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Vanilloid receptor agonists and antagonists are mitochondrial inhibitors: How vanilloids cause non-vanilloid receptor mediated cell death

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Abstract

Time-lapse photomicroscopy of human H460 lung cancer cells demonstrated of the transient receptor potential V1 (TRPV1) channel agonists, (*E*)-capsaicin and resiniferatoxin, and the TRPV1 antagonists, capsazepine, and SB366791, were able to bring about morphological changes characteristic of apoptosis and/or necrosis. Immunoblot analysis identified immunoreactivity for the transient receptor potential V1 (TRPV1) channel in rat brain samples, but not in rat heart mitochondria or in H460 cells. In isolated rat heart mitochondria, all four ligands caused concentration-dependent decreases in oxygen consumption and mitochondrial membrane potential. (*E*)-Capsaicin and capsazepine evoked concentration-dependent increases and decreases, respectively, in mitochondrial hydrogen peroxide production, whilst resiniferatoxin and SB366791 were without significant effect. These data support the hypothesis that (*E*)-capsaicin, resiniferatoxin, capsazepine, and SB366791 are all mitochondrial inhibitors, able to activate apoptosis and/or necrosis via non-receptor mediated mechanisms, and also support the use of TRPV1 ligands as anti-cancer agents.

Keywords: TRPV1; Vanilloid; Capsaicin; Resiniferatoxin; Capsazepine; SB366791; Apoptosis; Necrosis; Mitochondria; Cell death; Chemotherapy; Cancer

Ligands of the transient receptor potential V1 channel (TRPV1; also known as the vanilloid receptor) have been shown to have anti-proliferative and/or pro-apoptotic effects both *in vitro*, and in animal models *in vivo* [1]. However, the molecular mechanisms involved in the anti-cancer actions of vanilloid receptor agonists, such as capsaicin and antagonists, such as capsazepine, are complex, and their targets and the molecular mechanisms by which they initiate cancer cell death are incompletely understood. This is in part due to the complexity of the TRP channel family and the expanding list of ligands which act as agonists and/or antagonists [2]. In addition, recent studies have shown that vanilloid receptors are present on a more diverse range of cells than at first suspected, such as thymocytes [3]. Furthermore, vanilloid receptors may be transiently expressed

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on certain cell types in response to physiological stimuli [4]. The finding that vanilloid compounds may be ligands for other receptor types such as cannabinoid receptors [5], and conversely, that molecules such as polyamines [6] and endogenous cannabinoids, like anandamide [7], are suggested to be vanilloid receptor agonists has served to confound attempts to understand how vanilloid ligands cause cell death. Data from many studies using vanilloid ligands are made more difficult to interpret, as many of the cell types investigated have not been demonstrated to express functional vanilloid receptors, leading to the suggestion that the involvement of other, non-vanilloid receptor mediated mechanisms. There are indications that mitochondria could be involved in capsaicin-induced cell death, although some of these studies did not include the study of vanilloid receptor antagonists [8,9]. Recent studies have demonstrated that the tricyclic antidepressant chlorimipramine has tumour selective, pro-apoptotic effects in a variety of human cancer glioma cell lines, an action due to its ability to bind to mitochondrial complex III [10]. The aim of this study was, therefore, to investigate whether vanilloid agonists and antagonists also have pro-apoptotic and/or pro-necrotic actions on cancer cells, and if so, to further clarify their mechanism of action using highly purified preparations of isolated mitochondria.

Materials and methods

Cells and chemicals. H460 cells (a human non-small cell lung cancer line) were obtained from the American Type Culture Collection, Manassas, VA. Drugs were dissolved in 100% ethanol at a stock concentration of 10 mM. All chemicals used were of the highest grade available and were from Sigma Chemical Company, Merck Biosciences, Tocris, or InVitrogen. For all vanilloid receptor ligands used, the diluent (ethanol) was never present at >1.0%, and control culture flasks with H460 cells or mitochondrial incubations having the same concentration of diluent showed no statistically significant effects.

Time-lapse photomicroscopy of H460 cells. H460 cells were grown on Iwaki 35 mm glass based dishes to 60–70% confluency maintained in a water jacketed thermostatically controlled incubator at 37 °C in 95% O_2/5% CO_2 in RPMI 1640 with 10% foetal calf serum (FCS) and then treated with the drugs. Digital images were captured every 1 min for 2 h using a Leica DMIRE2 microscope and a Hamamatsu ORCA II BT 1024 CCD camera.

Isolation of rat heart mitochondria. Rat heart mitochondria was prepared from male 250 g Lister rats as previously described [11].

Measurement of mitochondrial oxygen consumption. Rat heart mitochondrial oxygen consumption was measured poloragraphically at 37 °C with 10 mM malate + 10 mM glutamate as previously described [12].

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was measured fluorimetrically (using rhodamine 123) as previously described [10] using either (1) 10 mM glutamate + 10 mM malate or (2) 10 mM succinate + 1 μM rotenone (to block complex I). Mitochondrial hydrogen peroxide production was measured fluorimetrically (using amplex red) at 37 °C in a Hitachi F2500 fluorimeter as previously described [13]. Fluorimeters were calibrated daily using fresh hydrogen peroxide solutions of known (spectrophotometrically determined) concentration.

SDS–PAGE and immunoblot analysis. H460 cells and isolated rat heart mitochondria were re-suspended in lysis buffer, then electrophoresed through 10% polyacrylamide gels and electroblotted to nitrocellulose membranes according to standard procedures [14]. TRPV1 receptor protein was detected with rabbit anti-TRPV1 polyclonal antibodies (Tocris Bioscience, Bristol, UK, product number 2233) according to the suppliers' instructions. Bound anti-TRPV1 antibodies were detected with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Dako, Ely, UK, code number P0048) and visualised by enhanced chemiluminescence (ECL).

Protein assay. Protein concentrations were determined using a microplate Lowry assay with bovine serum albumin used as a concentration standard [10].

Statistical analysis. All experiments were repeated $n = 3$–8 times. Statistical analysis was performed using Student's t-tests. Significance was attributed when $P < 0.05$.

Results

TRPV1 ligands induce morphological features of apoptosis and necrosis in H460 cells

Figs. 1A and B show single images taken at time = 0 and time = 2 h in the time-lapse microscope in the presence of 50 μM (*E*)-capsaicin or 50 μM resiniferatoxin, respectively. There was a general loss of cells in the fields of view (due to their detaching from the growing surface of the cell culture

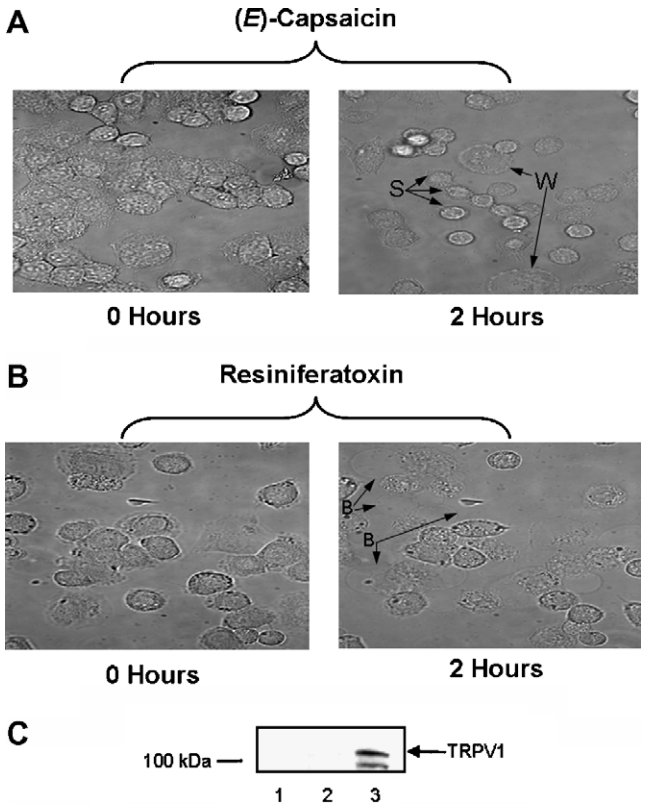

Fig. 1. Photomicroscopy of H460 cells at time = 0 and time = 2 h in the presence of (A) 50 μM (*E*)-capsaicin or (B) 50 μM resiniferatoxin. Rounding of some individual cells is indicated by an S on the images, swelling of other individual cells is indicated by a W on the images, "ballooning out" of the cytoplasm in some cells exposed to resiniferatoxin is indicated by a B on the images. (C) Shows an immunoblot of isolated rat heart mitochondria (lane 1), H460 cells (lane 2), and rat brain homogenate (lane 3). The arrow TRPV1 indicates immunoreactivity, corresponding to the molecular weight indicated by the supplier.

plate); rounding of some individual cells (indicated by an S on the images), swelling of other individual cells (indicated by a W on the images), with an increase in cytoplasmic granularity, particularly in the cells exposed to resiniferatoxin. After 2 h in the case of resiniferatoxin, there was also marked "ballooning out" of the cytoplasm in some cells, (indicated by a B on the images). Parallel experiments using 50 μM capsazepine or 50 μM SB366791 produced similar morphological changes to those observed with (*E*)-capsaicin (data not shown).

TRPV1 channels are not expressed in isolated rat heart mitochondria or in H460 cells

Fig. 1C shows an immunoblot of rat brain homogenate (used as a positive control), in which a band of immunoreactivity at approximately 108 kDa (corresponding to the supplier's specifications) was detected. In contrast, neither H460 cells nor isolated rat heart mitochondria displayed immunoreactivity at the same molecular weight.

TRPV1 agonists and antagonists inhibit mitochondrial oxygen consumption

In order to identify a potential mechanism for the TRPV1 ligand-induced apoptosis, we investigated the effects of these agents on isolated mitrochondria. Fig. 2A shows plots of mitochondrial oxygen consumption versus drug concentration for (*E*)-capsaicin, resiniferatoxin, capsazepine, and SB366791. All four vanilloid ligands caused a concentration dependent decrease in mitochondrial oxygen consumption.

TRPV1 ligands can decrease or increase mitochondrial hydrogen peroxide production

Fig. 2B shows mitochondrial hydrogen peroxide production in the presence of various concentrations of the vanilloid ligands. (*E*)-Capsaicin caused a concentration-dependent decrease in mitochondrial hydrogen peroxide production, whilst capsazepine caused a concentration-dependent increase in mitochondrial hydrogen peroxide production. Resiniferatoxin and SB366791 did not cause any significant change in mitochondrial hydrogen peroxide production.

TRPV1 ligands decrease mitochondrial membrane potential

Fig. 3 (Supplementary Material) shows annotated fluorimeter traces of rhodamine 123 fluorescence before and after subsequent additions of the various vanilloid ligands to isolated rat heart mitochondria. Supplementary Fig. 3A shows that two additions of 10 μM (*E*)-capsaicin (final concentration of 20 μM) caused a cumulative decrease in membrane potential. The addition of the uncoupler CCCP caused a further small increase in fluorescence, demonstrating that the mitochondria only had a relatively small membrane potential after the drug additions. Supplementary Figs. 3B–D show the changes in rhodamine 123 fluorescence observed in the presence of two sequential 10 μM additions (final concentration of 20 μM) of resiniferotoxin, capsazepine, and SB366791, respectively. It is notable that resiniferatoxin and both TRPV1 antagonists caused a more rapid and greater decrease in mitochondrial membrane potential than (*E*)-capsaicin, with only a small residual membrane potential being left at the end of the incubation period, as indicated by the smaller increase in fluorescence on addition of CCCP.

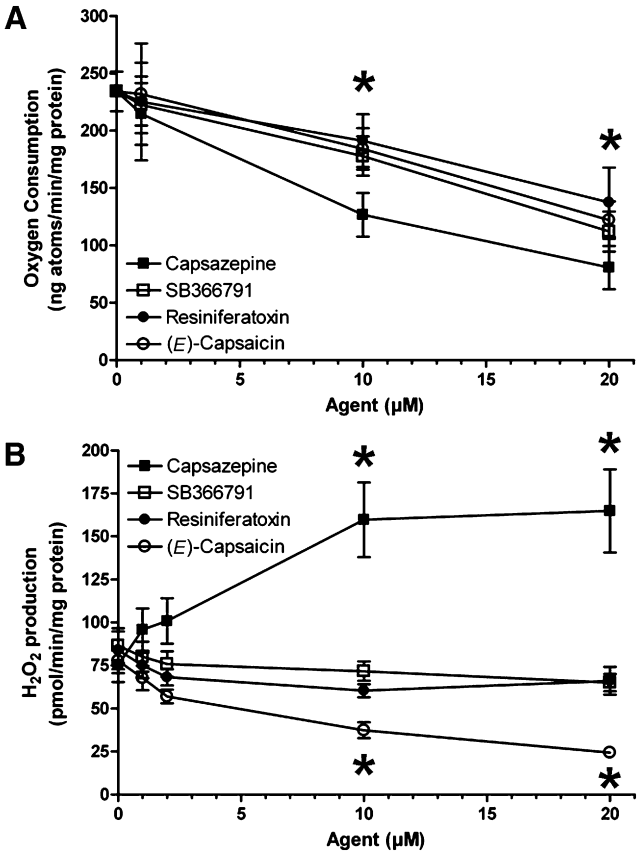

Fig. 2. (A) Shows plots of mitochondrial oxygen consumption versus drug concentration for (*E*)-capsaicin, resiniferatoxin, capsazepine, and SB366791. (B) Shows plots of mitochondrial hydrogen peroxide production versus drug concentration for (*E*)-capsaicin, resiniferatoxin, capsazepine, and SB366791. The asterisk symbol indicates statistical significance relative to control values at $P < 0.05$.

(E)-Capsaicin does not inhibit succinate-linked mitochondrial membrane potential generation

In order to define more closely the locus of vanilloid receptor ligand action, we investigated activity of complex II. Fig. 4 (Supplementary Material) shows that when mitochondrial complex I was blocked with rotenone and the mitochondria were using the complex II-linked substrate succinate, mitochondrial membrane potential was still decreased by resiniferatoxin, capsazepine, and SB366791, but not by (*E*)-capsaicin. The order of efficacy was different

to that in the presence of glutamate + malate, such that SB366791 appeared to have the most rapid and largest effect on mitochondrial membrane potential, capsazepine was the second most active, with resiniferatoxin having a relatively smaller and slower effect on membrane potential.

Discussion

In this report, we have identified a novel mechanism for vanilloid receptor-mediated cancer cell death, which targets mitochondrial function rather than the cell-surface vanilloid receptor.

Our data showing an absence of TRPV1 immunoreactivity in mitochondria are consistent with data from MitoP2, the mitochondrial proteome database [15], which show an absence of TRPV1 channel-like proteins in the mitochondrial proteomes of man, mouse, or the model laboratory organisms *Saccharomyces cerevisiae*, *Neurospora crassa* or *Arabidopsis thaliana*, (see http://141.39.186.157:8080/mitop2/).

The present data may also help to explain conflicting data from other studies. There are reports of capsaicin-evoked apoptosis, which may be blocked by omission of extracellular calcium and by TRPV1 antagonists, indicating the requirement for a functional vanilloid receptor [3]. The situation is made more complex by the observation that both vanilloid receptor agonists and antagonists cause apoptosis (in H460 cells in our studies and in other studies [16]) implicating a mechanism other than the cell-surface TRPV1 receptor. It is our contention that both these agents are able to induce apoptosis by causing mitochondrial depolarisation. Therefore, vanilloid receptor antagonists may indeed antagonise the binding of vanilloid receptor agonists to plasma-membrane associated TRPV1 receptors, but at the same time may cause mitochondrial depolarisation. As TRPV1 receptor activation evokes gating of calcium at the plasma membrane to increase cytosolic calcium, it is likely that in cells with active TRPV1 channels, vanilloid ligands could bring about cell death through a ''vicious cycle'' which entails increased cytosolic calcium [17,18], decreased mitochondrial membrane potential due to direct inhibition of mitochondrial membrane potential and consequent decreased ability of mitochondria to take part in regulation of cytosolic calcium levels [19]. The fact that the ligand binding site for the vanilloid receptor is located on the cytosolic face of the plasma membrane, also suggests that vanilloid ligands that bind to TRPV1 will also be available to bind to mitochondria and thereby able to elicit effects on mitochondria similar to those seen in our studies.

The finding that vanilloid receptor agonists and antagonists both cause death of H460 cancer cells, depolarisation of mitochondrial membrane potential and changes in mitochondrial hydrogen peroxide production are intriguing, and further studies are underway to characterise their pharmacology at the level of the mitochondrion. It appears from our data that, unlike the classical inhibitor rotenone, vanilloid receptor ligands act as relatively weak inhibitors of complex I, similar to the Parkinsonian-inducing agent MPP^+ (the 1 methyl-4 pheny-1,2,3,6, tetrahydropyridinium on), which is thought to be concentrated to around 10 mM within brain mitochondria in order to elicit its pathophysiological effects *in vivo* [12]. This has been demonstrated in the case of (*E*)-capsaicin, which is known to be a weak complex I inhibitor [20] but has not been shown in the cases of the other ligands.

Drugs can either decrease or increase mitochondrial hydrogen peroxide production, depending on where they bind to the electron-transport chain. The ability of (*E*)-capsaicin to decrease mitochondrial hydrogen peroxide production is, therefore, consistent with its ability to bind to mitochondrial complex I [20], and also with studies that have demonstrated the ability of the complex I inhibitor MPP^+ to decrease mitochondrial hydrogen peroxide production [12].

The capsazepine-induced increase in mitochondrial hydrogen peroxide production is consistent with its ability to decrease membrane potential with either complex I-linked substrates or complex II-linked substrates, and indicates a binding site that is common to both complex I-linked respiration and complex II-linked respiration. This binding site could be either complex III, which is the major site of superoxide production in mitochondria, or complex IV. Binding to either complex III or complex IV produces an electrochemically reduced respiratory chain with a concomitant increase in the production of superoxide and other ROS. Resiniferatoxin and SB366791 did not significantly change mitochondrial hydrogen peroxide production, but were able to decrease mitochondrial membrane potential in the presence of either complex I-linked or complex II-linked respiration. Therefore, these compounds may have binding sites on complex III that are proximal to the antimycin a binding site and therefore are able to decrease membrane potential in the absence of a significant change in mitochondrial hydrogen peroxide production.

The vanilloid receptor agonists decreased mitochondrial hydrogen peroxide production, but also decreased mitochondrial membrane potential, such that the mitochondrial oxidative phosphorylation is inhibited. ATP is needed for the terminal step in the glutathione synthesis pathway, and whether vanilloid ligands increase or decrease ROS production in the cell is perhaps less important than the fact that mitochondrial membrane potential is decreased with consequent change in mitochondrial and cytosolic glutathione redox status [14], and mitochondrial cytochrome *c* release, which is likely to result in apoptosis [21].

The concentrations of capsaicin that we have shown to alter function in isolated mitochondria (1–20 μM) are within the range that has been shown to be toxic to proliferating tumour cells (0.1–100 μM) [1]. In addition, we used isolated rat heart mitochondria, which have extremely high

activities of all of the mitochondrial respiratory chain complexes [22], with the result that in our experiments the drug/mitochondrial protein ratio is relatively low. Moreover, in contrast to the majority of studies on anti-cancer agents, where effects of drugs are studied usually in the absence of foetal calf serum (FCS), the experiments we performed on cells were in the presence of 10% FCS and the mitochondria experiments in the presence of BSA. The presence of FCS or BSA provides a source of protein which drugs are known to bind to, and thus the actual concentrations at which significant effects are elicited are likely to be much lower than those used in the present experiments. In addition, it is possible that pharmacokinetic influences can alter local drug concentrations considerably. *In vivo* administration of these relatively hydrophobic drugs could lead to active accumulation within the cell or within mitochondria, as in the case of MPP^+ [12], which would serve to raise the effective concentrations to which mitochondria were exposed.

The fact that the ligand binding site for the vanilloid receptor is located on the cytosolic face of the plasma membrane [23], also suggests that vanilloid ligands that bind to TRPV1 will also be available to bind to mitochondria and thereby able to elicit effects on mitochondria in cells similar to those seen in our studies. In fact, most of the TRPV1 channels seem to be located at internal membranes [24] although the exact nature of their distribution needs further clarification.

The extent of the mitochondrial response to exposure to vanilloid ligands such as the ones we have used will depend on cell type, with cancer cell mitochondria probably being more affected at any given ligand concentration, due to them having lower mitochondrial enzyme activities [25] than normal tissues. Depending on the concentration of drug that reaches the mitochondria in any given cell type, compounds such as vanilloid ligands used in this study could cause a cellular stress response [26], an apoptotic response [10] or a necrotic response [21]. Other workers have found that the same compounds we have used can produce either cytoprotection or cytotoxicity depending on concentration. Thus, capsaicin toxicity was antagonised by a range of receptor antagonists including capsazepine at 0.5 μM, but enhanced by capsazepine at 50 μM [17]. In prostate cancer cells, 200 μM capsazepine also increased cytotoxicity [18]. Our present observations agree well with these findings, and provide a second mechanism that explains this unexpected toxicity at higher doses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.179.

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