25 μM DCFDA for 45 min. After the incubation, cells were exposed to test agents as described in figure legends. Fluorescence was detected using a Victor 3 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA) or cells were photographed at 400× magnification using a Zeiss Axiovert 510 Meta Confocal Laser Scanning Microscope (LSM) (Carl Zeiss, Oberkochen, Germany).

2.8. Mitochondrial membrane potential

Mitochondrial membrane potential was measured using a TMRE—Mitochondrial Membrane Potential Assay Kit (Abcam). Cells were cultured in 6-well plates and then exposed to test agents for the indicated time points. Afterwards, cells were incubated in the presence 100 nM TMRE for additional 20 min. Cells were then trypsinized and the fluorescence signals were detected by FACSCalibur-device (BD Biosciences, Heidelberg, Germany) and the results were analyzed by CellQuest Software (BD Biosciences). At least 10,000 cells were analyzed in triplicates. In parallel experiments, cells were cultured in 4 chamber tissue culture slides and treated with tested agents, as described in figure legends. Thereafter cells were photographed at 400× magnification using a Zeiss Axiovert 510 Meta Confocal Laser Scanning Microscope Fluorescence and intensity was quantified using an ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/).

2.9. ATP content

Intracellular ATP level was analyzed by ATP Determination Kit (Biaffin GmbH & Co KG, Kassel, Germany). BON cells were cultured in white 96-well plates. After treatment with test agents, ATP was extracted by adding 50 μl of Somatic Cell ATP Releasing Reagent (Sigma-Aldrich). ATP extract was mixed with 50 μl reagent mix included in the kit. The luminescent signals were measured in 10 min using a Victor 3 1420 Multilabel Counter (PerkinElmer). To calculate ATP content a standard curve for a series of defined ATP concentrations was prepared.

2.10. Western blot

For cleaved caspase 3 determination, BON cells were cultured in 6-well plates with test agents. Cells were washed with ice-cold PBS, and then lysed for 10 min on ice in RIPA buffer (50 mmol/l Tris–HCl, pH 8.0 with 150 mmol NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mmol/l NaF, 1 mmol/l $Na_3(VO_4)$) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics). Lysates were centrifuged (14,000 ×g, 10 min) and supernatants were collected, and stored at −80 °C. To study changes in intracellular distribution of Bcl-1 and cytochrome c cytosolic and mitochondrial proteins were prepared, as previously described [19].