



HAL
open science

Upregulation of TRPV1 in mononuclear cells of end-stage kidney disease patients increases susceptibility to -arachidonoyl-dopamine (NADA)-induced cell death

Cassandra I. Saunders, Robert G. Fassett, Dominic P. Geraghty

► To cite this version:

Cassandra I. Saunders, Robert G. Fassett, Dominic P. Geraghty. Upregulation of TRPV1 in mononuclear cells of end-stage kidney disease patients increases susceptibility to -arachidonoyl-dopamine (NADA)-induced cell death. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2009, 1792 (10), pp.1019. <10.1016/j.bbadis.2009.07.008>. <hal-00562919>

HAL Id: hal-00562919

<https://hal.science/hal-00562919v1>

Submitted on 4 Feb 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Accepted Manuscript

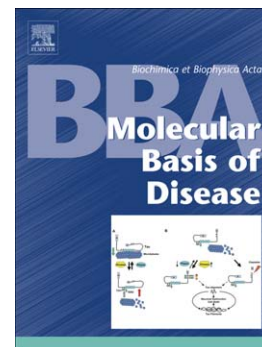
Upregulation of TRPV1 in mononuclear cells of end-stage kidney disease patients increases susceptibility to *N*-arachidonoyl-dopamine (NADA)-induced cell death

Cassandra I. Saunders, Robert G. Fassett, Dominic P. Geraghty

PII: S0925-4439(09)00151-3
DOI: doi:[10.1016/j.bbadis.2009.07.008](https://doi.org/10.1016/j.bbadis.2009.07.008)
Reference: BBADIS 62981

To appear in: *BBA - Molecular Basis of Disease*

Received date: 30 April 2009
Revised date: 19 June 2009
Accepted date: 13 July 2009



Please cite this article as: Cassandra I. Saunders, Robert G. Fassett, Dominic P. Geraghty, Upregulation of TRPV1 in mononuclear cells of end-stage kidney disease patients increases susceptibility to *N*-arachidonoyl-dopamine (NADA)-induced cell death, *BBA - Molecular Basis of Disease* (2009), doi:[10.1016/j.bbadis.2009.07.008](https://doi.org/10.1016/j.bbadis.2009.07.008)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Upregulation of TRPV1 in mononuclear cells of end-stage kidney disease patients increases susceptibility to *N*-arachidonoyl-dopamine (NADA)-induced cell death

Cassandra I. Saunders^a, Robert G. Fassett^{b,c}, Dominic P. Geraghty^{a,*}

^aSchool of Human Life Sciences, University of Tasmania, Launceston, Tasmania, Australia

^bRoyal Brisbane and Women's Hospital, Brisbane, Queensland, Australia

^cSchool of Medicine, The University of Queensland, Brisbane, Queensland, Australia

Keywords: cell death; end-stage kidney disease; mononuclear cells; *N*-arachidonoyl-dopamine; TRPV1; TRPV2

*Corresponding author. School of Human Life Sciences, University of Tasmania, Locked Bag 1320, Launceston, Tasmania, 7250, Australia. Tel. +613 63245488; fax: +613 63243658.

E-mail address: D.Geraghty@utas.edu.au (D.P. Geraghty).

Summary

Transient receptor potential vanilloid (TRPV) 1 channels function as sensors for a variety of noxious and inflammatory signals, including capsaicin, heat and protons, and are up-regulated under inflammatory conditions. As end-stage kidney disease (ESKD) is associated with chronic inflammation, impaired immunity and depressed lymphocyte numbers, we sought to determine whether altered TRPV1 (and related TRPV2) expression in immune cells might be a contributing factor. TRPV1 and TRPV2 mRNA expression in peripheral blood mononuclear cells (PBMC) was similar in controls and ESKD patients by quantitative real-time RT-PCR. However, using immunocytochemistry, TRPV1-immunoreactivity was significantly higher and TRPV2-immunoreactivity was significantly lower in PBMC from ESKD patients compared to controls. The plant-derived TRPV1 agonists, capsaicin and resiniferatoxin (RTX) and the putative endovanilloid/endocannabinoids, *N*-arachidonoyl-dopamine (NADA) and *N*-oleoyl-dopamine (OLDA), induced concentration-dependent death of PBMC from healthy donors with a rank order of potency of RTX>NADA>OLDA>>capsaicin. TRPV1 (5'-iodoresiniferatoxin) and cannabinoid (CB₂; AM630) receptor antagonists blocked the cytotoxic effect of NADA. In subsequent experiments, PBMC from ESKD patients exhibited significantly increased susceptibility to NADA-induced death compared to PBMC from controls. The apparent up-regulation of TRPV1 may be a response to the inflammatory milieu in which PBMC exist in ESKD and may be responsible for the increased susceptibility of these cells to NADA-induced death, providing a possible explanation as to why ESKD patients have reduced lymphocyte counts and impaired immune function. Thus, TRPV1 (and possibly CB₂) antagonists may have potential for the

treatment of immune dysfunction in ESKD.

ACCEPTED MANUSCRIPT

1. Introduction

The transient receptor potential (TRP) protein superfamily is a diverse group of voltage-independent, calcium-permeable cation channels expressed in mammalian cells that can be classified into six related subfamilies [1, 2]. The most well known member of the vanilloid (TRPV) subfamily, TRPV1, is directly activated by vanilloids, such as capsaicin, moderate heat ($\geq 43^{\circ}\text{C}$), protons ($\text{pH} \leq 5.9$), bases ($\text{pH} > 8$), bradykinins and some arachidonic acid metabolites, including the endogenous vanilloid/cannabinoid *N*-arachidonoyl-dopamine (NADA) [2-5]. TRPV1 is also (indirectly) sensitised by a diverse range of substances including protons, lipoxygenase products, bradykinins, prostaglandins and a number of cytokines and chemokines, all generated by inflammation [2, 6-8]. Hence, TRPV1 acts as a polymodal detector of noxious and inflammatory signals [9]. TRPV2 (also referred to as vanilloid receptor-like 1, VRL-1) shares ~49% homology with TRPV1 and is also a heat-gated channel, albeit with a higher temperature threshold of $\sim 52^{\circ}\text{C}$ [10]. TRPV2, however, is not activated by capsaicin or protons [2].

TRPV1 and TRPV2 were once believed to be located exclusively on neurons, where their function has been studied in great detail. However, more recent studies have demonstrated TRPV1 in a variety of non-neuronal cells, including neutrophil granulocytes, keratinocytes and mast cells from human skin as well as cells of epithelial origin in the bladder and urethra, gastrointestinal tract, lung and prostate [11-16]. TRPV2 has also been reported in urothelial cells, mast cells and parenchymal cells of the spleen, lung and intestine [10, 17-19]. Moreover, disease-associated increases in TRPV1 mRNA and/or protein expression have been demonstrated in the cervix (cervical cancer), urinary bladder

(detrusor hyperreflexia), gastrointestinal tract (inflammatory bowel disease), respiratory tract (chronic airway inflammatory disease) and leukocytes (hyposensitive individuals) [20-28], suggesting a pathological association between TRPV1 and inflammation. Studies also suggest that cells aberrantly expressing TRPV1 display increased susceptibility to apoptotic agents [13, 29, 30].

We have recently reported that both TRPV1 and TRPV2 are expressed by peripheral blood mononuclear cells (PBMC), consisting of lymphocytes (~83%) and monocytes [31]. Whilst the precise roles of these receptors remain unclear in these cells, lymphocytes are crucial cells of the immune system with T cells being associated with activation of the inflammatory response. TRPVs may also be an important component of the Ca^{2+} release-activated- Ca^{2+} (CRAC) channel, necessary to cause productive T cell activation, influence T cell signalling and/or mediate apoptosis due to Ca^{2+} overload [32-34]. Thus, the presence of TRPV1 (and possibly TRPV2) in PBMC suggests that these receptors play a role in the detection of inflammatory signals by lymphocytes and monocytes.

Patients with end-stage kidney disease (ESKD), requiring regular dialysis treatment, are in a constant state of inflammation and oxidative stress, reflected by elevated pentraxin levels [e.g., pentraxin-3 (PTX3) and C-reactive protein (CRP)] and other indicators of inflammation [e.g., interleukins (IL)-1, 4, 6 and 10 and tumour necrosis factor alpha (TNF- α)] [35]. In addition, depressed and aberrant cell-mediated immunity and altered lymphocyte function in chronic kidney disease has been well documented [36, 37], making this patient population an ideal model to investigate the effects of chronic inflammation on

TRPVs in immune system cells. Thus, the aims of the present study were to compare TRPV1 and TRPV2 mRNA expression and immunoreactivity in PBMC from healthy controls and ESKD patients, and to compare the susceptibility of PBMC from these subjects to vanilloid-induced death.

ACCEPTED MANUSCRIPT

2. Materials and Methods

2.1. Participants

Peripheral blood (8mL lithium-heparin, 8mL EDTA) was obtained from 32 ESKD patients [18 females, 14 males; aged 59 ± 12 years (mean \pm SD)] undergoing haemodialysis at Launceston General Hospital Renal Unit (Launceston, Tasmania, Australia) from either a fistula or vascular access catheter and 29 age- and gender-matched control participants (16 females, 13 males; aged 59 ± 11 years) from either the median or cephalic vein, under resting conditions. Control participants had no history of kidney disease or recent infection and were not currently taking anti-inflammatory medications. The study was approved by the Human Research Ethics Committee Network, Tasmania (H8897). All participating individuals gave informed written consent.

The primary causes of kidney disease were diabetic nephropathy (n=9), glomerulonephritis (n=5), reflux nephropathy (n=4), polycystic kidney disease (n=4), hypertension (n=3), miscellaneous (n=6) and uncertain diagnosis (n=1). Sixty-one percent of ESKD patients were taking at least one, often a combination of, prescribed anti-hypertensive medication(s) [calcium channel antagonist (n=10), angiotensin-converting enzyme inhibitor (n=9), β -blocker (n=8), angiotensin II receptor blockers (n=5), α -adrenergic blocker (n=2) and/or endothelin receptor antagonist (n=1)]. Other concurrent medications were: statin (n=8), erythropoietin (n=24), calcium supplement (n=23), inhaled glucocorticoid (n=4, low doses as necessary for asthma) and anticoagulant/antiplatelet agents (n=15; warfarin, aspirin, clopidogrel). Some patients had undergone prior kidney

transplantation (n=8), which remained *in situ*, however, due to rejection of the graft these individuals were no longer receiving immunosuppressive therapy.

All ESKD patients had a urea reduction ratio of >76% (target >70%). Pre-dialysis serum creatinine was $752 \pm 181 \mu\text{mol/L}$; white cell count was $7.4 \pm 2.4 \times 10^9/\text{L}$; lymphocyte count was $1.5 \pm 0.5 \times 10^9/\text{L}$ (normal reference range values are: $50\text{-}105 \mu\text{mol/L}$; $4.0\text{-}11.0 \times 10^9/\text{L}$; $1.5\text{-}4.0 \times 10^9/\text{L}$, respectively) [38]. 82% of patients had CRP >5mg/L, an indicator of inflammation.

2.2. Peripheral blood mononuclear cell preparation

PBMC were isolated as previously described by Duthie *et al.* [39]. Briefly, peripheral blood was diluted 1:1 with phosphate-buffered saline (PBS) and separated by centrifugation over Histopaque (Sigma-Aldrich, St. Louis, USA) at $700 \times g$ for 30 min at 20°C . The mononuclear layer was collected and centrifuged in RPMI 1640 (Invitrogen, Carlsbad, USA) at $700 \times g$ for 15 min at 20°C . The pellet was resuspended in RPMI containing 10% foetal calf serum (FCS, Invitrogen, Carlsbad, USA). Ten microlitres of cell suspension was diluted 1:1 with Trypan Blue (Sigma-Aldrich, St. Louis, USA) and 5 large squares counted using an Improved Neubauer Haemocytometer (Merck, Lutterwork, UK). The remaining cell suspension was centrifuged at $854 \times g$ for 15 min at room temperature and the pellet resuspended in 0.5mL aliquots at a density of 3×10^6 cells/mL in a freezing mix of 90% v/v heat-inactivated FCS and 10% v/v dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA). Suspensions were frozen at -80°C using a Nalgene Cryo 1°C Freezing Container (Thermo Fisher Scientific, Rochester, USA). The isolated PBMC suspension consisted predominantly of lymphocytes (control participants $87 \pm 3\%$; ESKD

patients 85±4%), determined using a Coulter MaxM haematology analyser (Beckman Coulter, Gladesville, Australia).

2.3. RNA extraction from isolated PBMC and reverse transcription

Frozen cell suspensions from EDTA blood were thawed and centrifuged at 854 X *g* for 3 min. Pellets were resuspended with 0.01M PBS and re-centrifuged. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, NRW, Germany) according to the manufacturer's instructions. The concentration of RNA was measured spectrophotometrically (Eppendorf Biophotometer, North Ryde, Australia) and samples stored at -20°C until use. Five microlitres of RNA suspension were reverse transcribed using an Omniscript Reverse Transcriptase (RT) Kit (Qiagen, Hilden, Germany) containing 10X RT buffer, dNTP-Mix, oligo (dT) primer (Promega, Madison, USA), RNase Inhibitor (Promega, Madison, USA) and Omniscript RT polymerase. Tubes with reaction mixtures were incubated at 44°C for 1 hour with 10 mins inactivation of enzyme at 92°C and the resultant cDNA was stored at -20°C until used for PCR amplification.

2.4. Quantitative real time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed using iQ SYBR Green Supermix (BioRad, Hercules, CA, USA). The following primer pairs (Geneworks, Adelaide, Australia) were used: human TRPV1, forward primer 5'-CAG CAG CGA GAC CCC TAA-3' (position 1209-1226 in the sequence of human TRPV1) and reverse primer 5'-CCT GCA GGA GTC GGT TCA-3' (position 1256-1273 in the sequence of human TRPV1) and human TRPV2, forward primer 5'-CTA CAG TGT CAT GAT CCA GAA

GGT-3' (position 1933-1956 in the sequence of human TRPV2) and reverse primer 5'-CCG AAA AGG AAG ACT AAG TAG ATC A-3' (position 1986-2010 in the sequence of human TRPV2). qRT-PCR assays for each gene target were performed on cDNA samples and genomic DNA standards in 96-well optical plates on a MyIQ Single-Colour Real-Time PCR Detection System (BioRad, Hercules, USA). Each amplification program consisted of: one cycle of 95°C for 15 mins, followed by 30 cycles of 95°C at 30 secs and 61°C (TRPV1) or 59°C (TRPV2) for 30 secs and a single cycle of 72°C for 30 secs, 65°C for 1 min and 65°C for 10 mins. A parallel assay, with an annealing temperature of 55°C, was run for the housekeeping gene GAPDH, in addition to target mRNA of interest.

2.5. TRPV1 and TRPV2 immunocytochemistry

TRPV1- and TRPV2-immunoreactivity present in PBMC was visualised by indirect immunofluorescence as previously described [31]. Briefly, one 3×10^6 cells/mL aliquot from each participant, isolated from lithium-heparinised blood (n=20 controls and 20 ESKD patients), was thawed at 37°C and centrifuged at 854 x g for 3 mins. Pellets were resuspended in 0.01M PBS and re-centrifuged. Non-specific antibody binding sites on the mononuclear cells were blocked by incubating for 1 hr at room temperature on a platform mixer (OM6 Orbital Mixer, Ratek, Boronia, Australia) in a solution of 5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA) followed by re-centrifugation. Pellets were then incubated for 30 mins at room temperature on a platform mixer with either rabbit polyclonal anti-capsaicin receptor (TRPV1) antisera (1:500, Chemicon International, Billerica, USA) or goat polyclonal anti-VRL-1 (TRPV2) antisera (1:250, Santa Cruz Biotechnology, Santa Cruz, USA), followed by incubation at 4°C overnight. After three

washes in 0.01M PBS, pellets were incubated with either goat Texas red-labelled anti-rabbit antibody for TRPV1 detection (1:1000, Molecular Probes, Eugene, USA) or a donkey fluorescein-isothiocyanate-labelled anti-goat antibody for TRPV2 detection (1:1000, Invitrogen, Carlsbad, USA) for 1.5 hrs at room temperature on a platform mixer, followed by another three washes in 0.01M PBS. The final supernatants were discarded and the pellets counterstained with nuclear yellow (0.001% in PBS, Sigma-Aldrich, St. Louis, USA) for 5 mins, to assess cell viability. After three washes in 0.01M PBS, the final supernatants were discarded and the pellets were resuspended in 85 μ L low melting point agarose (LMP, 1% in PBS, Invitrogen, Carlsbad, USA), mounted onto standard agarose (1% in PBS, Invitrogen, Carlsbad, USA) coated slides and cover-slipped. To test for non-specific immunofluorescence produced by the secondary antibodies, primary antibody was excluded in negative controls. No immunolabelling was observed on these slides.

Immunostaining of TRPV1 and TRPV2 in PBMC was viewed using an Olympus IX71 inverted fluorescence microscope (Olympus, Center Valley, USA). Fifty cells from each participant were randomly selected and photographed using an Optronics digital camera (Optronics, Model 60800, Goleta, USA). Exposure times were 800 millisecs for TRPV1 staining and 5.154 secs for TRPV2 staining. Images were captured and saved electronically as tiff files using MagnaFire software (version 2.1, Optronics, Goleta, USA). Unmodified images were converted to grey scale and inverted so that fluorescence appeared as black on a white background. TRPV1- and TRPV2-immunoreactivity in small (~5-8 μ m) and large (~10-17 μ m) mononuclear cells was quantitated using image analysis software (AIS, version 3.0, Imaging Research, St. Catharines, Canada). Detection was set above background. The intensity of immunoreactivity was defined as the product of relative

optical density (ROD) and area of immunoreactivity.

2.6. *Vanilloid/endovanilloid-induced PBMC death*

Peripheral blood from healthy, adult donors (n=3) was collected into EDTA tubes and PBMC isolated as previously stated. Frozen cell suspensions were thawed at 37°C and centrifuged at 854 X g for 3 min. Pellets were washed twice in 500µL RPMI containing 10% FCS at 854 X g for 3 min. Cell viability was determined by Trypan Blue exclusion [40]. Briefly, 10µL of cell suspension was diluted 1:1 with Trypan Blue and 5 large squares counted using an Improved Neubauer Haemocytometer. Samples with less than 80% viability were discarded. Cells were resuspended in 500µL complete RPMI containing 2mM L-glutamine, 100U Penicillin, 100U Streptomycin (Invitrogen, Carlsbad, USA) and 10% FCS in 24-well cell culture plates (Greiner Bio-One, Frickenhausen, Germany).

NADA, capsaicin, resiniferatoxin (RTX) and *N*-oleoyl-dopamine (OLDA) (Tocris Bioscience, Ellisville, USA) were reconstituted in ethanol to a stock concentration of 11mM, 50mM, 10mM and 2mM, respectively. Suspensions of PBMC (~1x10⁵ cells/mL) were incubated with NADA (3.4-100µM), capsaicin (15-500µM), RTX (1.5-50µM), OLDA (10-100µM) or vehicle (0.2% ethanol) for 24 hrs at 37°C in 8% CO₂ in a Heraeus HERAcell 150 incubator (Thermo Fisher Scientific, Rochester, USA). Cell suspensions were then centrifuged at 854 X g for 3 min. Pellets were washed twice in 500µL RPMI containing 10% FCS at 854 X g for 3 min. Cell viability was determined by Trypan Blue exclusion. Cell viability, normalised to pre-exposure cell viability (expressed as a percentage) was plotted against log [agonist] and the concentration of each agonist required

to reduce cell viability by 50% (EC_{50}) determined.

The EC_{50} for NADA-induced PBMC death was $29\mu\text{M}$ (see results). As NADA is an endogenous activator of TRPV1 and also relatively soluble in aqueous media, we employed $25\mu\text{M}$ NADA in all subsequent experiments including antagonist studies and comparisons between healthy controls and ESKD patients.

The receptors responsible for NADA-induced cytotoxicity were further investigated using TRPV1 and cannabinoid (CB) antagonists. Frozen cell suspensions from EDTA blood of healthy adult donors used above were thawed, washed, viability determined and resuspended in incubation medium as previously described. The TRPV1 antagonists, 5'-iodoresiniferatoxin (IRTX) and capsazepine (CPZ) (Tocris Bioscience, Ellisville, USA), were dissolved in ethanol. The CB_1 antagonist, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) and CB_2 antagonist, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl(4-methoxyphenyl) methanone (AM630) (Tocris Bioscience, Ellisville, USA), were dissolved in DMSO to a stock concentration of 3mM and 3.5mM, respectively. The final concentrations of IRTX (100nM), CPZ ($1\mu\text{M}$), AM251 (500nM) and AM630 ($1\mu\text{M}$) employed were based on previous studies [41-48]. NADA ($25\mu\text{M}$), vehicle (0.2% ethanol or 0.03% DMSO), IRTX (100nM), CPZ ($1\mu\text{M}$), IRTX + NADA (100nM and $25\mu\text{M}$, respectively), CPZ + NADA ($1\mu\text{M}$ and $25\mu\text{M}$, respectively), AM251 (500nM), AM630 ($1\mu\text{M}$), AM251 + NADA (500nM and $25\mu\text{M}$ respectively), AM630 + NADA ($1\mu\text{M}$ and $25\mu\text{M}$ respectively) or IRTX + AM630 + NADA (100nM, $1\mu\text{M}$ and $25\mu\text{M}$ respectively) were added to the cell suspensions ($n=3$ for all combinations). NADA was added 30 mins after antagonists. Samples were incubated for 24 hrs at 37°C in 8% CO_2 in a Heraeus

HERAcell 150 incubator. Cell suspensions were processed as described earlier. Post-exposure cell viability was normalised to pre-exposure cell viability (expressed as a percentage).

Finally, frozen cell suspensions from EDTA blood from ESKD patients and age- and gender-matched controls were thawed, washed, viability determined and resuspended in incubation medium as previously described. NADA (25 μ M, n=7 controls and 7 ESKD patients) or vehicle (0.2% ethanol, n=3 controls and 3 ESKD patients) was added to cell suspensions. Samples were incubated for 24 hrs at 37°C in 8% CO₂ in a Heraeus HERAcell 150 incubator. Cell suspensions were processed as previously described. Post-exposure cell viability was normalised to pre-exposure cell viability (expressed as a percentage).

2.7. Statistical analysis

All qRT-PCR data were captured using MyIQ Single-Colour Real-Time PCR Detection System Software (version 1.0, BioRad, Hercules, USA). Using the standard curve generated with DNA plasmid templates, the threshold cycle value for each cDNA sample was used to calculate the initial quantity of cDNA template per well. Data from each well was normalised by dividing the quantity of target gene cDNA by the quantity of GAPDH cDNA and expressed as copies per 10⁶ copies of GAPDH to correct for differences in RNA quantity and quality.

Simple linear regression analysis (Prism, v4.0, GraphPad Software, San Diego, USA) was performed to determine relationships between TRPV1/2 mRNA and immunoreactivity, and age in both controls and ESKD patients, and TRPV1/2 mRNA and

immunoreactivity, and time spent undergoing dialysis treatment, estimated glomerular filtration rate (eGFR), CRP, urea and creatinine levels in ESKD patients. General linear modelling and stepwise regression (STATA, version 9.0, StataCorp LP, College Station, USA), with adjustment for potential confounders by inclusion of covariates, was also employed. Unpaired t-tests were used to determine whether there were any significant differences in TRPV1 and TRPV2 mRNA/immunoreactivity between controls and ESKD patients, gender in both groups and the mode of blood collection, CRP levels and prescribed medications in the ESKD patients.

Nonlinear regression was used to determine the EC_{50} value for TRPV1 agonists (Prism). Unpaired t-test was used to determine the effect of TRPV1 antagonists on NADA-induced PBMC death. $P < 0.05$ was considered statistically significant for regression and comparison analyses and $p < 0.01$ was considered statistically significant for associations. Data are presented as mean and standard error of mean (SEM).

3. Results

3.1. *TRPV1 and TRPV2 mRNA expression in PBMC*

TRPV1 expression was ~130-fold lower than TRPV2 expression in both control participants and ESKD patients (Fig. 1). However, there were no significant differences in TRPV1 (Fig. 1A) or TRPV2 (Fig. 1B) mRNA expression between controls and ESKD patients. Expression of the housekeeping gene, GAPDH, was also not significantly different between control participants and ESKD patients (Fig. 1C).

Interestingly, TRPV1 mRNA was inversely related to age (Fig. 2A) in both controls ($p=0.047$) and ESKD patients ($p=0.056$). However, there was no significant relationship between TRPV2 mRNA and age in either the control group ($p=0.064$) or ESKD patients ($p=0.607$; Fig. 2B). Statistical analysis demonstrated no significant relationship between TRPV1 or TRPV2 mRNA and gender in either group. In addition, for ESKD patients, there were no associations between TRPV1 or TRPV2 mRNA copy number and the duration of dialysis therapy, whether the blood sample was obtained from a fistula or vascular access catheter, prescribed medications, biochemical parameters, such as serum creatinine and CRP, or cause of kidney disease.

3.2. *TRPV1- and TRPV2- immunoreactivity*

Non-specific immunofluorescence produced by the secondary antibodies was negligible for both the TRPV1 and TRPV2 antibodies (negative control, Fig. 3). The intra-sample intensity of TRPV1-immunoreactivity in PBMC varied greatly in both controls and ESKD patients (ROD x area ranged from >0.001 –13.82 and >0.001 –20.41, respectively).

Up to 42% of cells from individual control participants and up to 60% of cells from individual ESKD patients ($15\pm 14\%$ and $28\pm 19\%$, respectively) demonstrated intensely fluorescent puncta. In contrast, whilst the intra-sample level of TRPV2-immunoreactivity also varied in both controls and ESKD patients (<0.001 – 0.08 in both groups), fluorescence was much less intense and not localised to a definable cellular compartment or structure. A representative image of the variation in TRPV1- and TRPV2-immunoreactivity in PBMC from a control participant is provided (Fig. 3).

The intensity of TRPV1-immunoreactivity in PBMC from patients with ESKD was significantly ($p<0.001$) greater (92%) than that of controls (Fig. 4A). TRPV2-immunoreactivity was significantly ($p=0.003$) lower (28%) in PBMC from patients with ESKD when compared to controls (Fig. 4B).

The isolated PBMC suspensions, after thawing, consisted predominantly of lymphocytes (control participants $87 \pm 3\%$; ESKD patients $85 \pm 4\%$). Two distinct subsets of lymphocytes exist, large lymphocytes and small lymphocytes. The majority of large lymphocytes are natural killer cells and the small lymphocytes consist of T and B cells. A small lymphocyte may also become a large lymphocyte as it is stimulated to proliferate (e.g., by mitogens such as PHA or binding of a specific antigen) [49]. To determine whether these observed changes in TRPV1- and TRPV2-immunoreactivity were limited to a specific PBMC size, the intensity of TRPV1- and TRPV2-immunoreactivity in the two cell subgroups was compared based on their size (small PBMC ~ 5 – $8\mu\text{m}$; large PBMC ~ 10 – $17\mu\text{m}$; Figure 4). TRPV1-immunoreactivity in both small and large PBMC of controls and ESKD patients was similar (Fig. 4A). In contrast, the lower overall TRPV2-

immunoreactivity observed in PBMC from ESKD patients was due primarily to a decrease in immunoreactivity expressed by small PBMC (Fig. 4B).

Regression analysis revealed no relationship between TRPV1 (n=20 controls and 20 ESKD patients; aged 35 to 78 years) or TRPV2 (n=10 controls and 10 ESKD patients; aged 46 to 78 years) immunoreactivity with age in controls ($r^2=0.001$, $p=0.953$ and $r^2=0.041$, $p=0.574$, respectively) or ESKD patients ($r^2=0.001$, $p=0.902$ and $r^2=0.285$, $p=0.112$, respectively). There was no statistically significant relationship between TRPV1 or TRPV2 mRNA and gender in either group. Similarly, there were no associations between TRPV1 or TRPV2-immunoreactivity and duration of dialysis therapy, the mode of blood collection, prescribed medications, biochemical parameters or cause of kidney disease in ESKD patients.

There was no relationship between TRPV1 mRNA and TRPV1-immunoreactivity or TRPV2 mRNA and TRPV2-immunoreactivity in either controls (TRPV1, $p=0.775$; TRPV2, $p=0.072$) or ESKD patients (TRPV1, $p=0.374$; TRPV2, $p=0.987$).

3.3. Vanilloid/endovanilloid-induced PBMC death

Exposure of PBMC from healthy adult donors to putative endogenous TRPV1 activators (NADA and OLDA), and the 'classical' plant-derived activators, capsaicin and RTX, for 24 hrs produced concentration-dependent decreases in cell viability (Fig. 5A). The rank order of potency of these agents (EC_{50} , μM , n=3-4 experiments) was RTX (21 ± 1)>NADA (29 ± 1)>OLDA (40 ± 1)>>capsaicin (240 ± 1). The slopes of the concentration-response curves for all four TRPV1 activators were extremely steep, ranging

from -3 to -9.

Exposure of PBMC suspensions to TRPV1 and CB receptor antagonists alone had no effect on cell viability (Fig. 5B). The TRPV1 selective and CB₂ selective antagonists, IRTX and AM630, respectively, almost completely blocked NADA (25 μ M)-induced cell death. Incubation with both IRTX and AM630 produced no additional inhibition. However, the cytotoxic effect of NADA was not antagonised by the functional TRPV1 antagonist, CPZ, or the selective CB₁ antagonist, AM251 (Fig. 5B). In fact, NADA combined with CPZ or AM251 appeared to be more cytotoxic to PBMC ($p=0.029$ and $p=0.007$, respectively) when compared to NADA alone (Fig. 5B).

PBMC viability after incubation with NADA was determined in seven ESKD patients and age- and gender-matched control participants. TRPV1-immunoreactivity in the PBMC from ESKD patients used in these NADA studies was significantly ($p=0.015$) greater than that of corresponding controls. Cell viability after 24 hrs exposure to NADA was significantly ($p=0.003$) lower (17%) in ESKD patients compared with controls (Fig. 5C)

When data from both controls and ESKD patients were combined, there was a highly significant ($p=0.006$) negative association between TRPV1-immunoreactivity and cell viability ($r^2 = 0.484$).

4. Discussion

We have previously reported that both TRPV1 and TRPV2 are present in PBMC of healthy adults [31]. In the present study, we extended that work and used qRT-PCR, immunocytochemistry and an *in vitro* cell viability assay to determine whether TRPV1 (and TRPV2) in PBMC are affected by the inflammatory state associated with ESKD.

There was no difference in TRPV1, TRPV2 or GAPDH mRNA expression in PBMC from ESKD patients compared to controls. TRPV1 mRNA expression decreased with age in both controls and ESKD. It is well-documented that immune, in particular lymphocyte, function declines with age [50-52]. However, whether this result is merely coincidental or there is a true link between TRPV1 and declining immune function with age has yet to be investigated.

The intensity of both TRPV1 and TRPV2 immunofluorescence varied between PBMC from the same individual. This may be due to unique subsets of mononuclear cells, e.g., lymphocytes and monocytes, expressing TRPV1 or TRPV2 protein to differing degrees. Indeed, we have preliminary data that show monocyte TRPV1 and TRPV2 mRNA expression and immunoreactivity are significantly lower in monocytes compared to lymphocytes in healthy individuals (Kunde D, Whitmore B, Geraghty D, unpublished observations). In addition, detailed studies into TRPV expression by cell subsets (T, B and natural killer cells and monocytes) using flow cytometric analysis is underway.

TRPV1-immunoreactivity in both small and large PBMC of ESKD patients was significantly higher compared to controls. In contrast, TRPV2-immunoreactivity was significantly lower in ESKD patients compared to controls and was confined to small PBMC. Given that these differences in TRPV1- and TRPV2-immunoreactivity in PBMC

from ESKD patients were not related to TRPV1 and TRPV2 mRNA expression, respectively, TRPV1 and TRPV2 protein may be reciprocally regulated in PBMC of ESKD patients. Interestingly, Ji et al. [53] have reported that inflammation increases cell surface expression of TRPV1 by rat DRG neurons, with no change in TRPV1 mRNA expression. Thus, inflammation/disease may affect translation, rather than transcription, of TRPV1 and TRPV2.

TRPV1 or TRPV2 mRNA expression and immunoreactivity were unrelated to the time (months) spent undergoing dialysis treatment, the mode of blood collection, gender, prescribed medications, biochemical parameters or cause of kidney disease. Thus, the changes reported in TRPV1- (and TRPV2-) immunoreactivity in ESKD patients may be a consequence of chronic inflammation. Indeed, the high CRP levels (>5mg/L) recorded in the majority (82%) of ESKD patients in this study is indicative of an inflammatory state. Inflammation associated with ESKD may trigger up-regulation of TRPV1 and supports previous studies that demonstrated increases in TRPV1 mRNA and/or immunoreactivity in a variety of inflammatory conditions [20-27], thus, providing further evidence for a pathological association between increased TRPV1 expression and inflammation. In order to confirm TRPV1 and TRPV2 protein expression in PBMC of control participants and ESKD patients, a Western Blot analysis protocol is currently being designed in our laboratory.

Changes to cell surface receptors and signalling events in PBMC from ESKD patients on haemodialysis have previously been reported [54-56]. Depressed proliferative responses to the T cell mitogens phytohemagglutinin and concanavalin A and increased

expression of a distinct V-region of the β -chains of the T-cell receptor antigen receptor (V β 6.7) have shown to be increased in CKD patients compared to controls [55, 56]. In addition, several groups have demonstrated an increased expression of the interleukin 2 receptor (IL-2R), critical for T cell proliferation, on PBMC from patients undergoing dialysis. Evidence that heightened expression of IL-2R is linked to immunodeficiency has also been reported [54], supporting the depressed and aberrant cell-mediated immunity and altered lymphocyte function in CKD, which is well documented [36, 37].

Due to the paucity of studies addressing the function(s) and regulation of TRPV2, we cannot currently explain the mechanism behind, or the functional consequences of, TRPV2 down-regulation in PBMC of ESKD patients. TRPV1, however, is a vital component of mammalian sensory function and while definitive physiological functions of TRPV1 and the consequences of activation in PBMC have not been established, TRPV1 has been implicated as a key mediator of various cellular responses to vanilloids *in vivo* and *in vitro* and several studies have demonstrated that TRPV1 mediates cell death in a variety of cell types, including human neuroblastoma and lymphoma cell lines and TRPV1-transfected human kidney cells [13, 29, 30, 57].

In preliminary experiments, concentration-dependent PBMC death following exposure to the endovanilloid/endocannabinoids, NADA and OLDA, and the plant-derived TRPV1 activators, capsaicin and RTX, was demonstrated, further supporting the presence of TRPV1 in these cells. Moreover, RTX was >10-fold more potent than capsaicin at inducing PBMC death, indicative of the 'classical' TRPV1 receptor. The relatively high

EC₅₀s for NADA (~30µM) and OLDA (40µM) should not be seen as evidence against a physiological role for TRPV1 on PBMC. *N*-arachidonylethanolamine (AEA), the first endovanilloid/cannabinoid to be described, induces apoptosis with an EC₅₀ ranging from 2-20µM, depending on the cell type/line [13, 21, 58, 59]. Moreover, the threshold for TRPV1 activation by AEA and other agonists is significantly lowered by several other regulatory factors, including protein kinase C, which can be activated by a diverse range of substances including bradykinins, acidic pH and prostaglandins, all generated by inflammation [60, 61].

NADA-induced PBMC death was mediated by TRPV1 and CB₂ receptors, as prior exposure to the TRPV1 antagonist, IRTX, and the CB₂ receptor antagonist, AM630 (but not the CB₁ receptor antagonist, AM251), almost completely blocked the cytotoxic effect. Whilst NADA is 50-fold more potent at CB₁ compared with CB₂ receptors (K_i values are 0.25 and 12µM, respectively), CB₁ receptors are primarily expressed in the brain, while CB₂ receptors are known to be expressed on various types of cells in the immune system including B-lymphocytes, natural killer cells and macrophages (Malfitano et al., 2007). An involvement of CB₂ receptors in the regulation of inflammation and immunity is widely accepted [62]. Furthermore, activation of CB₂ receptors by AEA has been shown to suppress immune function [63]. The present study suggests that a combination of TRPV1- and CB₂ receptor-mediated mechanisms may be required for NADA-induced PBMC death.

Interestingly, CPZ (at the concentration employed; 1µM) was without effect on NADA-induced PBMC death. TRPV1 agonist-induced increases in intracellular Na⁺ and tetramerisation of TRPV1 subunits at the plasma membrane level have both been suggested

as possible mechanisms of cell death. However, they have both been shown to be blocked by CPZ [64, 65]. Reilly et al. [66] have also presented data that highlight the existence of a TRPV1-mediated process that ultimately leads to cell death in a TRPV1-overexpressing bronchial epithelial cell line, which can be inhibited by IRTX (0.1-1 μ M), but not CPZ (0.01~50 μ M). The authors proposed that cell death may also occur via the activation of intracellular, ER-bound TRPV1, accessible only by IRTX, to promote vanilloid-induced toxicity.

Given the markedly increased expression of TRPV1-immunoreactivity in PBMC from ESKD patients, the effect of NADA on PBMC viability in ESKD patients was compared to control participants. PBMC from the ESKD patients demonstrated significantly increased susceptibility to *in vitro* NADA-induced cytotoxicity compared to controls, which was significantly related to an increase in TRPV1-immunoreactivity. This is comparable to other studies which have reported that cells over-expressing TRPV1 are more susceptible to vanilloid-induced cell death [13, 21, 58, 67, 68]. Whilst construction of full concentration-effect curves for TRPV1 agonists, and the effects of TRPV1, CB₁ and CB₂ receptor antagonists on NADA-induced PBMC death, in both ESKD and controls would have been appropriate, due to the limited supply of PBMC from both groups and the fact that several ESKD patients died within six months of PBMC being obtained, PBMC death was assessed using only a single (~EC₅₀) concentration of NADA and antagonist studies were not performed. As NADA-induced PBMC death in healthy donors could be blocked by the TRPV1 and CB₂ receptor antagonists, IRTX and AM630, respectively, without CB₂ immunocytochemistry a contribution of increased CB₂ receptor expression

cannot be ruled out in the present study. Nevertheless, there is minimal amino acid sequence homology between TRPV1 (particularly the sequence used to raise the antibody) and the CB₂ receptor. Moreover, the direct relationship between TRPV1 over-expression and NADA cytotoxicity is supported by the fact that there was a strong negative association between TRPV1-immunoreactivity and cell viability following exposure to NADA. Whilst it is not possible to compare the effect of NADA exposure on PBMC of ESKD patients with equivalent TRPV1-immunoreactivity to controls to definitively determine whether the increase in PBMC death is due to an increase in TRPV1-immunoreactivity, it would be prudent to perform Ca²⁺ flux studies to further elucidate the mechanism of NADA-induced PBMC death.

In conclusion, the present study suggests that the endovanilloid/cannabinoid, NADA, induces PBMC death by a mechanism that involves both TRPV1 and CB₂ receptors. PBMC from ESKD patients over-express TRPV1 protein (increased TRPV1-immunoreactivity) but not message, which in turn enhances NADA-induced death of these cells. This finding may provide a novel explanation as to why ESKD patients have reduced lymphocyte counts and impaired immune function. Thus, TRPV1 or CB₂ antagonists may have potential for the treatment of immune dysfunction in ESKD patients.

Acknowledgements

Many thanks to the Clifford Craig Medical Research Trust, in particular Lisa Anderson and Marianne Smith for their help in setting up the study and obtaining the informed consent of ESKD patients. The generosity of the ESKD patients from the Launceston General Hospital Renal Unit and controls for participating in the study was much appreciated. Thanks also to the Staff at the Launceston General Hospital Renal Unit for their assistance in blood sample collection, Drs Kiran Ahuja and Iain Robertson for their assistance with statistical analysis, and Ms Jodi Almond for her help in consenting control participants. This study was supported in part by a University of Tasmania Institutional Grant to DPG and Clifford Craig Medical Research Trust Grant to DPG and RGF.

References

- [1] A. Ferrer-Montiel, C. Garcia-Martinez, C. Morenilla-Palao, N. Garcia-Sanz, A. Fernandez-Carvajal, G. Fernandez-Ballester, R. Planells-Cases, Molecular architecture of the vanilloid receptor: Insights for drug design, *Eur. J. Biochem.* 271 (2004) 1820-1826.
- [2] C. Montell, Physiology, phylogeny, and functions of the TRP superfamily of cation channels, *Sci. STKE* 90 (2001) RE1-RE17.
- [3] O. Gohar, The transient receptor potential (TRP) ion channels: A remarkable multifunctional superfamily, *Modulator* 20 (2005) 20-23.
- [4] C. Huang, The transient receptor potential superfamily of ion channels, *J. Am. Soc. Nephrol.* 15 (2004) 1690-1699.
- [5] A. Dhaka, V. Uzzell, A. Dubin, J. Mathur, M. Petrus, M. Bandell, A. Patapoutian, TRPV1 is activated by both acidic and basic pH, *J. Neurosci.* 29 (2009) 153-158.
- [6] N. Zhang, J. Oppenheim, Crosstalk between chemokines and neuronal receptors bridges immune and nervous systems, *J. Leuk. Biol.* 78 (2005) 1210-1214.
- [7] P. Holzer, TRPV1 and the gut: from a tasty receptor for a painful vanilloid to a key player in hyperalgesia, *Eur. J. Pharmacol.* 500 (2004) 231-241.
- [8] A. Szallasi, D. Cortright, C. Blum, S. Eid, The vanilloid receptor TRPV1: 10 years from channel cloning to antagonist proof-of-concept, *Nature Rev.* 6 (2007) 357-372.
- [9] D. Clapham, TRP channels as cellular sensors, *Nature* 426 (2003a) 517-524.
- [10] M. Gunthorpe, C. Benham, A. Randall, J. Davis, The diversity in the vanilloid (TRPV) receptor family of ion channels, *Trends Pharmacol. Sci.* 23 (2002) 183-191.
- [11] L. Birder, A. Kanai, W. de Groat, S. Kiss, M. Nealen, N. Burke, K. Dineley, S. Watkins, I. Reynolds, M. Caterina, Vanilloid receptor expression suggests a sensory role

for urinary bladder epithelial cells, *Proc. Nat. Acad. Sci. USA* 98 (2001) 13396-13401.

[12] I. Heiner, J. Eisfeld, A. Luckhoff, Role and regulation of TRP channels in neutrophil granulocytes, *Cell Calcium* 33 (2003b) 533-540.

[13] C. Reilly, J. Taylor, D. Lanza, B. Carr, D. Crouch, G. Yost, Capsaicinoids cause inflammation and epithelial cell death through activation of vanilloid receptors, *Toxicol. Sci.* 73 (2003) 170-181.

[14] M. Sanchez, A. Sanchez, B. Collado, S. Malagarie-Cazenave, N. Olea, M. Carmena, J. Prieto, I. Diaz-Laviada, Expression of the transient receptor potential vanilloid 1 (TRPV1) in LCaP and PC-3 prostate cancer cells and in human prostate tissue, *Eur. J. Pharmacol.* 515 (2005) 20-27.

[15] M. Southall, T. Li, L. Gharibova, Y. Pei, G. Nicol, J. Travers, Activation of epidermal vanilloid receptor-1 induces release of proinflammatory mediators in human keratinocytes, *J. Pharmacol. Exp. Therap.* 304 (2003) 217-222.

[16] S. Stander, C. Moormann, M. Schumacher, J. Buddenkotte, M. Artuc, V. Shpacovitch, T. Brzoska, U. Lippert, B. Henz, T. Luger, D. Metze, M. Steinhoff, Expression of vanilloid receptor subtype 1 in cutaneous sensory nerve fibers, mast cells, and epithelial cells of appendage structures, *Exp. Dermatol.* 13 (2004) 129-139.

[17] S. Caprodossi, R. Lucciarini, C. Amantini, M. Nabissi, G. Canesin, P. Ballarini, A. Di Spilimbergo, M. Cardarelli, L. Servi, G. Mammana, G. Santoni, Transient receptor potential vanilloid type 2 (TRPV2) expression in normal urothelium and in urothelial carcinoma of human bladder: Correlation with the pathologic stage, *Eur. Urol.* 54 (2008) 612-620.

[18] M. Caterina, T. Rosen, M. Tominaga, A. Brake, D. Julius, A capsaicin-receptor

homologue with a high threshold for noxious heat, *Nature* 398 (1999) 436-441.

[19] A. Stokes, L. Shimoda, M. Koblana Huberson, C. Adra, H. Turner, A TRPV2-PKA signaling module for transduction of physical stimuli in mast cells, *J. Exp. Med.* 200 (2004) 137-147.

[20] A. Akbar, Y. Yiangou, P. Facer, J. Walters, P. Anand, S. Ghosh, Increased capsaicin receptor TRPV1-expressing sensory fibres in irritable bowel syndrome and their correlation with abdominal pain, *Gut* 57 (2008) 923-929.

[21] E. Contassot, M. Tenan, V. Schnuriger, M. Pelte, P. Dietrich, Arachidonyl ethanolamide induces apoptosis of uterine cervix cancer cells via aberrantly expressed vanilloid receptor-1, *Gynecol. Oncol.* 93 (2004a) 182-188.

[22] P. Facer, M. Casula, A. Smith, C. Benham, I. Chessel, C. Bountra, M. Sinisi, R. Birch, P. Anand, Differential expression of the capsaicin receptor TRPV1 and related novel receptors TRPV3, TRPV4 and TRPM8 in normal human tissues and changes in traumatic and diabetic neuropathy, *BMC Neurol.* 23 (2007) 7-11.

[23] P. Gepetti, S. Materazzi, P. Nicoletti, The transient receptor potential vanilloid 1 receptor: Role in airway inflammation and disease, *Eur. J. Pharmacol.* 533 (2006) 2047-2214.

[24] Y. Jia, R. McLeod, J. Hey, TRPV1 receptor: A target for the treatment of pain, cough, airway disease and urinary incontinence, *Drug News Perspect.* 18 (2005) 165-171.

[25] P. Mathews, A. Qasim, P. Facer, J. Davis, D. Thompson, P. Anand, Increased capsaicin receptor TRPV1 nerve fibres in the inflamed human oesophagus, *Eur. J. Gastroenterol. Hepatol.* 16 (2004) 897-902.

[26] P. Tympanidis, M. Casula, Y. Yiangou, G. Terenghi, P. Dowd, P. Anand, Increased

vanilloid receptor VR1 innervation in vulvodynia, *Eur. J. Pain* 8 (2004) 129-133.

[27] Y. Yiangou, P. Facer, N. Dyer, C. Chan, C. Knowles, N. Williams, P. Anand, Vanilloid receptor 1 immunoreactivity in inflamed human bowel, *The Lancet* 357 (2001) 1338-1339.

[28] G. Spinsanti, R. Zannolli, C. Panti, I. Ceccarelli, L. Marsili, V. Bachiocco, F. Frati, A. Aloisi, Quantitative real-time PCR detection of TRPV1-4 gene expression in human leukocytes from healthy and hyposensitive subjects, *Molecular Pain* 4 (2008).

[29] J. Chow, M. Norng, J. Zhang, J. Chai, TRPV6 mediates capsaicin-induced apoptosis in gastric cancer cells: Mechanisms behind a possible new "hot" cancer treatment, *Biochim. Biophys. Acta* 1773 (2007) 565-576.

[30] M. Johansen, C. Reilly, G. Yost, TRPV1 antagonists elevate cell surface populations of receptor protein and exacerbate TRPV1-mediated toxicities in human lung epithelial cells, *Toxicol. Sci.* 89 (2006) 278-286.

[31] C. Saunders, D. Kunde, A. Crawford, D. Geraghty, Expression of transient receptor potential vanilloid 1 (TRPV1) and 2 (TRPV2) in human peripheral blood, *Mol. Immunol.* 44 (2007) 1429-1435.

[32] Y. Anis, Involvement of Ca²⁺ in the apoptotic process - "friends or foes", *Pathways* 2 (2006) 2-7.

[33] N. Bronstein-Sitton, T cell signalling and activation: No simple matter, *Pathways* 2 (2006) 8-11.

[34] E. Schwarz, C. Kummerow, A. Wenning, K. Wagner, A. Sappok, K. Waggershauer, D. Griesemer, B. Straub, A. Quintana, M. Hoth, Calcium dependence of T cell proliferation following focal stimulation, *Eur. J. Immunol.* 37 (2007) 2723-2733.

- [35] M. Tarakçioğlu, A. Erbağcı, C. Usalan, R. Deveci, R. Kocabaş, Acute effect of hemodialysis on serum levels of the proinflammatory cytokines, *Med. Inflamm.* 12 (2003) 15-19.
- [36] B. Descamps-Latscha, The immune system in end-stage renal disease, *Curr. Op. Nephrol. Hypertension* 2 (1993) 883-891.
- [37] E. Kay, L. Raij, Immune abnormalities in renal failure and hemodialysis, *Blood Purif.* 4 (1986) 120-129.
- [38] Royal College of Pharmacologists, Australasia Royal College of Pharmacologists Annual Manual (version 4.0), Adelaide, 2004.
- [39] S. Duthie, L. Pirie, A. Jenkinson, S. Narayanan, Cryopreserved versus freshly isolated lymphocytes in human biomonitoring: endogenous and induced DNA damage, antioxidant status and repair capability, *Mutagenesis* 17 (2002) 211-214.
- [40] W. Hathaway, L. Newby, J. Githens, The acridine orange viability test applied to bone marrow cells I. Correlation with trypan blue and eosin dye exclusion and tissue culture transformation, *Blood* 23 (1963) 517-525.
- [41] J. Daher, The identity of the route of influx of calcium induced by caffeine in primary sensory neurons in mammals, *Master in Pathologia*, 2007.
- [42] M. Jhaveri, S. Elmes, D. Kendall, V. Chapman, Inhibition of peripheral vanilloid TRPV1 receptors reduces noxious heat-evoked responses of dorsal horn neurons in naïve, carrageenan-inflamed and neuropathic rats, *Eur. J. Neurosci.* 22 (2005) 361 - 370.
- [43] E. Rousseau, M. Cloutier, C. Morin, S. Proteau, Capsazepine, a vanilloid antagonist, abolishes tonic responses induced by 20-HET Am. *J. Physiol. Lung Cell Mol. Physiol.* 288 (2005) 460-470.

- [44] S. Skogvall, M. Berglund, M. Dalence-Guzmán, K. Svensson, P. Jönsson, C. Persson, O. Sterner, Effects of capsazepine on human small airway responsiveness unravel a novel class of bronchorelaxants, *Pul. Pharmacol. Ther.* 20 (2007) 273-280.
- [45] T. Brown, J. Brotchie, S. Fitzjohn, Cannabinoids decrease corticostriatal synaptic transmission via an effect on glutamate uptake, *J. Neurosci.* 3 (2003) 11073-11077.
- [46] S. Greenwood, S. Fitzjohn, Role of endocannabinoids in metabotropic glutamate receptor-mediated depression of excitatory synaptic transmission in the adult rat hippocampus *in vitro*, *J. Physiol.* 547P (2003) C29.
- [47] C. Michalski, M. Maier, M. Erkan, D. Sauliunaite, F. Bergmann, P. Pacher, S. Batkai, N. Giese, T. Giese, H. Friess, J. Kleeff, Cannabinoids reduce markers of inflammation and fibrosis in pancreatic stellate cells, *PLoS ONE* 3 (2008) e1701.
- [48] M. Rajesh, H. Pan, P. Mukhopadhyay, S. Bátkai, D. Osei-Hyiaman, G. Haskó, L. Liaudet, B. Gao, P. Pacher, Pivotal Advance: Cannabinoid-2 receptor agonist HU-308 protects against hepatic ischemia/reperfusion injury by attenuating oxidative stress, inflammatory response, and apoptosis, *J. Leukoc. Biol.* 82 (2007) 1382-1389.
- [49] A. Abbas, A. Lichtman, *Basic immunology: Functions and disorders of the immune system*, 3rd edn ed., Saunders Elsevier, China, 2009.
- [50] R. Hodes, The effects of aging on lymphocyte development and function: Introduction, *Springer Semin. Immunopathol.* 24 (2002) 1-5.
- [51] P. Linton, K. Dorshkind, Age-related changes in lymphocyte development and function, *Nature Immunol.* 5 (2004) 133-139.
- [52] K. Weinberg, R. Parkman, Age, the thymus, and T lymphocytes, *New Eng. J. Med.* 332 (1995) 182-183.

- [53] R. Ji, T. Samad, S. Jin, R. Schmoll, C. Woolf, p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia, *Neuron* 36 (2002) 57-68.
- [54] H. Dumann, S. Meuer, K. Meyer zum Buschenfelde, H. Kohler, Hepatitis B vaccination and interleukin 2 receptor expression in chronic renal failure, *Kidney Int.* 38 (1990) 1164-1168.
- [55] K. Raska, J. Raskova, G. Shea, G. Lifter, I. Ghobrial, R. Eisinger, L. Homer, T-cell subsets and cellular immunity in end-stage renal disease, *Am. J. Med.* 75 (1983) 734-740.
- [56] G. Sunder-Plassmann, G. Heinz, L. Wagner, B. Prychzy, K. Derfler, T-cell selection and T-cell receptor variable B-chain usage in chronic hemodialysis patients, *Clin. Nephrol.* 37 (1992) 252-259.
- [57] S. Kim, S. Kim, U. Oh, B. Jin, Transient receptor potential vanilloid subtype 1 mediates microglial cell death in vivo and in vitro via Ca²⁺-mediated mitochondrial damage and cytochrome *c* release, *J. Immunol.* 177 (2006) 4322-4329.
- [58] E. Contassot, R. Wilmotte, M. Tenan, M. Belkouch, V. Schnuriger, N. De Tribolet, K. Bourkhardt, P. Dietrich, Arachidonylethanolamide induces apoptosis of human glioma cells through vanilloid receptor-1, *J. Neuropathol. Exp. Neurol.* 63 (2004b) 956-963.
- [59] M. Giuliano, G. Calvaruso, O. Pellerito, P. Portanova, D. Carlisi, R. Vento, G. Tesoriere, Anandamide-induced apoptosis in Chang liver cells involves ceramide and JNK/AP-1 pathway, *Int. J. Mol. Med.* 17 (2006) 811-819.
- [60] R. Sancho, A. Macho, L. de La Vega, M. Calzado, B. Fiebich, G. Appendino, E. Munoz, Immunosuppressive activity of endovanilloids: *N*-arachidonoyl-dopamine inhibits activation of the NF- κ B, NFAT, and activator protein 1 signalling pathways, *J. Immunol.*

172 (2004) 2341-2351.

[61] V. Di Marzo, T. Bisogno, L. De Petrocellis, Anandamide: Some like it hot, *Trends Pharmacol. Sci.* 22 (2001) 346-349.

[62] T. Tanikawa, K. Kurohane, Y. Imai, Induction of preferential chemotaxis of unstimulated B-lymphocytes by 2-arachidonoylglycerol in immunized mice, *Microbiol. Immunol.* 51 (2007) 1013-1019.

[63] T. Eisenstein, J. Meissler, Q. Wilson, J. Gaughan, M. Adler, Anandamide and Delta9-tetrahydrocannabinol directly inhibit cells of the immune system via CB2 receptors, *J. Neuroimmunol.* 189 (2007) 17-22.

[64] E. Grant, A. Dubin, S. Zhang, R. Zivin, Z. Zhong, Simultaneous Intracellular Calcium and Sodium Flux Imaging in Human Vanilloid Receptor 1 (VR1) - Transfected Human Embryonic Kidney Cells: A Method to Resolve Ionic Dependence of VR1-Mediated Cell Death, *J. Pharmacol. Exp. Therap.* 300 (2002) 9-17.

[65] N. Kedei, T. Szabo, J. Lile, J. Treanor, Z. Olah, M. Iadarola, P. Blumberg, Analysis of the native quaternary structure of vanilloid receptor 1, *J. Biol. Chem.* 276 (2001) 28613-28619.

[66] C. Reilly, M. Johansen, D. Lanza, J. Lee, K. Lim, G. Yost, Calcium-dependent and independent mechanisms of capsaicin receptor (TRPV1)-mediated cytokine production and cell death in human bronchial epithelial cells, *J. Biochem. Mol. Toxicology.* 19 (2005) 266-275.

[67] M. Caterina, M. Schumacher, M. Tominaga, T. Rosen, J. Levine, D. Julius, The capsaicin receptor: a heat-activated ion channel in the pain pathway, *Nature* 389 (1997) 816-824.

[68] K. Thomas, A. Sabnis, M. Johansen, D. Lanza, P. Moos, G. Yost, C. Reilly, Transient receptor potential vanilloid 1 agonists cause endoplasmic reticulum stress and cell death in human lung cells, *J. Pharmacol. Exp. Therap.* 321 (2007) 830-838.

ACCEPTED MANUSCRIPT

Figure Legends

Fig. 1. TRPV1, TRPV2 and GAPDH mRNA expression in PBMC of controls and ESKD patients. (A) TRPV1 (B) TRPV2 and (C) GAPDH mRNA expression in PBMC of controls (n=29) and ESKD patients (n=32) was analysed by quantitative real time RT-PCR. mRNA for TRPV1 and TRPV2 is expressed as copies per 10^6 copies of the housekeeping gene, GAPDH. Data are presented as mean \pm SEM.

Fig. 2. Relationship between age and TRPV1 or TRPV2 mRNA expression in PBMC of controls and ESKD patients. The correlation between age and (A) TRPV1 or (B) TRPV2 mRNA expression in PBMC of controls (n=29) and ESKD patients (n=32) was analysed using linear regression (r^2 , correlation coefficient). mRNA is expressed as copies per 10^6 copies of the housekeeping gene, GAPDH.

Fig. 3. Photomicrographs of TRPV1- and TRPV2-immunoreactivity in PBMC of a control participant. Photomicrographs are black and white conversions of coloured (unmodified) TRPV1 and TRPV2 fluorescent images. Lanes 1-4 demonstrate the variation in TRPV1- and TRPV2-immunoreactivity from low (lane 1) to high (lane 4). (A) TRPV1-immunoreactivity in PBMC was greater and more concentrated than (B) TRPV2-immunoreactivity in both controls and ESKD patients. Scale bar = $10\mu\text{M}$.

Fig. 4. Quantitation of TRPV1- and TRPV2-immunoreactivity in PBMC of controls and ESKD patients. The intensity of (A) TRPV1- and (B) TRPV2-immunoreactivity (relative optical density x area) in PBMC from control participants (white bars) and ESKD patients

(black bars). Fifty cells from each participant were randomly selected and photographed. PBMC were subsequently classified as either small (~5-8 μ m) or large (~10-17 μ m) and the intensity of (A) TRPV1- and (B) TRPV2-immunoreactivity calculated. TRPV1; n=20 controls and 20 ESKD patients, TRPV2; n=10 controls and 10 ESKD patients,* denotes $p<0.05$ compared to controls. Data are presented as mean \pm SEM.

Fig. 5. Vanilloid-induced PBMC death. (A) Log-concentration-response curves for *N*-arachidonoyl-dopamine (NADA)-, *N*-oleoyl-dopamine (OLDA)-, capsaicin (CAP)- and resiniferatoxin (RTX)-induced death of PBMC from healthy adult donors. PBMC were exposed to increasing concentrations of NADA (3.4-100 μ m), CAP (15-500 μ m), RTX (1.5-50 μ m) or OLDA (10-100 μ m) for 24 hrs. Each point represents the mean \pm SEM of n=3 experiments. Where error bars are absent they are within symbols. (B) Effect of TRPV1, CB₁ and CB₂ antagonists on NADA-induced PBMC death. Cell viability studies were performed with the TRPV1 antagonists, 5'-iodoresiniferatoxin (IRTX, 100nM) and capsazepine (CPZ, 1 μ M), a CB₁ antagonist, AM251 (500nM), and a CB₂ antagonist, AM630 (1 μ M). PBMC from healthy adult donors were incubated for 24 hrs with either vehicle (0.2% ethanol, V, white bar), NADA (25 μ M, N, black bar), antagonist alone (white bar) or antagonist in the presence of NADA (black bar). Bars represent the mean \pm SEM (vehicle and NADA; n=6, antagonists and antagonists + NADA; n=3). * denotes $p<0.05$ compared with vehicle; # denotes $p<0.05$ compared with antagonist alone; † denotes $p<0.05$ compared with NADA alone. (C) NADA-induced PBMC death in controls and ESKD patients. Cell viability studies were performed on PBMC from controls and ESKD

patients using 24 hrs exposure to either vehicle (0.2% ethanol, white bars) or NADA (25 μ M, black bars). Bars represent the mean \pm SEM (vehicle; n=3 controls and 3 ESKD patients, NADA; n=7 controls and 7 ESKD patients, * denotes p<0.05 compared with vehicle; # denotes p<0.05 compared with controls. In all experiments, cell viability was determined by Trypan Blue exclusion and normalised to pre-exposure cell viability (expressed as a percentage).

Figure 1

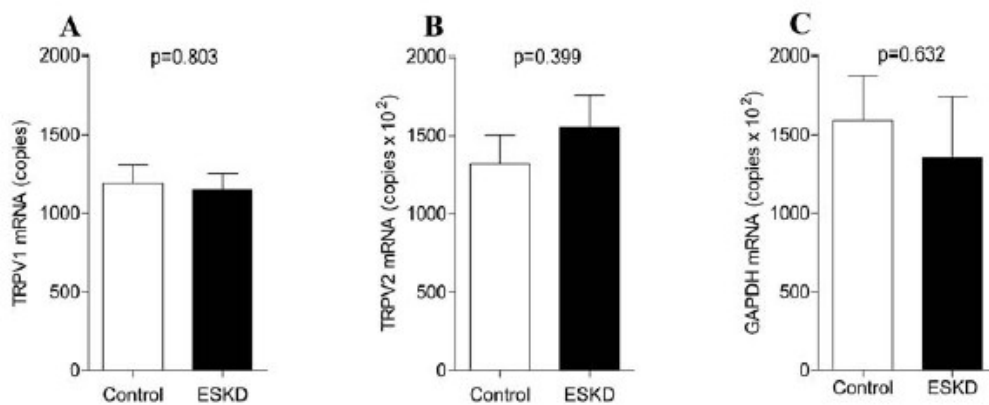


Figure 2

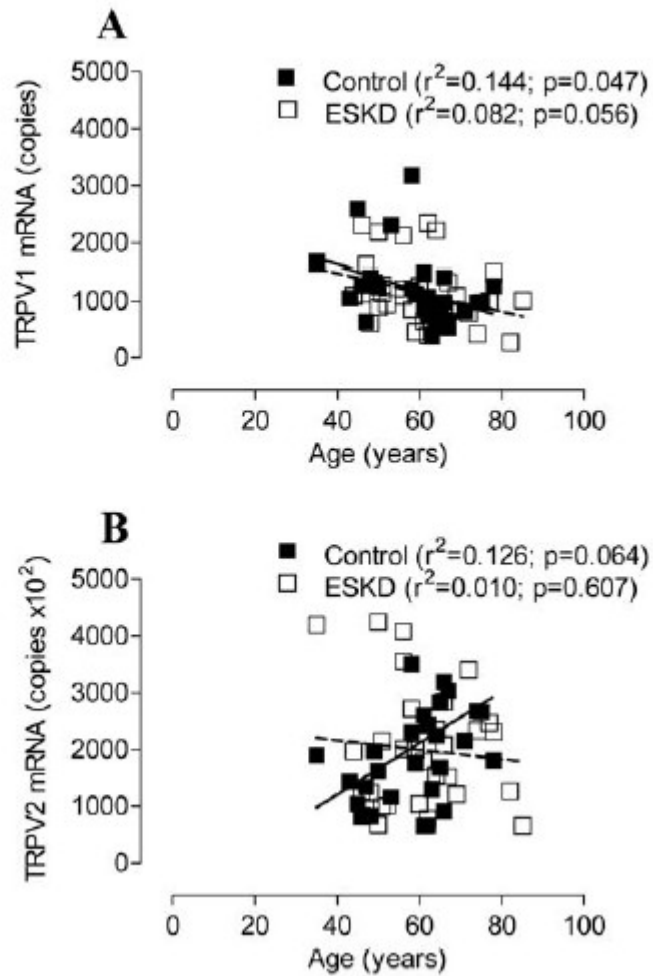
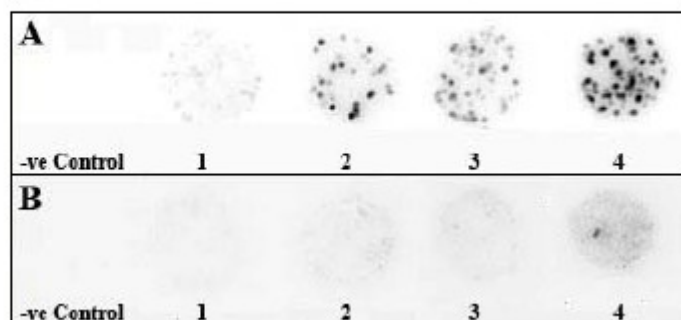
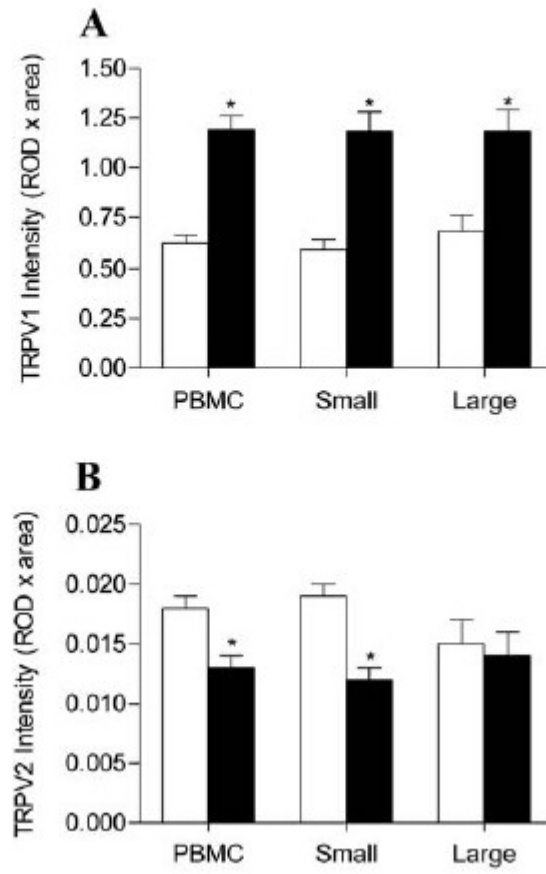


Figure 3



ACCEPTED MANUSCRIPT

Figure 4



A

Figure 5

