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A biochemical assessment of Ochratoxin A stress responses *in vitro*, with resveratrol as a possible therapeutic intervention.

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DEDICATION

To my parents. Thank you for giving me opportunities you did not have; it is only through your hard work and sacrifice that I stand where I am today. Thank you for instilling in me the importance of education and fortitude. Thank you most of all for your love and support.

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PUBLICATIONS

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ABBREVIATIONS

AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APAF1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
ARE	Antioxidant response element
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
Bad	B-cell lymphoma 2-associated death promoter
Bax	B-cell lymphoma 2-associated X-protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BEN	Balkan Endemic Nephropathy
BER	Base excision repair
BiP	Binding immunoglobulin protein
BSA	Bovine serum albumin
C/EBP	CCAAT-enhancer binding protein
cAMP	Cyclic adenosine monophosphate
Caspase	Cysteine-aspartic residue
CCM	Complete culture media
CHD	Coronary heart disease
CHOP	CCAAT-enhancer binding protein homologous protein
CIN	Chronic interstitial nephritis
CKD	Chronic kidney disease

CO_2	Carbon dioxide
COX	Cyclooxygenase
CR	Calorie restriction
cREB	Cyclic adenosine monophosphate response element binding
CYP_{450}	Cytochrome P450
DCFDA	Dichlorodihydrofluorescein diacetate
dH_20	Deionised water
DMEM	Dulbecco's minimum essential media
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DNPH	Dinitrophenylhydrazine
EGFR	Epidermal growth factor receptor
eIF1α	Eukaryotic initiation factor 2 alpha
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential media
EMT	Epithelial-mesenchymal transition
EPO	Erythropoietin
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ETC	Electron transport chain
FCS	Foetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GIT	Gastrointestinal tract
GPx	Glutathione peroxidase
GRP78	78kDa glucose-related protein
GSH	Glutathione

GSSG	Glutathione disulfide
H^+	Hydrogen
H_20	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HEK293	Human embryonic kidney cells
HIF1	Hypoxia inducing factor-1
HO-1	Heme oxygenase
Hr	Hours
HRP	Horse-radish peroxidase
HSP	Heat shock protein
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
IKK	Inhibitory kappa B kinase
IL-1β	Interleukin-1 beta
IRE1	Inositol requiring enzyme-1
IκB	Inhibitory kappa B
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
JECFA	Joint Food and Agriculture Organisation of the United Nations/WHO Expert Committee on Food Additives
Keap1	Kelch-like ECH-associated protein 1
LDL	Low-density lipoprotein
LLC-PK1	Porcine kidney epithelial cells
LonP1	Lon protease
MDCK	Madin-Darby canine kidney cells

mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NQO-1	Nicotinamide adenine dinucleotide phosphate quinone oxidoreductase 1
Nrf2	Nuclear factor-erythroid 2-related factor 2
O_2	Oxygen
O_2^-	Superoxide anion
OAT	Organic anion transporters
OGG1	8-oxoguanine-DNA-glycosylase
OH^-	Hydroxyl radical
OP-OA	Open-ring Ochratoxin A
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
OTHQ	Ochratoxin A hydroquinone
OTα	Ochratoxin alpha
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase-1
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivation 1-alpha

Phe	Phenylalanine
Phe-Tyr	Phenylalanine-tyrosine
pVHL	von Hippel Lindau protein
qPCR	Quantitative polymerase chain reaction
RBI	Relative band intensity
RER	Rough endoplasmic reticulum
RK13	Rabbit kidney cells
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulphate
Ser	Serine
SER	Smooth endoplasmic reticulum
Sir2	Silent information regulator 2
SIRT	Sirtuin
SMAC	Second mitochondrial-derived activator of caspases
SOD	Superoxide dismutase
SP1	Site-1 protease
SP2	Site-2 protease
TCA	Trichloroacetic acid
TEMED	Tetramethylenediamine
TGFβ	Transforming growth factor beta
TNF	Tumour necrosis factor
TRAILR	Tumour necrosis factor-related apoptosis inducing ligand receptor
TTBS	Tween-20 Tris buffered saline

TWI	Tolerable weekly intake
UPP	Ubiquitin proteasome pathway
UPR	Unfolded protein response
UTT	Urinary tract tumour
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
XBP1	X-box-binding protein 1

ABSTRACT

Ochratoxin A (OTA), a fungal-derived toxin, contaminates foods consumed by humans (grains, fruit, coffee, spices) and animals. This toxin has been implicated as a possible aetiological agent in nephropathies across Europe (Balkan endemic nephropathy) and North Africa (chronic interstitial nephritis), often causing tumour formation and eventual organ failure. Research has also identified OTA as hepatotoxic and immunomodulatory. OTA disrupts redox homeostasis by affecting cellular responses to oxidative stress, DNA damage and reactive oxygen species (ROS)-related dysfunction. Although tumour formation and renal fibrosis have been reported, a molecular mechanism of action, to date, has not been elucidated.

This study evaluates a range of concentrations of OTA (plasma level variations and IC_{50}) over 24hr and 48hr in human kidney (HEK293) and liver (HepG2) cell models. HEK293 IC_{50} concentrations of OTA at 24hr (1.5µM) and 48hr (9.4µM) increased oxidative stress by modulating the expression of endogenous antioxidants, Nrf2 and GSH; furthermore, OTA induced DNA fragmentation at both time periods. Resveratrol, a naturally produced phytoalexin found in berries and grapes, was introduced as an antioxidant compound. The co-treatment of OTA and resveratrol significantly ameliorated the toxicity of OTA; it reduced intracellular ROS, decreased DNA fragmentation, increased GSH concentrations and upregulated DNA repair enzyme, *OGG1*. Resveratrol further upregulated expression of repair and maintenance proteins, SIRT1 and LonP1.

A sub-IC_{50} (0.5µM) and supra-IC_{50} (2µM) concentration of OTA was assessed to determine its acute (24hr) influence on inflammatory processes that promote transformation (epithelial-mesenchymal transition) and aberrant replication. The canonical pathway of inflammation was activated by the sub-IC_{50} – protein expression of phosphorylated (p)-NFκB (Ser536) was significantly upregulated ($p<0.05$). OTA (0.5µM) induced inflammation (increased pNFκB and pIKK), 1.2µM OTA decreased inflammation (decreased caspase 1 and IL-1β, with increased IκBα protein) and 2µM OTA activated both the intrinsic and extrinsic pathways of apoptosis, and induced cell death (increased caspases 8, 9, 3/7). This established that acute exposure to OTA induces inflammation and cell death, possibly resulting in renal dysfunction and fibrosis.

Plasma range concentrations (0.125µM – 0.5µM) were used to observe the effects of OTA on tumour promoting pathways – ER stress, hypoxia and apoptosis, pathways often dysregulated in tumour suppression. OTA increased protein oxidation in HEK293 cells; ER stress-induced cell survival was observed in both kidney and liver cells. The protein expression of PERK and peIF2α was increased by OTA exposure in both cell lines, indicating protein misfolding and activation of the unfolded protein

response (UPR). ATF4 was upregulated; however, CHOP and executioner caspases (6 and 3/7) were significantly down-regulated, indicating cell survival. OTA's effect on pNrf2 and pNFκB indicated increased oxidative stress and inflammatory induction. The lowest OTA concentration (0.125μM) induced acute hypoxia (increased HIF1α expression) in kidney and liver cells, accompanied by increased HSP90 expression. The mRNA expression of *VEGF* and *TGFβ* was upregulated over prolonged exposure to OTA in both cell lines. Increased HIF1α and HSP90 produces a highly mutagenic environment.

The data shows that OTA disrupts several pathways, induces oxidative stress, protein damage, inflammation and hypoxia simultaneously. Lower concentrations of OTA induce more robust toxic effects and tumour promoting environments. Concurrent modulation of these cellular response pathways by OTA could promote cancer formation and organ dysfunction.

UKUQALA

I-Ochratoxin A (OTA), i-toxin etholakala ngogwayi, ihlamba ukudla okudliwa ngabantu (okusanhlamvu, izithelo, ikhofi, izinongo) nezilwane. Le toxin ifakwe njenge-etiological agent e-nephropathies kulo lonke elaseYurophu (i-Balkan nephropathy ephilayo) naseNyakatho Afrika (i-nephritis engapheliyo ye-interstitial interstitial), ngokuvamile edala ukubunjwa kwe-tumor kanye nokuhluleka kwesinye isikhathi. Ucwaningo luye lwaphawula i-OTA njenge-hepatotoxic kanye ne-immunomodulatory. I-OTA iphazamisa i-redox homeostasis ngokuthinta izimpendulo zamangqamuzana ekucindezelekeni kwe-oxidative, ukulimala kwe-DNA kanye nezinhlobo ze-oksijeni ezisebenzayo (i-ROS) -kusebenza okungasebenzi. Nakuba ukwakheka kwe-tumor kanye ne-renal fibrosis kuye kwabikwa, indlela yokusebenza yamangqamuzana, kuze kube yimanje, ingakacacisiwe.

Lolu cwaningo luhlola ububanzi bezinombolo ze-OTA (ukuhluka kwezinga le-plasma ne-IC_{50}) ngaphezu kwamahora angu-24 no-48h ezinso zomuntu (HEK293) namamodeli eseli (HepG2). I-HEK293 I-IC_{50} yokugxila kwe-OTA ngamahora angu-24 (1.5μM) no-48hr (9.4μM) yandisa ukucindezeleka kwe-oxidative ngokumisa ukubonakaliswa kwama-antioxidant okungapheli, i-Nrf2 ne-GSH; Ngaphezu kwalokho, ukuhlukaniswa kwe-DNA okwenziwe i-OTA kuzo zombili izikhathi zesikhathi. I-Resveratrol, i-phytoalexin eyakhiwe ngokwemvelo etholakala kumajikijolo namagilebhisi, yasungulwa njenge-antioxidant compound. Ukusebenzisana kwe-OTA kanye nokuvuselela kabusha kwamandla kakhulu kwalungisa uketshezi lwe-OTA; yanciphisa i-ROS ye-intracellular, iyanciphisa ukuhlukaniswa kwe-DNA, ukukhushulwa kwe-GSH okwandisiwe kanye ne-DNA yokulungisa i-enzyme ye-DNA, OGG1. I-Resveratrol iboniswa ngokuqhubekayo okusetshenziselwa ukuguqulwa kwamaprotheni, i-SIRT1 ne-LonP1.

I-sub-IC_{50} (0.5μM) kanye ne-supra-IC_{50} (2μM) yokuhlushwa kwe-OTA yahlolwa ukuze inqume ithonya layo elibi (24hr) ngezinqubo zokuvuvukala ezikhuthaza ukuguqulwa (ukuguqulwa kwe-epithelial-mesenchymal) nokuphindaphindiwe okungahambi kahle. Umzila we-canonical of inflammation wavulwa yi-IC_{-50} - amaprotheni ukubonakaliswa kwe-phosphorylated (p) -NFKB (Ser536) yayiphakanyisiwe kakhulu ($p < 0.05$). I-OTA (0.5μM) yenza ukuvuvukala (ukukhuphuka kwe-pNFκB ne-pIKK), i-1.2μM i-OTA yehle ukuvuvukala (ukwehla kwe-caspase 1 ne-IL-1β, nge-protein ye-IκBα eyandisiwe) no-2μM i-OTA yasebenzisa kokubili indlela yangaphakathi neyinkimbinkimbi ye-apoptosis, futhi yaholela ekufeni kweseli (amakhansela akhuphukile 8, 9, 3/7). Lokhu kwafakazela ukuthi ukuvezwa okunamandla ku-OTA kwenza kube nokuvuvukala nokufa kweseli, okungenzeka kubangele ukukhubazeka kwama-renal kanye ne-fibrosis.

Ukugxila kwamaplasma (0.125μm - 0.5μM) kwasetshenziswa ukugcina imiphumela ye-OTA nge-tumor ekhuthaza izindlela - ukucindezeleka kwe-ER, i-hypoxia ne-apoptosis, izindlela ezivame

ukuhlukunyezwa ekucindezelweni kwesisu. U-OTA ukwandise uketshezi lweprotheyini kuma-HEK293 amaseli; Ukusinda kwengqamuzana yokucindezeleka kwe-ER kubonwe emavikini amabili nezinso zesibindi. Inkulumo yamaphrotheni ye-PERK ne-peIF2α yanyuswa ukuvezwa kwe-OTA kokubili emigqeni yamaseli, okubonisa amaprotheni ukuhlukumeza nokusebenza kwempendulo yamaphrotheni (UPR) ebonakalayo.

I-ATF4 yayinganiselwe; Kodwa-ke, i-CHOP kanye ne-executioner caspases (6 no-3/7) yayinciphise kakhulu, ekhombisa ukuthi kusinda iseli. Umphumela we-OTA ku-pNrf2 futhi i-pNFκB ikhombise ukucindezeleka okwenyuka kwe-oxidative nokufakelwa okuvuthayo. I-concentration ye-OTA ephansi kunazo zonke (i-0.125μm) yenza i-hypoxia enobuchopho (inkulumo ekhulisiwe ye-HIF1α) emaqenjini ezinso nezinso, ehambisana nenkulumo ekhulisiwe ye-HSP90. Ukubonakaliswa kwe-MRNA ye-VEGF ne-TGFβ kwahlukunyezwa ngaphezu kokuvezwa isikhathi eside ku-OTA kumabili imigqa yeseli. Ukwanda kwe-HIF1α ne-HSP90 kuveza imvelo eningi kakhulu.

Idatha ibonisa ukuthi i-OTA iphazamisa izindlela eziningana, inciphisa ukucindezeleka okwenziwe nge-oxidative, ukulimala kwamaprotheni, ukuvuvukala ne-hypoxia ngesikhathi esisodwa. Izibalo ezingezansi ze-OTA zenza imiphumela enobuthi enamandla kakhulu kanye nokugqugquzela izimo. Ukuguqulwa kwesimo samanje kwezimpendulo zamagciwane nge-OTA kungakhuthaza ukwakhiwa komdlavuza kanye nokusebenza komzimba.

CHAPTER 1
INTRODUCTION

Fungal contamination of foods poses a significant world-wide health problem. Mycotoxins, fungal secondary metabolites formed during normal metabolism, which contaminate foods consumed by humans and animals (Bhat, 2010). Species of *Aspergillus* and *Penicillium* fungi produce various mycotoxins, one of the most prevalent being Ochratoxin A (OTA) (Reddy and Bhoola, 2010), which frequently contaminates grains, meat products, fruit, coffee, and alcoholic beverages (Ostry et al., 2013). Fungal and resultant mycotoxin contamination of food can be attributed to poor harvesting techniques, inadequate packaging and storage, and poorly regulated transport systems (Bhat, 2010). This toxin has a high affinity for protein binding (specifically albumin), resulting in a half-life of 35 days in humans (Petzinger and Ziegler, 2000). OTA can also affect the health of nursing infants, as it has been detected in human breast milk (Jonsyn-Ellis, 2012).

The International Agency for Research on Cancer (IARC) has classified OTA as a group 2B carcinogen, indicating that this toxin is possibly carcinogenic to humans ((IARC), 1993). Renal carcinogenicity in rodents exposed to OTA is well characterised (Cavin et al., 2007; Gautier et al., 2001). The toxin is resistant to high temperatures and pressures, therefore persisting in the food chain (Boudra et al., 1995; Trivedi et al., 1992). OTA is a known renal toxicant; it has also been shown to possess hepatotoxic, genotoxic, and immunotoxic characteristics (Gayathri et al., 2015; Pfohl-Leszkowicz et al., 1998; Thuvander et al., 1995). OTA has been linked as a possible contributing factor in Balkan endemic nephropathy (BEN), and chronic interstitial nephritis (CIN) in North African countries (Castegnaro et al., 2006; Zaied et al., 2011). These diseases present as progressive renal dysfunction, with eventual renal failure and possible tumour formation (Castegnaro et al., 2006; Wafa et al., 1998). Repeated exposure to OTA results in alterations to renal cell structure, consequently affecting renal efficiency (Heussner et al., 2007). The structurally altered cells become more fibroblast-like in nature, losing renal cell characteristics, and progressing to fibrosis (Heussner et al., 2007). Dopp et al. (1999) also showed that OTA disrupts cellular structure in Syrian hamster embryo (SHE) fibroblasts by binding to actin (Dopp et al., 1999).

OTA-induced disruptions in redox homeostasis and antioxidant response have been researched extensively. Ferrante et al. (2006) reported that OTA increased oxidative stress in murine liver and kidney (Ferrante et al., 2006). The master regulator of the antioxidant response, nuclear-erythroid 2-related factor 2 (Nrf2), has been shown to decrease in activity upon OTA exposure (Boesch-Saadatmandi et al., 2009). Redox disruptions compromise cell functionality and can lead to cell death.

Free radical interaction with proteins causes altered structure and function, resulting in misfolded or unfolded proteins. Conformational changes result in protein aggregates, which need to be cleared by appropriate quality control mechanisms (Gregersen and Bross, 2010). If this aggregation is not cleared and protein damage is not repaired, the cell undergoes apoptosis directed by the endoplasmic reticulum (ER) and the unfolded protein response (UPR) (Mirzaei and Regnier, 2008). Gan et al. (2017) showed that OTA induced ER stress in pig kidney and spleen, while Sheu et al. (2017) reported that OTA increased oxidative stress, induced ER stress and eventual cell death in mouse and rat mesangial cells (Gan et al., 2017; Sheu et al., 2016). Oxidative damage to DNA results in mutations and possible tumour formation, as seen by OTA-promotion of DNA damage in rat liver cells (Kamp et al., 2005a). A hypoxic environment is often highly mutagenic, and can contribute to DNA damage and genetic instability (Yuan and Glazer, 1998). Stachurska et al. (2011) showed that acute exposure to OTA increased the activity of hypoxia inducible factors, transcription factors responsible for cellular response to fluctuations in oxygen tension, and decreased vascular endothelial growth factor (VEGF) in porcine kidney epithelial (LLC-PK1) cells (Stachurska et al., 2011). This indicates that OTA compromised kidney functionality, as VEGF is required for efficient glomerular filtration.

Consumption of mycotoxins frequently occurs in underprivileged communities where food supply and storage conditions are inadequate. This becomes especially relevant in poorer communities, such as Sub-Saharan Africa, where the disease burden is high (Bankole et al., 2006; Jonsyn-Ellis, 2012). Many individuals are exposed to multiple illnesses, such as tuberculosis and HIV, as well as high levels of pollution, and mycotoxin ingestion. Furthermore, mycotoxins, such as OTA, have been detected in pet food and animal feed, posing a threat to the health of pets and livestock (Denli and Perez, 2010; Singh and Chuturgoon, 2017). It is important to determine how mycotoxins, such as OTA, affect biochemical outcomes in order to establish targeted therapeutic interventions.

Naturally occurring antioxidants provide easily accessible therapy to circumvent OTA toxicity. Resveratrol is a phytoalexin and potent antioxidant that is naturally produced in berries, grapes, peanuts, and cocoa plants (Burns et al., 2002) in response to stress or injury. Resveratrol decreases oxidative damage (lipid peroxidation), scavenges free radicals, and augments antioxidant defence (Fremont, 2000; Leonard et al., 2003; Miller and Rice-Evans, 1995). Resveratrol also possesses anti-cancer properties. Jang et al. (1997) showed resveratrol-induced inhibition of tumour initiation, promotion, and progression in rats (Jang et al., 1997). OTA and resveratrol affect the same biochemical pathways; thus, resveratrol could potentially reduce or inhibit OTA-induced toxicity and cellular damage.

OTA has been linked to the progression of renal failure and tumour formation in individuals with BEN/CIN, it also contributes to hepatotoxicity and immune dysfunction. The toxin acts on multiple pathways, however, there is little knowledge about these biochemical outcomes in humans. This study

aimed to determine the biochemical mechanisms by which OTA exerts its toxicity. The effects of OTA on human kidney (HEK293) and human liver (HepG2) cells was determined by observing how this toxin interacted with several stress pathways. Resveratrol was included to determine if the compound could augment the antioxidant response during co-exposure with OTA.

The specific objectives of this study were to determine the following:

- OTA-induced oxidative stress in kidney cells over 24hr and 48hr, and the potential therapeutic effects of resveratrol in this model
- Expression of inflammatory and apoptotic markers during acute exposure to OTA in kidney cells
- Activation of the UPR and ER stress in human kidney and liver cells, in response to plasma levels of OTA over 24hr and 48hr
- Effects of plasma level OTA concentrations on cellular hypoxia response and potential cancer biomarkers in kidney and liver cells over 24hr and 48hr

CHAPTER 2
LITERATURE REVIEW

2.1 Mycotoxins

Mycotoxins are low-molecular weight compounds produced as secondary metabolites by various species of filamentous fungi around the world. These compounds contaminate a variety of food and feedstuffs, ranging from crops and fruit, to meat, beverages, and animal feed. Fungal contamination of grains occurs frequently, and often results in vast financial losses (Bhat, 2010). Human and animal ingestion of mycotoxins can result in illness and death. Ochratoxins, Fumonisins, and Aflatoxins represent a few of the most studied and prevalent toxins encountered in human foods. Fungal contamination and accompanying mycotoxin production can occur during harvesting, handling, packaging and storage (Bhat, 2010).

Some species of fungi can produce multiple mycotoxins, while several fungal species may produce the same mycotoxin (Hussein and Brasel, 2001), as such it is important to determine the effects of toxin interaction within animal and human systems. Ingestion of these toxins can affect different communities of people. Financially disadvantaged communities would be exposed to higher levels of mycotoxins than wealthy communities, as foods of lower quality are likely to be cheaper and freely available. Mycotoxins frequently contaminate grains and cereals which make up a staple food group for many disadvantaged communities, such as maize and cassava consumption in Sub-Saharan Africa (Bankole et al., 2006; Jonsyn-Ellis, 2012). Children would be more susceptible to the effects of Patulin, a toxin produced by several fungal species, as it contaminates apple juice and fruit products, frequently consumed by young children (Fernández-Cruz et al., 2010).

Certain mycotoxins have been linked as aetiological agents in human diseases – Aflatoxin and Fumonisin involvement in hepatocellular carcinoma, oesophageal cancer and immune dysfunction (Wild and Gong, 2010; Zain, 2011). Ochratoxins have been associated with the incidence of renal dysfunction and renal tumour formation, specifically in Europe and North Africa (Abid et al., 2003; Castegnaro et al., 2006), generating much speculation into the toxin's DNA-damaging effects.

2.1.1 Ochratoxin A

Ochratoxins are a group of mycotoxins consisting of several members, the most common being Ochratoxin A (OTA), B (OTB) and C (OTC). OTA is the most prevalent and potent (Reddy and Bhoola, 2010). Ochratoxin A, or (*R*)-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-

yl)carbonyl)-L-phenylalanine, is produced by various species of *Aspergillus* and *Penicillium* fungi, frequently contaminating food consumed by humans and animals (Marquardt and Frohlich, 1992; Walker, 2002). OTA has been found in grains, alcoholic beverages, coffee, and meat products, such as pork and poultry (Ostry et al., 2013).

OTA was discovered in 1965 in South Africa; it has since been encountered in foods around the world (van der Merwe et al., 1965). The toxin has been classified as a group 2B (possibly carcinogenic to humans) compound by the International Agency for Research on Cancer (IARC) ((IARC), 1993). The Joint Food and Agriculture Organisation of the United Nations (FAO)/World Health Organisation (WHO) Expert Committee on Food Additives (JECFA) recommends a maximum tolerable weekly intake (TWI) of 112ng/kg body weight (WHO, 2002). OTA is primarily known for its nephrotoxicity (Creppy et al., 1995; Loboda et al., 2017; Schwerdt et al., 1999; Zanic-Grubisic et al., 2000) and has been linked to Balkan Endemic Nephropathy (BEN) and chronic interstitial nephritis (CIN) – progressive renal diseases resulting in kidney failure and possible tumour formation (Castegnaro et al., 2006; Zaied et al., 2011). It also displays hepatotoxicity (Capraro and Rossi, 2012; Ferrante et al., 2006; Gayathri et al., 2015), immunotoxicity (Creppy et al., 1983; Dwivedi and Burns, 1985; Thuvander et al., 1995), and genotoxicity (Hibi et al., 2013b; Pfohl-Leszkowicz et al., 1998; Zeljezic et al., 2006).

The chemical structure of OTA (Fig 2.1) enables it to participate in toxic biochemical reactions. OTA is a chlorinated isocoumarin compound; it consists of a dihydroisocoumarin moiety carboxyl-linked to L-Phenylalanine (Phe). The presence of L-Phe allows the toxin to participate in protein synthesis reactions, disrupting protein homeostasis (Pfohl-Leszkowicz et al., 2007). OTA is resistant to both high pressures (Trivedi et al., 1992) and temperatures (Boudra et al., 1995), making it difficult to remove or degrade the toxin by cooking.

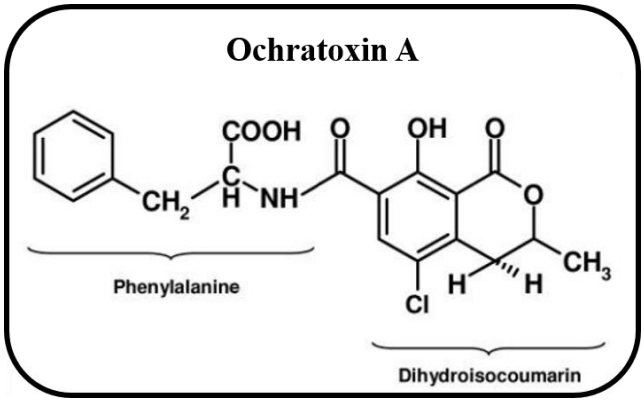

Figure 2.1: Chemical structure of Ochratoxin A (Hundhausen et al., 2008).

2.1.2 Absorption, Metabolism and Excretion

Since OTA is a common food and beverage contaminant, the main route of OTA absorption is through oral ingestion. Once ingested, mono-anion and di-anion forms of the toxin are present in the duodenum, favouring passive absorption along the gastrointestinal tract (GIT) (Pfohl-Leszkowicz et al., 2007). Once in circulation, OTA readily binds to serum albumin and other proteins; up to 99% of the ingested toxin binds to plasma proteins (Hagelberg et al., 1989). This high affinity for protein increases the half-life of the toxin – which varies across species. The half-life in humans is 35 days and OTA can still be detected in plasma samples 280 days post-exposure (Petzinger and Ziegler, 2000). The half-life was found to reach 4 hours (hr) in chickens (Galtier et al., 1981), 120hr in rats and 21 days in Rhesus Macaque monkeys (Hagelberg et al., 1989). Persistence *in vivo* allows for prolonged interaction and a greater potential for harmful outcomes.

Differences in absorption rates, half-lives and toxic effects depend on differences in species, sex, toxin dose, health status and general diet of the animal (Pfohl-Leszkowicz et al., 2007). Organic anion transporters (OAT) transport OTA into the kidney. Literature has shown that OTA participates in enterohepatic circulation (Kumagai and Aibara, 1982; Roth et al., 1988) and kidney reabsorption (Ringot et al., 2006), resulting in toxin deposits located at the renal papilla and medulla, increasing cell-toxin interaction time. Since OTA is protein-bound, it can be reabsorbed at the proximal and distal tubules, avoiding excretion, and accumulating in the kidneys. Distribution *in vivo* has been recorded extensively, with OTA found in the milk of rats and cows (Breitholtz-Emanuelsson et al., 1993a; Breitholtz-Emanuelsson et al., 1993b). It has also been detected in human breast milk, posing a significant health risk to breast-feeding infants (Jonsyn-Ellis, 2012).

Biotransformation provides an alternative mechanism to combat toxicity and persistence of OTA, however each metabolic derivative possesses different characteristics and levels of toxicity. Protease-mediated cleavage of the peptide bond separates the Phe moiety from the chlorinated dihydroisocoumarin moiety yielding Ochratoxin alpha (OTα) and Phe (Fig 2.2) (Sorrenti et al., 2013). OTα is unable to disturb protein homeostasis without the Phe moiety, resulting in a less toxic compound. This reaction is performed in the large intestine by gut microbiota. Hohler et al. (1999) proposed that ruminants (cows, sheep) would possess increased resistance to OTA toxicity as the rumen would contain greater communities of microorganisms (Hohler et al., 1999). This theory extends to the possibility of using ruminants to remove OTA from the environment by feeding contaminated grains to ruminants for biotransformation to less toxic metabolites, such as OTα (Hohler et al., 1999).

OTA can be converted into several metabolites via phase I reactions with cytochrome P_{450} isoforms (CYP_{450} 1A1/1A2/2B1/3A1/3A2). The kidney and liver express high levels of cytochromes, thus are

heavily involved in biotransformation, increasing susceptibility to OTA toxicity during excretion of the toxin (Ringot et al., 2006). Protein-OTA binding, however, prevents successful elimination, hence the need for chemical modification of the parent molecule into more soluble products. The cytochrome interaction yields hydroxylated derivatives – 4(R)-OH OTA, 4(S)-OH OTA, 10-OH OTA (Fig 2.2) – which are less toxic than OTA and can be easily eliminated (Pitout, 1969). The removal of OTA's chlorine atom produces OTB (Fig 2.2), a less toxic derivative which is unable to participate in iron chelation or reactive oxygen species (ROS) generation via Fenton reactions (Fig 2.4), OTA can also be transformed into more toxic metabolites, such as OTA hydroquinone (OTHQ) and open-ring OTA (OP-OA) – these compounds undergo clearance at a slower rate than OTA and research has shown increased OP-OA toxicity in rats (Xiao et al., 1996). OTA excretion occurs via biliary (faeces) and renal (urine) routes, as such the toxin is in close contact with both the liver and kidney, resulting in toxin accumulation.

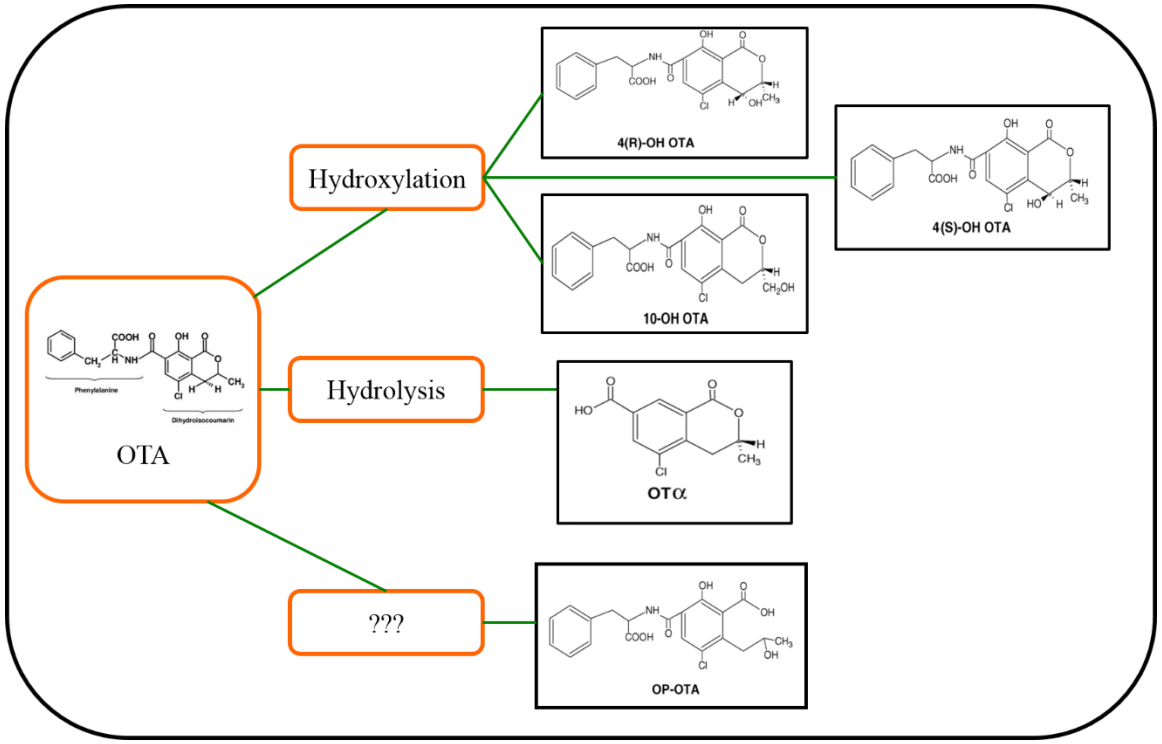

Figure 2.2: Biotransformation of OTA into various metabolites by hydroxylation and hydrolysis. (Adapted from Ringot et al. (2006), prepared by author (Ringot et al., 2006)).

2.1.3 Related Nephropathies

OTA has been identified as a possible contributing factor in nephropathies around the world, most prominently Balkan endemic nephropathy (BEN) – a renal disease experienced by individuals in the Balkan region of Europe (Fig 2.3) (Barnes et al., 1977; Castegnaro et al., 2006; Elling and Krogh, 1977).

The disease presents as progressive renal dysfunction and eventual failure, occasionally with the development of urinary tract tumours (UTT). This region experiences high levels of OTA contamination with inhabitants consuming OTA levels approaching and sometimes exceeding the TWI of 112ng/kg body weight (Abouzied et al., 2002; Vrabcheva et al., 2004). Chronic interstitial nephritis (CIN) presents with similar kidney dysfunction and tumour formation, and has been recorded in Northern Africa (Fig 2.3) (Abid et al., 2003; Wafa et al., 1998; Zaied et al., 2011). Heussner et al. (2007) reported alterations to renal cellular structure and function when repeatedly exposed to OTA (Heussner et al., 2007). The research suggested conversion from renal epithelia to fibroblast-like cells in an attempt to adapt and resist OTA toxicity. During conversion, collagen levels increase, renal cells proliferate, migrate, and become more fibroblast-like, ultimately losing renal structure and function (Heussner et al., 2007). These changes present as progressive fibrosis seen in OTA-induced nephrotoxicity (BEN/CIN).

Figure 2.3: Maps showing Balkan Peninsula (Google, 2009) and North African regions (Google, 2017) where incidences of Balkan endemic nephropathy (BEN) and chronic interstitial nephritis (CIN) have been reported.

2.2 Ochratoxin A Toxicity and Biochemical Responses

OTA is known to exert toxic effects on the kidney (Creppy et al., 1995), liver (Capraro and Rossi, 2012) and immune system (Thuvander et al., 1995). It is also a known carcinogen in rodents and possibly contributes to various nephropathies and tumour formation (Castegnaro et al., 2006). However, the exact mechanism of action is elusive; the toxin acts on several signalling pathways and alters cellular functioning at a biochemical level. Many mechanisms of action have been proposed, all of which could act in concert to contribute to overall OTA toxicity.

2.2.1 Mitochondrial Toxicity and Oxidative Stress Induction

2.2.1.1 Mitochondrial ROS Production

The mitochondrion regulates cellular metabolism, apoptosis and, most importantly, produces adenosine triphosphate (ATP) via respiration. Electrons are transferred along the electron transport chain (ETC) located on the inner mitochondrial membrane, driving ATP synthesis, some electrons combine with oxygen (O_2) prematurely and yield superoxide anions (O_2^-) (Semenza, 2011). Electron transport is optimised in a healthy cell to ensure efficient production of ATP without excessive ROS production, but disturbances in mitochondrial homeostasis destabilise this balance and result in overproduction of ROS. OTA has been reported to affect mitochondrial functioning, contributing to defective ATP synthesis, disruptions in the ETC, and increased production of ROS (O_2^-, hydroxyl radicals (OH^-)) (Cooke et al., 2003). The toxin possesses a chlorine atom, which can chelate iron and promote Fenton reactions (Fig 2.4), further increasing cellular ROS production. ROS are very reactive with biological molecules, increased production of these radicals culminates in deoxyribonucleic acid (DNA) strand breakage, calcium disruption, lipid peroxidation and cellular dysfunction. OTA has been shown to induce oxidative damage in several *in vitro* (Arbillaga et al., 2007; Raghubeer et al., 2015b) and *in vivo* (Cavin et al., 2007; Ferrante et al., 2006; Kamp et al., 2005a) models. Cells respond to this toxic insult by mobilising defence and repair mechanisms, most importantly, nuclear factor-erythroid 2-related factor 2 (Nrf2) and the antioxidant response element (ARE) would be activated to upregulate transcription of defensive proteins (Nguyen et al., 2009) – catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD) 1 in the cytoplasm and SOD2 in the mitochondria. Ferrante et al. (2006) reported increased oxidative stress and concomitant lipid peroxidation in the liver and kidneys of mice exposed to OTA (Ferrante et al., 2006).

$$\mathbf{Fe\ (II) + H_2O_2 \rightarrow OH^- + OH + Fe\ (III)}$$

Figure 2.4: Hydroxyl radical production by iron chelation during Fenton reaction (Halliwell and Chirico, 1993).

2.2.1.2 Nuclear factor-erythroid 2-related factor 2 (Nrf2): The Master Regulator

Nrf2, a transcription factor closely associated with its repressor, Kelch-like ECH-associated protein 1 (Keap1), is responsible for mounting the antioxidant defence response. Keap1 acts as an oxidative stress sensor (Kobayashi et al., 2004). During absences of stress stimuli, Keap1 sequesters Nrf2 in the cytoplasm and prevents its translocation to the nucleus (Fig 2.5). In this complex, Nrf2 is rapidly

degraded by the ubiquitin-proteasome pathway (UPP) (Itoh et al., 1999; McMahon et al., 2003; Stewart et al., 2003). Upon interaction with free radicals, Keap1 breaks the association with Nrf2 allowing the transcription factor to translocate to the nucleus, heterodimerize with a small Maf protein, and bind to the ARE in the promoter regions of antioxidant enzyme genes (Fig 2.5), such as NADPH quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) (Motohashi and Yamamoto, 2004; Vomhof-DeKrey and Picklo, 2012). The activation of this element upregulates transcription of a battery of antioxidant genes, such as SOD, catalase, and GPx, attempting to restore homeostasis and neutralise excessive ROS. SOD cleaves mitochondrial ROS produced from the ETC and transforms it into hydrogen peroxide (H_2O_2) and O_2 (Pias et al., 2003). Thereafter, catalase, a cytosolic selenoprotein, facilitates the reduction of H_2O_2 to water (H_2O) (Alfonso-Prieto et al., 2012). GPx and its cofactor, tripeptide thiol glutathione (GSH), confer protection against ROS by scavenging free radicals. GSH, consisting of glutamate, cysteine, and glycine, scavenges free radicals and is oxidised during this reaction, forming glutathione disulphide (GSSG) (Wu et al., 2004). The ratio of GSH:GSSG indicates the extent of cellular oxidative stress (Zitka et al., 2012). Research has shown that OTA downregulates Nrf2 activity affecting overall antioxidant defence responses (Boesch-Saadatmandi et al., 2009; Cavin et al., 2007; Stachurska et al., 2013). Disruptions in this pathway would render the cell vulnerable to further oxidative damage, protein synthesis impairment, and DNA strand breaks, contributing to disease manifestation. Manipulation of the Nrf2-Keap1 pathway could hold the key to combatting OTA toxicity, and research has shown that natural compounds, such as resveratrol and sulforaphane, could be used to modulate Nrf2 activity, enhancing the antioxidant response (Bishayee et al., 2010; Cheung and Kong, 2010).

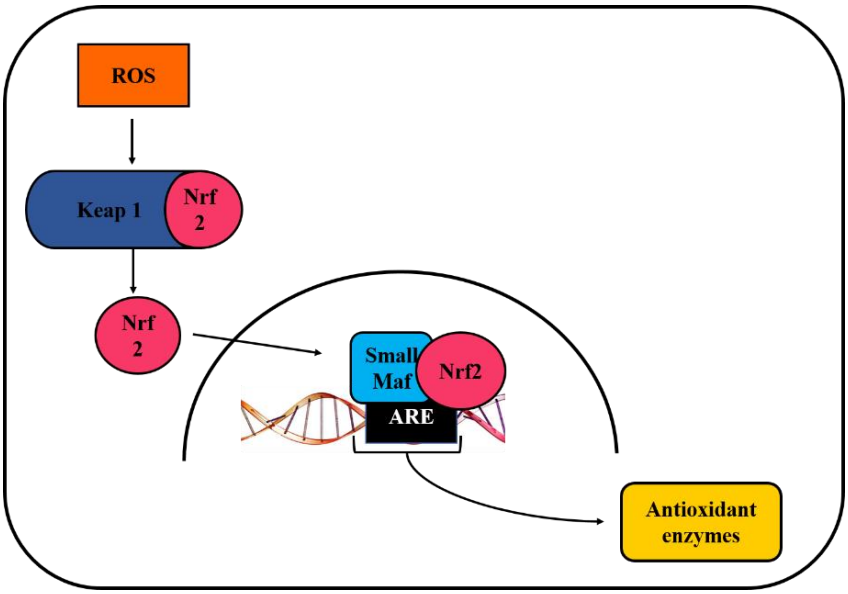

Figure 2.5: Representation of Nrf2-Keap1 interaction and activation of antioxidant response (Adapted from Kim et al. (2010), prepared by author (Kim and Vaziri, 2010)).

2.2.2 Protein Synthesis and Apoptosis

2.2.2.1 Calcium Homeostasis during Endoplasmic Reticulum (ER) Stress

Disruptions in calcium homeostasis indicate an early event of ROS-induced damage. Calcium plays an important role as a secondary messenger, mediating the transfer of biological information; if not tightly regulated disturbances in calcium homeostasis could be fatal to the cell. Altered homeostasis causes increased calcium release from cellular stores, such as the endoplasmic reticulum (ER) (Brini et al., 2013). This response jeopardises functionality of calcium-sensitive channels, causing depolarisation of the mitochondrial membrane, release of cytochrome c, and activation of the caspase cascade, driving the cell toward apoptosis. Dopp et al. (1999) showed that OTA disrupted intracellular calcium levels in Syrian hamster embryo (SHE) fibroblasts by releasing intracellular stores and increasing extracellular influx (Dopp et al., 1999). As a result, this alteration disrupted the cytoskeleton by directly binding to actin, ultimately compromising cellular structure and function (Dopp et al., 1999). Structural damage could be the cause of OTA-induced renal cell impairment and the progression to fibrosis.

Disruptions in ER homeostasis would further increase calcium release; these disruptions occur when OTA affects protein synthesis. OTA contains a Phe moiety, which allows the toxin to compete with Phe during protein interactions (Fig 2.6). OTA competitively inhibits phenylalanine-tyrosine (Phe-Tyr) synthase interaction with Phe, disrupting peptide elongation and amino acylation (Creppy et al., 1990; Zanic-Grubisic et al., 2000). Phe hydroxylase mediates Phe metabolism by converting Phe to Tyr using an irreversible hydroxylation reaction (Zanic-Grubisic et al., 2000). OTA could disrupt this process by competitive inhibition as a substrate for the enzyme, leading to decreased synthesis of Phe-requiring proteins and accumulation of Phe in the blood (Ringot et al., 2006). In this way, OTA would induce protein synthesis stress, activating several quality control pathways.

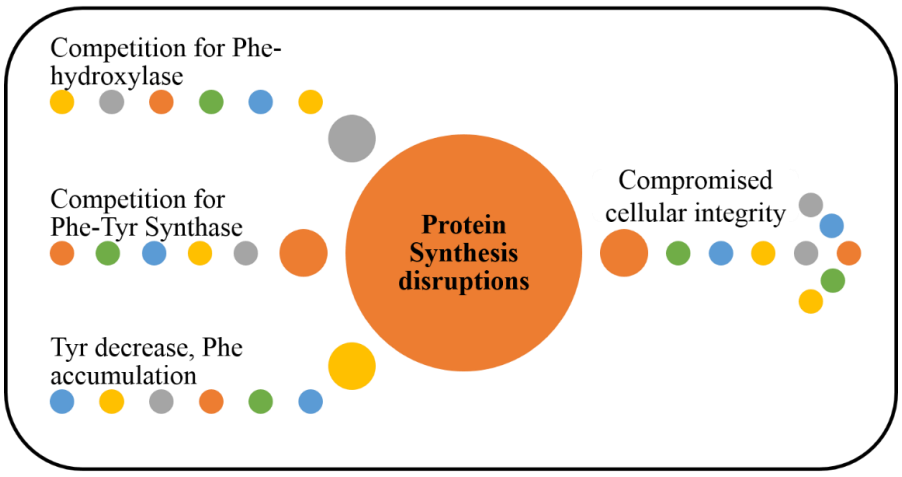

Figure 2.6: Protein synthesis disruptions resulting from phenylalanine moiety toxicity (prepared by author).

2.2.2.2 Lon Protease1 (LonP1)-mediated quality control

Proteins have unique structures dependent on their functionality; disruptions in this structure would lead to misfolded and dysfunctional proteins. Oxidative stress impacts protein structure, consequently affecting protein function (Gregersen and Bross, 2010). Proteins can be affected by ROS in several ways – oxidation of the protein backbone causing fragmentation, oxidation of amino acid side chains, and formation of protein carbonyl derivatives (Berlett and Stadtman, 1997). ROS-induced alterations to protein conformation lead to changes in hydrophobicity and protein aggregation – failure to remove these aggregates by proteolytic means would disrupt metabolism and even lead to cell death (Mirzaei and Regnier, 2008). Lon protease-1 (LonP1) is involved in mitochondrial quality control and the maintenance of mitochondrial DNA turnover (Quirós et al., 2014). This protease degrades damaged proteins and promotes cellular survival. LonP1 activity is increased by hypoxia, oxidative and ER stress, and has also been linked to tumourigenesis if overexpressed (Quiros et al., 2014). Sirtuin 3 (SIRT3), is a mitochondrially located deacetylase which functions in mitochondrial responses to oxidative stress and protein misfolding (Giralt and Villarroya, 2012). SIRT3 has been reported to regulate the acetylation of LonP1, affecting its stability and expression (Hebert et al., 2013). Accumulation of specific proteins is often linked to disease conditions, such as Alzheimer's, and aging (Mirzaei and Regnier, 2008).

2.2.2.3 ER Stress and Protein Repair

During oxidative stress the incidence of unfolded proteins increases, initiating the unfolded protein response (UPR) to restore protein conformation or induce apoptosis if the damage is too significant. The ER is a specialised organelle responsible for the synthesis, folding and transport of proteins to appropriate destinations. It also manufactures phospholipids and steroids, and serves as a calcium reservoir (Bravo et al., 2013). The ER is divided into two types – smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER). RER contains ribosomes for protein synthesis, while SER is ribosome-free and specialises in lipid manufacture. The localisation of SER and RER depends on cell function (i.e., protein or steroid synthesis specialisation) (Bravo et al., 2013). ER stress is induced by circumstances that alter ER homeostasis and function, such as nutrient deprivation, toxic insult, and protein misfolding. This in turn, activates the UPR – a three-pronged mechanism of protein repair. The UPR seeks to restore protein homeostasis by simultaneously decreasing global protein synthesis, increasing the folding capacity by increasing chaperone proteins, and degrading excess damaged proteins (Rozpędek et al., 2016).

Protein kinase RNA-like endoplasmic reticulum kinase (PERK), a marker for ER stress, phosphorylates eukaryotic initiation factor 2 alpha (eIF2α), which inhibits global protein synthesis to decrease client

load in the ER lumen (Fig 2.7). eIF2α also selectively translates activating transcription factor 4 (ATF4) to mediate expression of genes relating to amino acid metabolism, redox homeostasis, and cell survival (Ye et al., 2010). ATF4 can upregulate expression of CCAAT-enhancer binding protein (C/EBP) homologous protein (CHOP), a mediator of apoptosis (Nishitoh, 2012). The PERK-eIF2α-ATF4-CHOP pathway aims to decrease overall protein synthesis to alleviate accumulation of non-native proteins in the ER lumen (Bravo et al., 2013).

The inositol requiring enzyme-1 (IRE1) pathway, the second branch of the UPR, senses the accumulation of unfolded proteins in the ER lumen (Shamu and Walter, 1996) and increases the expression of ER chaperone proteins (such as heat shock proteins (HSP)), in this manner promoting cell survival during ER stress (Fig 2.7) (Cox et al., 1993). IRE1α and IRE1β are two paralogs present in mammalian cells. During ER stress IRE1α catalyses X-box-binding protein 1 (XBP1) RNA splicing (Tirasophon et al., 1998), yielding a protein which regulates genes responsible for ER protein synthesis, protein folding, redox metabolism, and degradation of misfolded proteins via ER-associated degradation (ERAD – ubiquitin-proteasome pathway) and autophagy (lysosomal pathway) (Walter and Ron, 2011).

The third branch of the UPR is the activating transcription factor 6 (ATF6) pathway. During ER stress activation this transcription factor is cleaved by Site-1 protease (SP1 – yielding p50ATF6α and site-2 protease (SP2 – yielding p60ATF6β) at the Golgi apparatus. p50ATF6α (the N-terminal fragment) enters the nucleus and upregulates UPR gene transcription to increase protein folding capacity (Fig 2.7). This activity leads to increased binding immunoglobulin (BiP) or 78kDA glucose-regulated protein (GRP78) (Schroder and Kaufman, 2005; Yamamoto et al., 2004), CHOP (Ma et al., 2002), and XBP1 (Yoshida et al., 2001). PERK, IRE1 and ATF6 are all rendered inactive during periods of low stress by BiP/GRP78, an ER chaperone (Wang et al., 2009). Upon sensing ER stress BiP/GRP78 releases the ER transmembrane signal transducers (PERK/IRE1/ATF6), inducing their activation and subsequent UPR activity. The pro-apoptotic function of CHOP is activated during periods of excessive ER stress, and if the UPR cannot restore homeostasis (Li et al., 2014). CHOP promotes the expression of pro-apoptotic genes, resulting in the progression of apoptosis.

Gan et al. (2017) reported that OTA induced ER stress in pig kidney and spleen, by upregulation of GRP78 protein (Gan et al., 2017). Sheu et al. (2017) further reported that OTA increased expression of phosphorylated PERK, phosphorylated eIF2α, and CHOP in mouse and rat mesangial cells (Sheu et al., 2016). This research showed OTA-induced ER stress, ROS production and resultant apoptosis (Sheu et al., 2016). OTA is known for renal and hepatic toxicity, however, research on ER stress in human kidney and liver cells is lacking, this leaves a gap in knowledge with regards to OTA-induced ER stress and UPR activity in human cells.

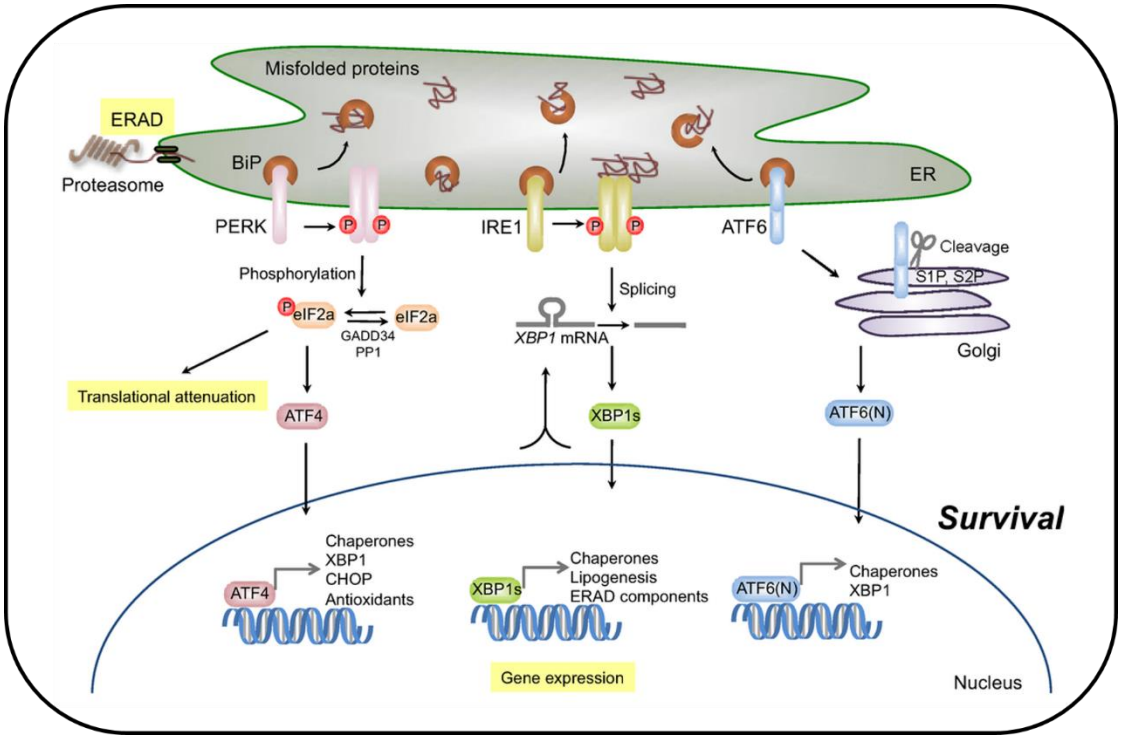

Figure 2.7: Representation of molecular mechanisms involved in ER stress induction of the UPR leading to protein repair and cell survival (Kadowaki and Nishitoh, 2013).

2.2.2.4 OTA and Apoptosis Induction

Apoptosis or programmed cell death is a carefully controlled process that is fundamental to long term survival in multicellular organisms. Apoptosis regulates cellular proliferation during development or injury (Orrenius et al., 2011). This process is carried out in a clinical manner, minimising leakage of cell contents, which in turn prevents mass inflammation. Apoptosis occurs during periods of cellular stress, where the cell becomes damaged and cannot be repaired. Apoptosis is controlled by families of pro- or anti-apoptotic proteins. Induction is modulated by the pro-apoptotic family of B-cell lymphoma 2 (Bcl-2) proteins (Bcl-2-associated X protein (Bax), Bcl-2-associated death promoter (Bad)) (Tsujimoto, 1998). Bouaziz et al. (2011) showed that OTA can modulate the expression of these Bcl-2 proteins in various human cancer cells to induce apoptosis (Bouaziz et al., 2011).

Apoptosis can occur via two mechanisms; both require a cascade of cysteine-aspartic proteases (caspases). When cognate ligands bind to death receptors, such as the tumour necrosis factor (TNF)-related apoptosis-inducing ligand receptor (TRAILR) and Fas, initiator caspases 8 and 10 are activated and in turn cleave and activate executioner caspases 3 and 7, resulting in apoptosis (Fig 2.8) (Ichim and Tait, 2016). This is termed the extrinsic pathway. During the second pathway, certain cellular stimuli (DNA damage, ER stress, hypoxia) trigger the activity of pro-apoptotic proteins (Bax, Bad), these

proteins induce permeabilisation of the outer mitochondrial membrane releasing cytochrome c and second mitochondrial-derived activator of caspases (SMAC) into the cytosol (Fig 2.8), inducing the formation of an apoptosome when cytochrome c interacts with apoptotic protease activating factor 1 (APAF1) (Ichim and Tait, 2016). This activates caspase 9, which activates executioner caspases 3 and 7, completing the intrinsic, or mitochondrial, mechanism of apoptosis. Literature shows that OTA is capable of inducing apoptosis *in vitro* in neuronal cells (Zhang et al., 2009), MDCK-C7 cells (Gekle et al., 2000) and human kidney cells (Raghubeer et al., 2017), as well as *in vivo* in rats (Petrik et al., 2003) and mice (Atroshi et al., 2000). Excessive apoptosis could cause tissue wasting and compromise organ functionality, whereas insufficient apoptosis would allow unchecked cellular proliferation and possible cancer formation.

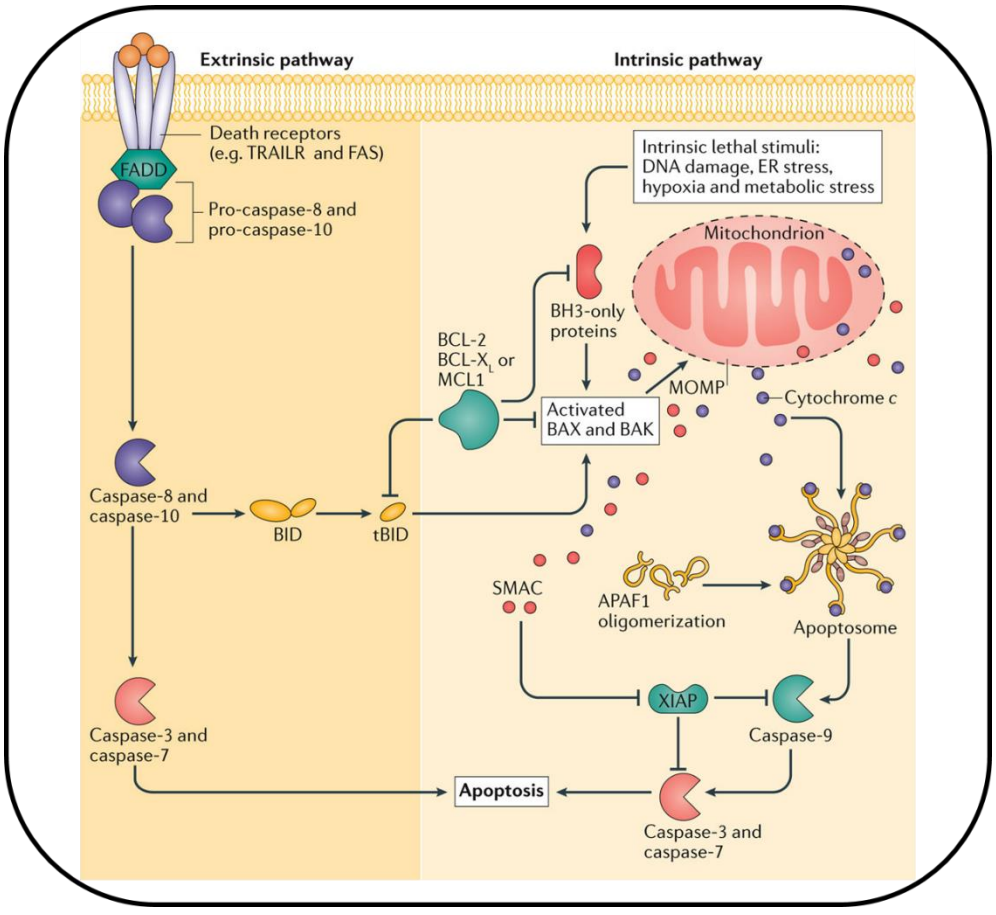

Figure 2.8: The extrinsic and intrinsic mechanisms of apoptosis (Ichim and Tait, 2016).

2.2.3 Genotoxicity

2.2.3.1 OTA-induced DNA damage

OTA-induced DNA damage is greatly debated, with some authors maintaining that OTA can directly induce genotoxicity, while others believe DNA damage is a secondary outcome due to OTA-induced oxidative stress (Sorrenti et al., 2013). DNA strand breakage is observed when OH^- radicals are inserted into DNA double bonds (yielding adduct radicals) or when hydrogen (H^+) is abstracted from thymine methyl groups (yielding allyl radicals) (Cooke et al., 2003). The radicals produce multiple DNA products, which can be removed if the cell is functioning optimally, however if the antioxidant and DNA repair responses are defective then the bases would persist and compromise genomic integrity. Genomic instability is a critical event in cancer initiation, with many studies reporting OTA-induced DNA fragmentation (Bouslimi et al., 2008; Golli-Bennour et al., 2010; Pfohl-Leszkowicz et al., 1991), although the method of this consequence is unknown. Raghubeer et al. (2015) demonstrated DNA fragmentation in human kidney cells exposed to OTA, and Kamp et al. (2005) showed DNA damage in rat liver cells (Kamp et al., 2005a; Raghubeer et al., 2015b). OTA has also been shown to upregulate genes related to DNA damage repair in primary rat hepatocytes (Chopra et al., 2010).

2.2.3.2 DNA Damage repair

Oxidative stress-induced DNA damage has been researched extensively, with a similar conclusion – excessive oxidative stress frequently results in DNA damage (Cerutti, 1989; Halliwell and Aruoma, 1991; Junod et al., 1989; Kleiman et al., 1990; Storz et al., 1987). DNA strand breakage can result in mutations and genomic instability (Cooke et al., 2003). Secondary measures are put into effect should the antioxidant defence systems fail, and oxidative DNA damage occurs. The cell imposes several check points to repair damage and prevent replication of aberrant DNA. A central tumour suppressor, p53, known as the "guardian of the genome" is involved in a number of DNA damage response pathways (Williams and Schumacher, 2016). p53 plays an important role in cell cycle control, by acting as a check point before allowing replication to occur. If DNA damage is detected, p53 arrests cell cycle progression to allow DNA repair (Williams and Schumacher, 2016). Wang et al. (1995) and Smith et al. (1995) showed that p53 is active in the nucleotide excision repair (NER) pathway, involved in repairing damage induced by ultraviolet (UV) irradiation (Smith et al., 1995; Wang et al., 1995b). p53 has been reported to participate in the base excision repair (BER) pathway, by augmenting the activity of base repair machinery via cell cycle control; should the BER pathway fail p53 could direct the cell towards apoptosis (Offer et al., 2001).

Another member of the BER process is 8-oxoguanine-DNA-glycosylase (OGG1), an enzyme which removes 8-oxoguanine base lesions which are commonly produced by ROS exposure (Cooke et al.,

2003). These highly mutagenic lesions result from adenine mispairs, inducing G:C to T:A transverse mutations (Abedin et al., 2013). OGG1 therefore, plays a fundamental role in preventing ROS-induced mutations, dysfunction of this enzyme would result in base lesion accumulation, increased mutation frequency and greater risk of carcinogenesis.

2.2.4 Inflammation, Hypoxia and the Cancer Microenvironment

2.2.4.1 Classical Inflammatory Pathway

Inflammation is a mechanistic response to cellular stressors by the innate immune system, it functions to remove the source of stress, repair the cellular damage, and restore homeostasis (Lawrence and Gilroy, 2007). However, prolonged or chronic inflammation can be a major determining factor in the onset and progression of diseases such as diabetes, cardiovascular diseases (CVD) and cancer (Khansari et al., 2009). The transcription factor, nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) consisting of two subunits (p65 and p50) (Fig 2.9), is responsible for both pro- and anti-inflammatory induction (Lawrence, 2009). During normal cellular conditions, NFκB is held inactive by its inhibitor, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), in the cytosol until specific signals are received to activate NFκB. At this point, IκB kinase (IKK) is activated and phosphorylates IκBα, causing its degradation by ubiquitination (Fig 2.9) (Israël, 2010). This results in IκBα dissociating from NFκB, allowing the transcription factor to translocate to the nucleus and transcribe numerous genes associated with inflammation, redox response, cellular repair, and apoptosis (Chaves et al., 2009; Pahl, 1999). Known inducers of NFκB are ROS, interleukin-1 beta (IL-1β), and TNFα (Denes et al., 2012; Fitzgerald et al., 2007; Renard et al., 1997). Caspase 1 (interleukin-1 converting enzyme (ICE)) processes inactive pro-IL-1β to the active form – IL-1β, which promotes NFκB activity.

Persistent inflammation could result in apoptosis if homeostasis cannot be reached; it could also alter cellular structure and function. Epithelial-mesenchymal transition (EMT) is characterised as the trans-differentiation of epithelial into mesenchymal cells, when uncontrolled it could lead to fibrotic conditions and cancer induction (Lamouille et al., 2014). Inflammation has been noted as an initiating factor in the progression of EMT (Li et al., 2016). Research has shown that inflammatory factors (TNFα, IL-1β) can exacerbate the effects of transforming growth factor beta (TGFβ) on EMT induction in bronchial cells (Borthwick et al., 2013; Doerner and Zuraw, 2009; Kamitani et al., 2011). Hennemeier et al. (2012) showed that OTA upregulates genes associated with inflammation and malignant transformation in human primary proximal tubular cells (Hennemeier et al., 2012). OTA induced oxidative stress and NFkB activation in HepG2 cells, with quercetin (a flavonoid found in fruits and vegetables) used as therapeutic intervention (Ramyaa and Padma, 2013). Little data exists detailing the inflammatory effects of OTA in human kidney cells.

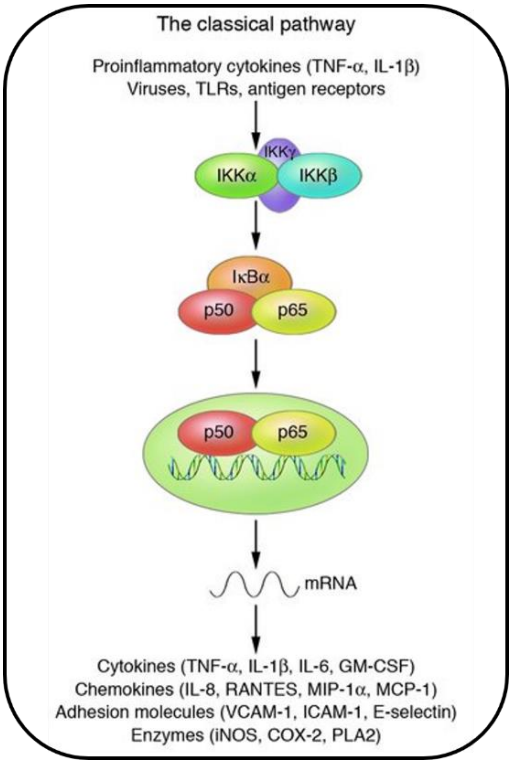

Figure 2.9: The classical pathway of NFκB activation by IKK-induced phosphorylation of IκBα to activate NFκB (Luo et al., 2005).

TGFβ is a cytokine which plays an important role in angiogenesis, apoptosis, proliferation and differentiation (Massagué et al., 2000). It can be activated by several stimuli, such as oxidative stress and inflammation. Cancer cells can exploit the function of TGFβ and escape proliferative control, resulting in malignancy and metastasis (Elliott and Blobe, 2005); overexpression of TGFβ has been linked with numerous human cancers (dos Reis et al., 2011; Higashi et al., 2001). Al Anati (2009) reported increased expression of TNFα and subsequent inflammation in rat liver post-OTA exposure (Al-Anati et al., 2009). Raghubeer et al. (2017) demonstrated that OTA induced inflammation in human kidney cells, by upregulation of active NFκB (Raghubeer et al., 2017). Furthermore, inhibition of inflammatory factors (TNFα) assists in reducing the occurrence of EMT (Borthwick et al., 2013), inhibiting NFκB reduced interstitial fibrosis (Miyajima et al., 2003), and upregulating the inhibitor of NFκB, IκBα, reduces fibrosis of the lung and kidney. Understanding EMT is particularly important for determining the mechanism underlying OTA toxicity and its ability to induce progressive fibrosis in the kidney (Lopez-Novoa and Nieto, 2009).

2.2.4.2 Hypoxia Inducing Factor-1 mediation of Hypoxia and Cancer

The cancer microenvironment represents the cellular surroundings in which cancer cells survive, this environment can be very hostile, but can also provide the perfect combination of factors for tumour cells to thrive. The environment contains areas of hypoxia, with an acidic pH, and nutrient deprivation (Vaupel, 2004). For tumours to develop into malignancies, they must pass through the hallmarks of cancer and acquire certain abilities to promote survival and dissemination (Mbeunkui and Johann, 2009). These abilities include moving within the tumour mass, as well as being able to survive transport in blood, the ability to degrade extracellular matrix, and re-establish growth in a new tissue environment (Weinberg, 2007). These characteristics all point towards malignant growth and metastasis. Hypoxia is often a common characteristic of the tumour microenvironment. Limited O_2 availability prompts tumour cells to adapt in order to survive, it also promotes genetic instability as hypoxic conditions are often mutagenic (Yuan and Glazer, 1998).

Cellular adaptive responses are mediated by hypoxia inducing factor-1 (HIF1), the master regulator of cellular hypoxia response, consisting of two subunits – HIF1α and HIF1β (Chun et al., 2002; Wang et al., 1995a). HIF1α is the O_2-dependent, cytoplasmic subunit; it is continually degraded during normoxia by the ubiquitin-proteasome system (Fig 2.10). HIF1β is O_2-independent and constitutively expressed in the nucleus (Semenza, 2001a). Specific prolyl residues of HIF1α are hydroxylated in well-oxygenated cells by O_2-dependent enzymes; these modified sites bind to the von Hippel-Lindau protein (pVHL), marking HIF1α for degradation (Cockman et al., 2000; Maxwell et al., 1999). During hypoxic conditions the prolyl sites are not hydroxylated, preventing pVHL binding and consequent proteasomal degradation; HIF1α is free to translocate to the nucleus to heterodimerize with HIF1β and activate hypoxia response genes (Fig 2.10), such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), growth factors, and glycolytic enzymes (Fig 2.11) (Vaupel, 2004). VEGF is the most potent angiogenic promoter, it is essential for efficient glomerular filtration, and the formation of new blood vessels (Pepper et al., 1991; Semenza, 2001b). EPO promotes the formation of new blood cells during hypoxia (Haase, 2013), while glycolytic enzymes (such as hexokinase) ensure that ATP levels are maintained in O_2-deprived cells (Marin-Hernandez et al., 2009).

Pyruvate dehydrogenase kinase-1 (PDK1) functions with HIF1α for cellular adaptation to hypoxia (Prigione et al., 2014). PDK1 inactivates pyruvate dehydrogenase (PDH) – which converts pyruvate into acetyl-CoA, used in the Krebs cycle to produce ATP. In this way PDK1 decreases pyruvate oxidation and increases lactate formation, called the "Pasteur Effect" (Liu and Yin, 2017). For cancer cell survival, HIF1α and PDK1 would produce a metabolic shift (Fig 2.11) to ensure an adequate supply of nutrients (Prigione et al., 2014). PDK1 could promote the Warburg effect, which would allow cancer cells to produce energy without pyruvate oxidation (Vander Heiden et al., 2009).

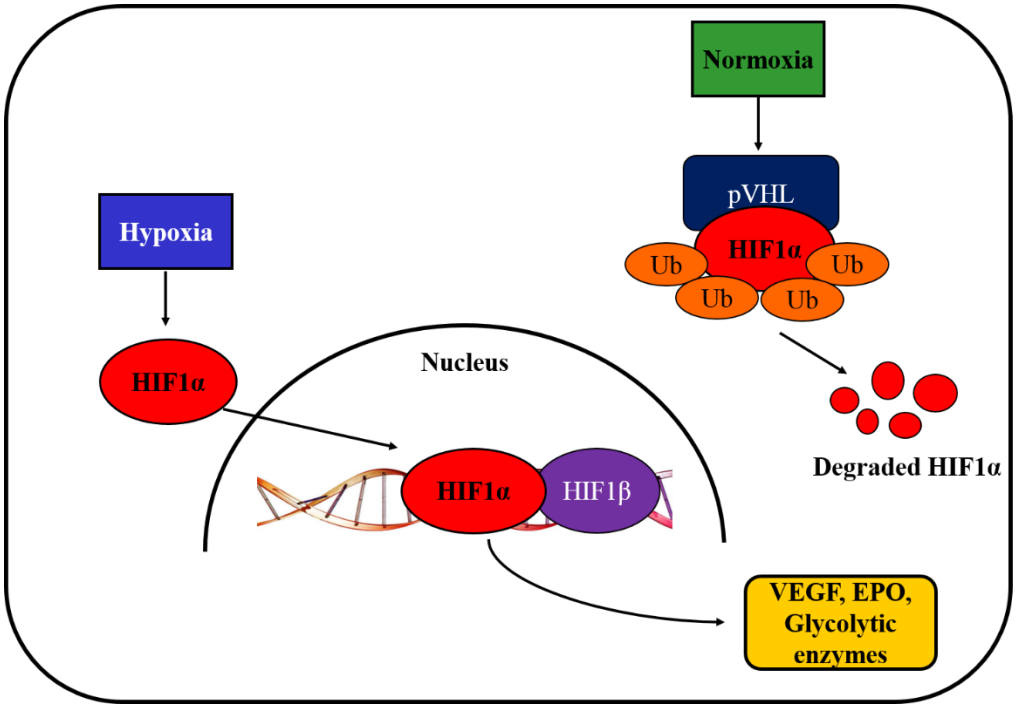

Figure 2.10: Representation of HIF1α-pVHL interaction during normoxia (degradation by ubiquitin-proteasome system) and hypoxia (nucleus translocation). (Adapted from Pires *et al* (2014), prepared by author (Pires, 2014)).

Heat shock protein 90 (HSP90) functions to stabilise protein conformation and degrade damaged proteins (Buchner, 1999). It interacts closely with HIF1α and is a crucial regulator of HIF1α activity (Isaacs et al., 2002). HSP90 participates in steroid receptor functioning; however, this ability can prove detrimental as HSP90 can modulate the functioning of growth factor receptors, such as epidermal growth factor receptor (EGFR), and alter apoptosis signalling (Lurje and Lenz, 2009). HSP90 has been linked to tumourigenesis as it influences growth signals, stabilises mutant proteins and induces VEGF (Fontana et al., 2002). These abilities indicate that HSP90 can select for cells with DNA mutations, as well as promote angiogenesis and cell proliferation of compromised cells. HSPs are often overexpressed in tumours, as such current research has focused on targeting HSPs in search of anti-cancer therapeutics (Whitesell and Lindquist, 2005).

The effects of OTA on hypoxia inducible factors (HIFs) in porcine kidney epithelial (LLC-PK1) cells has been reported by Stachurska et al. (2011), indicating that acute OTA exposure increases activity of HIFs and decreases VEGF production, compromising kidney functionality (Stachurska et al., 2011). This data poses an interesting question as to how OTA-induced modulation of hypoxia responses contributes to nephrotoxicity; how do these effects differ during periods of extended exposure to the

toxin? Presently, no data exists on the relationship between OTA and the hypoxia response in human kidney or liver cells.

Taken together, these factors contribute to tumour cell adaptation and metastasis in hypoxic environments. Yasuda et al. (2004) demonstrated that HIF1α induces a metabolic switch in cancer cells by upregulating VEGF and hexokinase expression in hepatocellular carcinoma (Yasuda et al., 2004), while Kallergi et al. (2009) reported increased levels of HIF1α and VEGF in metastatic breast cancer (Kallergi et al., 2009). Cancer cells are able to respond and adapt to hostile conditions with HIF1α as the mediator of adaptation, exerting selective pressure on malignant cells by selecting adapted (increased proliferation and apoptosis resistance) over non-adapted cells (Graeber et al., 1996).

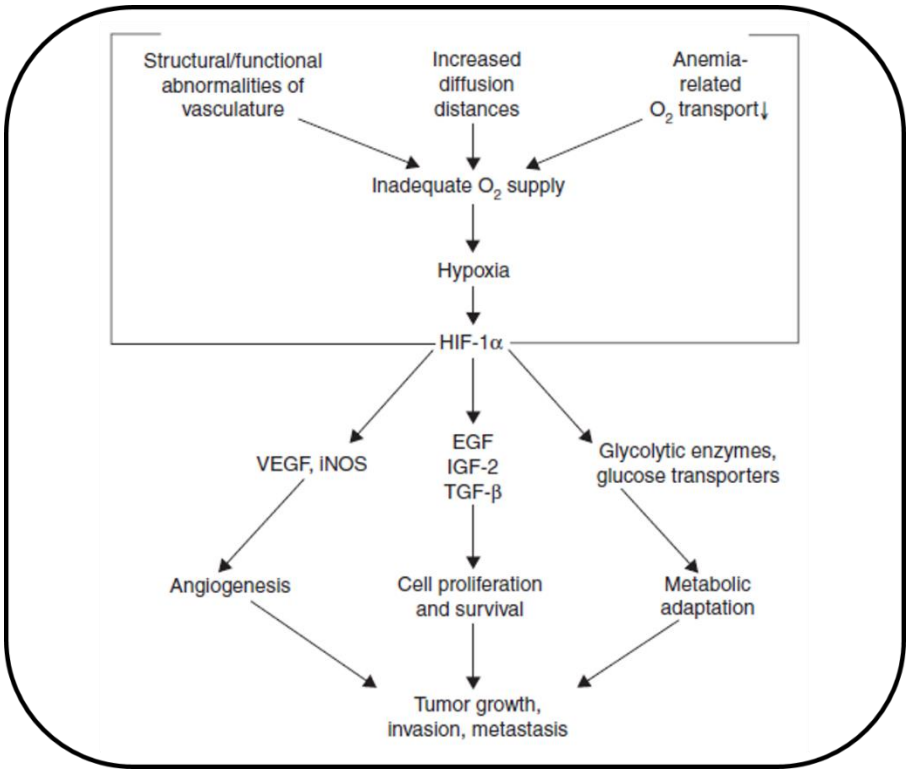

Figure 2.11: HIF1α activators and downstream targets, resulting in cellular adaptation of cancer cells to a hypoxic environment (Vaupel, 2004).

2.3 Resveratrol, a Possible Therapeutic

Resveratrol is a naturally produced plant compound reported to possess an array of medical benefits. It can be found in various foods, such as berries, cocoa products, peanuts, wine and grapes (Burns et al., 2002). Resveratrol was discovered in 1940 when it was isolated from the roots of *Veratum grandiflorum* or white hellebore (Takaoka, 1940). Large amounts of resveratrol are produced by *Vitis vinifera*

(common grape vine) in response to cellular stress or injury, UV radiation damage or fungal infection (specifically *Botrytis cinerea*) (Langcake et al., 1979). In 1976 trans-resveratrol (Fig 2.12) was discovered in grape skins by Langcake and Pryce (1976) (Langcake et al., 1979). This nutraceutical is frequently found in wines due to its presence in grape skin. The concentration of resveratrol in wine depends on several factors – the type of wine, the type of grape being used, the extent of fungal infection and the geographical region of the vineyards (Fremont, 2000). Red wine often contains greater concentrations of resveratrol because the skin, seed and stem (greatest sources of resveratrol production in grapes) are macerated during wine production (Gambini et al., 2015). Since resveratrol is poorly soluble in water, grape fermentation processes promote the extraction of resveratrol into the alcohol (Gambini et al., 2015).

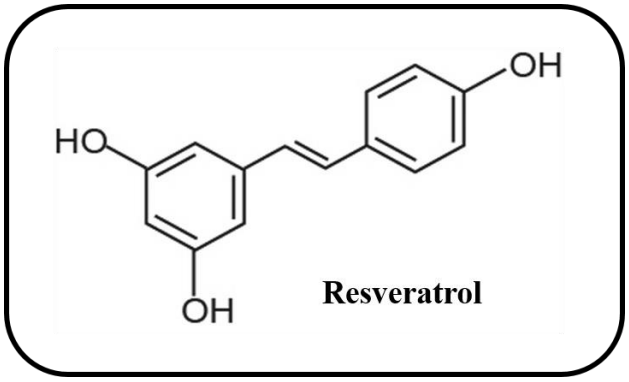

Figure 2.12: Chemical structure of resveratrol (Davinelli et al., 2012).

2.3.1 Absorption and Metabolism

Human ingestion of resveratrol varies as the content in food varies; grapes, berries and wine can be high in resveratrol; however, absorption and bioavailability pose a problem. The compound is readily absorbed from the GIT upon ingestion, thereafter it enters the bloodstream where several forms of resveratrol can be found. The liver carries out phase II metabolism on resveratrol by sulphate and glucuronic acid conjugation – yielding resveratrol-3-O-glucuronide and resveratrol-3-O-sulphate. Some resveratrol remains free in the blood and can bind to albumin, which could act as a reservoir to preserve bioavailability (Delmas et al., 2011). Enterohepatic cycling of resveratrol has been suggested in rats and humans, this process sees the majority of resveratrol being metabolised, reducing the concentration of free resveratrol reaching target tissues in the body (Marier et al., 2002).

2.3.2 Therapeutic Potential

2.3.2.1 Antioxidant Defence and Anti-inflammatory Action

Research has shown that resveratrol is able to augment the antioxidant defence response. This response is initiated when cells experience a redox shift, favouring free radical formation. The antioxidant defence attempts to restore homeostasis by reducing the damaging effects of ROS (Birben et al., 2012). Resveratrol has been shown to decrease lipid peroxidation, increase antioxidant activity and scavenge free radicals (Fremont, 2000; Leonard et al., 2003; Miller and Rice-Evans, 1995). It exerts these protective effects over both the liver and kidney by upregulating Nrf2 (Fig 2.14), thereby altering expression of antioxidant enzymes – GPx, catalase, and SOD (Bishayee et al., 2010; Kitada and Koya, 2013; Sharma et al., 2006). Literature has shown that OTA disrupts the Nrf2 pathway, thus resveratrol could be used as a therapeutic intervention to augment cellular antioxidant responses.

2.3.2.2 Cardiovascular Protection

The use of resveratrol against heart disease has been recorded as far as 2000 years ago, when it was used in Ayurvedic medication (Paul et al., 1999). In 1982, Arichi et al. (1982) reported that resveratrol reduced lipogenesis in the liver of rats, this research indicated metabolic effects of resveratrol, particularly with regards to cardio-protective qualities (Arichi et al., 1982). The "French Paradox" explains the observation that French people consume foods high in saturated fat, however, experience low incidence of coronary heart disease (CHD); it was observed that the individuals in this region also frequently consumed red wine (Catalgol et al., 2012; Kopp, 1998). It was thought that red wine (containing resveratrol) could contribute to the low incidence of CHD. Frankel et al. (1993) showed that resveratrol protected against oxidation of low-density lipoprotein (LDL), an indication that resveratrol could prevent atherosclerosis (Frankel et al., 1993).

Resveratrol decreases inflammation in a manner similar to non-steroidal anti-inflammatory drugs (NSAIDs). It increases vasodilation and inhibits the activity of cyclooxygenase (COX), preventing conversion of arachidonic acid to prostaglandins, which cause platelet aggregation (Fig 2.14) (Chen and Pace-Asciak, 1996; Guilford and Pezzuto, 2011; Jang et al., 1997). The antioxidant and anti-inflammatory properties of resveratrol would contribute to overall cardiac protection. Scavenging of free radicals decreases the oxidation of LDL, preventing its accumulation in atherosclerotic lesions (Luo et al., 2014; Martinet and Kockx, 2001). Decreased platelet aggregation coupled with mediation of the COX pathway would decrease vasodilation and protect against CHD (Bradamante et al., 2004; Momchilova et al., 2014).

2.3.2.3 Resveratrol and Sirtuins

Sirtuins (SIRTs) are histone deacetylases (HDACs) dependent on nicotinamide adenine dinucleotide (NAD^+), linking their function to the cellular nutrition state (Dang, 2014). There are seven SIRT members (SIRT1 – SIRT7) found in humans, SIRT function is linked to cellular longevity and the ability to protect against diseases associated with aging (Morris, 2013). The silent information regulator 2 (Sir2) protein, found in *Saccharomyces cerevisiae* (yeast), functions in transcriptional silencing, mediating cellular health and controlling lifespan (Brachmann et al., 1995; Gottlieb and Esposito, 1989; Tennen et al., 2012). SIRT1 is a mammalian homologue to Sir2, it is the most studied and functions in cellular stress response and metabolic alterations (Saunders and Verdin, 2007). SIRT1 has been reported to modulate peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and p53 in the nucleus, and NFκB and HIF1α in the cytosol (Morris, 2013), influencing cell survival and proliferation.

SIRT3 (mitochondrially located) is also activated by resveratrol, and participates in oxidative stress response, mitochondrial stress and cardio-protection (Fig 2.13) (Chen et al., 2015; Mathieu et al., 2016; Zhou et al., 2014). Conversely, OTA is reported to influence oxidative and mitochondrial stress, as well as inflammatory pathways; it would prove valuable to determine the effects of SIRT1 and resveratrol on these pathways in combination with OTA, since the two compounds are often found together (e.g. grapes/wine).

Aging is outlined as the progressive loss of cellular responsiveness, cellular homeostasis disruptions and increased dysfunction (López-Otín et al., 2013). Calorie restriction (CR) has been found to decrease the effects of aging and extend lifespan by activating SIRT1, this in turn modifies cellular stress response and DNA repair processes, reducing genomic instability and promoting cell survival (Fig 2.14) (Baur, 2010; Tennen et al., 2012). Resveratrol has been identified as a CR mimetic, activating SIRT1 and subsequently delaying cellular aging (Barger JL, 2008).

2.3.2.4 Chemotherapeutic Potential

The hallmarks of cancer are defined as acquired biochemical capabilities for the progression of tumourigenesis. There are six hallmarks: continuous proliferation, bypassing apoptosis, non-responsiveness to growth suppressive signals, immune system evasion, inducing angiogenesis and fostering metastatic characteristics (Hanahan and Weinberg, 2011). To attain these qualities, the cell requires a level of genomic instability and an inflammatory environment, which OTA toxicity provides. Research has shown that resveratrol is able to disrupt a number of these requirements. Together, the

antioxidant and anti-inflammatory capabilities of resveratrol could remedy genomic instability, chronic inflammation and angiogenesis (Delmas et al., 2006; She et al., 2003). Cyclooxygenase action can metabolically activate carcinogens, inducing DNA damage, while prostaglandins stimulate tumour growth and disrupt immune system functioning. By downregulating inflammatory responses resveratrol prevents COX and prostaglandin contribution to carcinogenesis (Jang et al., 1997).

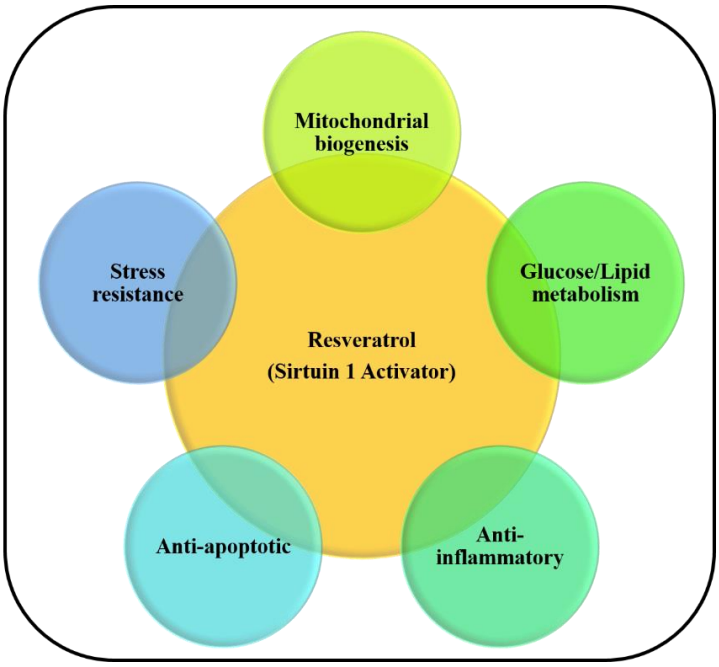

Figure 2.13: Beneficial effects of resveratrol as a SIRT1 activator (prepared by author).

Jang et al. (1997) proved the chemopreventive potential of resveratrol in rats, where resveratrol was shown to inhibit biochemical incidences of tumour initiation, promotion and progression (Jang et al., 1997). Carbo et al. (1999) noted that resveratrol induced apoptosis in a rat tumour model (Carbo et al., 1999), while Bishayee et al. (2010) showed reduced oxidative stress and inflammation in chemically induced hepatic tumours in rats exposed to resveratrol (Bishayee et al., 2010). Resveratrol has been shown to modulate inflammatory proteins to prevent 7,12-Dimethylben(a)anthracene (a tumour initiator) induced mammary carcinogenesis in rats (Banerjee et al., 2002). This evidence makes a compelling case for resveratrol's potential use in chemotherapy.

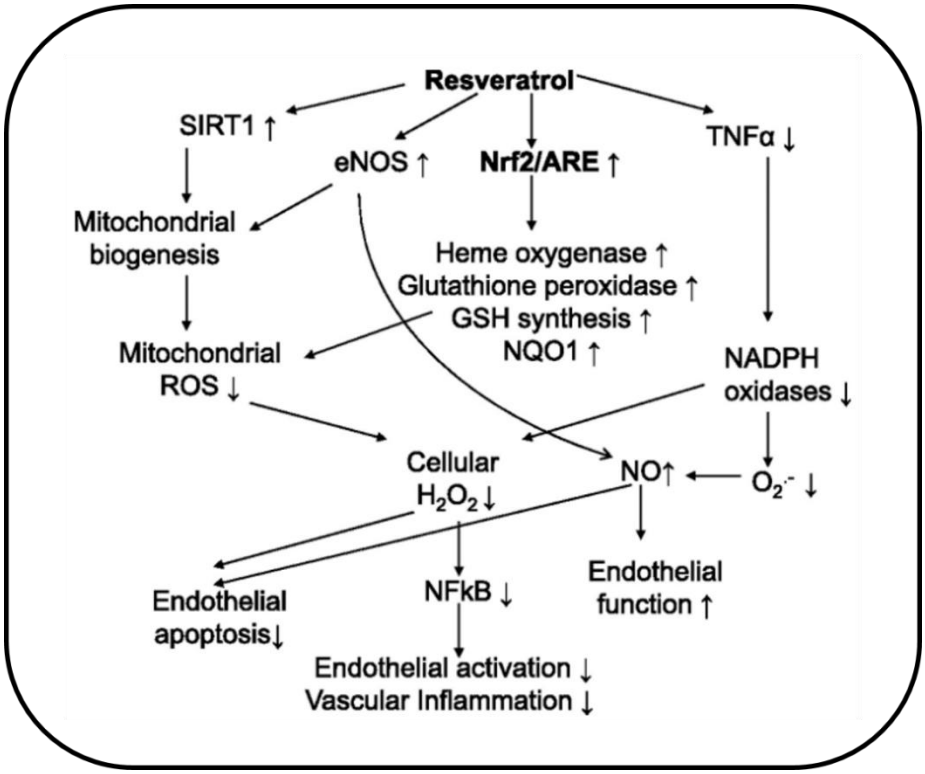

Figure 2.14: Potential resveratrol targets and the compound's involvement in inflammation, oxidative and mitochondrial stress, hypoxia, and cardiovascular protection (Haskó and Pacher, 2010).

It is important to note that inflammation, UPR activation, oxidative stress, and hypoxia all function in concert and influence cell survival. Pereira et al. (2014) demonstrated that ER stress was able to potentiate HIF1 activity and increase *VEGF* expression to higher levels than if hypoxia or ER stress had been induced alone (Pereira et al., 2014). The PERK-eIF2α branch has the ability to induce NFκB activation by inhibiting IκBα synthesis, thereby allowing transcription of NFκB targets in the nucleus (Deng et al., 2004). Lim et al. (2010) and Joo et al. (2015) proved that SIRT1 (activated by resveratrol) interacts with HIF1α and influences its activity during both normoxic and hypoxic conditions (Joo et al., 2015; Lim et al., 2010).

The effect of OTA on oxidative stress pathways is well documented, however, investigations into how hypoxia, ER stress and inflammation are affected together will be valuable in determining the carcinogenicity of OTA in humans. Integration of these pathways is fundamental in elucidating the mechanism of OTA toxicity and in establishing possible therapeutic interventions, such as resveratrol.

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CHAPTER 3

The Phytoalexin Resveratrol Ameliorates Ochratoxin A Toxicity in Human Embryonic Kidney (HEK293) Cells.

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Abstract

Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced by *Aspergillus* and *Penicillium* fungi. It contaminates human and animal food products, and chronic exposure is associated with renal fibrosis in humans (Balkan endemic nephropathy). Resveratrol, a phytoalexin, possesses anti-cancer and antioxidant properties. We investigated the mechanism of cellular oxidative stress induced by OTA, and the effect of resveratrol in human embryonic kidney (HEK293) cells over 24h and 48h. Cells were exposed to OTA [IC_{50} = 1.5 µM (24h) and 9.4µM (48h) determined using MTT assay] and 25µM resveratrol. Glutathione was quantified by luminometry and gene expression of Nrf2 and OGG1 was determined by qPCR. Protein expression of Nrf2, LonP1, SIRT3, and pSIRT1 was assessed by Western blot, DNA damage (comet assay), and intracellular reactive oxygen species (flow cytometry). At 24h, resveratrol increased mRNA expression of the DNA repair enzyme, OGG1 ($p<0.05$), whereas OTA and OTA+resveratrol significantly decreased OGG1 expression ($p<0.05$). OGG1 expression increased during 48h exposure to resveratrol and OTA+resveratrol ($p<0.05$). Comet tail lengths doubled in 48h OTA-treated cells, whereas at both time periods, OTA+resveratrol yielded shorter comet tails ($p<0.0001$). During 24h and 48h exposure, OTA, resveratrol, and OTA+resveratrol significantly decreased mRNA expression of Nrf2 ($p<0.05$). Luminometry analysis of GSH revealed an increase by OTA+resveratrol for 24h and 48h ($p<0.05$ and $p<0.001$, respectively). Western blot analysis showed decreased Nrf2 protein expression during 24h exposure, but increased Nrf2 expression during 48h. LonP1 protein expression increased during 24h exposure to OTA ($p<0.05$) and OTA+resveratrol ($p<0.0011$) and during 48h exposure to resveratrol ($p<0.0005$).

Keywords: Resveratrol, Ochratoxin A, Oxidative stress, LonP1, HEK293 cells

Introduction

Ochratoxin A (OTA) is a common contaminant of foods, such as grains, fruit, and animal feed [Marin-Kuan et al., 2008]. It is ubiquitously produced by fungal species of *Aspergillus* and *Penicillium*, specifically *Aspergillus ochraceus* (Schilter et al., 2005). Rural populations in developing countries rely heavily on grains as a staple source of sustenance; many of these communities lack proper storage facilities for harvested grains; this leads to increased fungal contamination and potentially increased production of OTA (Bankole et al., 2006). OTA was first discovered in South Africa (SA) in 1965 (van der Merwe et al., 1965) and has since been found in food sources around the world (Jonsyn-Ellis, 2012). Although first discovered in SA, there is still little documented information on OTA mycotoxicosis in SA. Understanding the mechanism of OTA toxicity is particularly important for African countries as grains, such as cassava and maize, contribute to a large part of the daily sustenance for a high percentage of the population (Jonsyn-Ellis, 2012).

Microorganisms in the gastrointestinal tract (GIT) hydrolyse OTA to the less toxic by-product, Ochratoxin α (OTα) (Hohler et al., 1999), which is then excreted via the liver and kidney (Cheng et al., 2013). Exposure to high levels of OTA has been linked to renal dysfunction and tumourigenesis in humans (Özçelik et al., 2004). OTA is nephrotoxic and results in destruction of the renal tubular epithelium, causing progressive renal failure (Pfohl-Leszkowicz and Manderville, 2007). This disease is particularly prominent in the Balkan regions, such as Bulgaria and Romania, and is known as Balkan endemic nephropathy (BEN) (Pfohl-Leszkowicz and Manderville, 2007).

OTA exerts its toxic effects via a number of mechanisms, the most prominent method is by alteration of the antioxidant potential of the cell (Meyer et al., 2013), resulting in oxidative stress induction, and increased reactive oxygen species (ROS) (Schaaf et al., 2002). During oxidative stress, nuclear factor-erythroid 2-related factor 2 (Nrf2) dissociates from its inhibitor, Kelch-like ECH-associated protein 1 (Keap-1), translocates into the nucleus, and binds to the antioxidant response element (ARE), resulting in the transcription of proteins associated with the antioxidant defence system (Nguyen et al., 2009). Therefore, we aimed to investigate the interactions of both OTA and resveratrol with the Nrf2 antioxidant defence system in cultured human kidney cells.

Resveratrol (3, 5, 4'-trihydroxy-trans-stilbene) is a polyphenol found in the skin of injured grapes and some fruit, and is, therefore, commonly present in wine and grape juice (Athar et al., 2007). Resveratrol, synthesized by plants as a defensive mechanism, is proposed to have antimicrobial, antioxidant, anti-carcinogenic, and anti-inflammatory properties (Athar et al., 2007). This compound is widely studied and holds great potential in disease treatment. Resveratrol possesses free radical scavenging potential, contributing to its antioxidant capacity [Borra et al., 2005]. The phytoalexin is also known to activate sirtuin 1 (SIRT1), a NAD^+-dependent deacetylase known for its ability to modulate transcription of

proteins that augment the oxidative stress response, as well as proteins that promote cell survival in times of stress (Borra et al., 2005; Timmers et al., 2012). These attributes could contribute to the compound's chemo-preventive ability, as well as the ability to diminish cellular damage due to oxidative stress (Baur and Sinclair, 2006). Both resveratrol and OTA have been found in grapes; therefore, it is important to have an understanding of the dynamic between these two compounds.

OTA exposure is widespread throughout Europe, through the ingestion of grape products, such as wine and juice. This is especially prominent in the grape producing regions of Italy (Visconti et al., 2008). The mechanisms of OTA toxicity and the cellular antioxidant response are relatively unknown. This study investigated the cytotoxic effects of OTA on HEK293 cells after 24h and 48h exposure. Further, the therapeutic effects of resveratrol in both untreated and OTA-treated HEK293 cells were investigated. We believe these results will provide valuable insight into OTA toxicity in human kidney cells, and whether resveratrol can be used as a potential treatment to combat this toxicity.

Materials

Tissue culture consumables were purchased from Whitehead Scientific (Johannesburg, South Africa). The HEK293 cells were obtained from Highveld Biological (Johannesburg, South Africa). Ochratoxin A and resveratrol were purchased from Sigma–Aldrich (St Louis, MO). Methylthiazoltetrazolium (MTT) salt, phosphate buffered saline (PBS) tablets, and agarose were purchased from Capital Laboratory Supplies (Johannesburg, South Africa). The bicinchoninic acid (BCA) assay kit and tris-chloride were purchased from Sigma–Aldrich. Kits and reagents used for luminometry (GSH-Glo™ Glutathione Assay kit) were purchased from Promega (Madison, USA). Reagents for qPCR were purchased from Bio-Rad (Hercules, CA) and the primer sequences were purchased from Inqaba Biotechnologies (Pretoria, South Africa). Western blotting reagents (Laemmli sample buffer and chemiluminescent reagents) were obtained from Bio-Rad. Primary antibodies (rabbit anti-Nrf2 (8882), rabbit anti-SIRT3 (C73E7), and rabbit antipSIRT1 (2314L) were purchased from Cell Signaling Technology (Danvers, MO). Rabbit anti-LonP1 (HPA002192-100UL) was purchased from Sigma-Aldrich (St. Louis, MO) and horse-radish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG) were purchased from Cell Signaling Technology. Proteins were normalized to anti-β-actin (A3854) from Sigma–Aldrich. All other solvents and salts were purchased from Merck Chemicals (Johannesburg, South Africa). Protein isolation was conducted using Cytobuster™ (Novagen), supplemented with protease inhibitors (Roche 05892791001) and phosphatase inhibitors (04906837001).

Methods

Cell Culture

HEK293 cells were cultured in monolayer (10^6 cells per 25 cm^3 culture flask). The cells were maintained in complete culture medium [CCM: Dulbecco's minimum essential media (DMEM), 10% fetal calf serum, 1% Penstrepfungizone, and 1% L-glutamine] and incubated overnight at 37°C in a humidified incubator supplied with 5% CO_2. Cells were washed with 0.1M PBS. Once flasks were confluent, cells were treated, removed by trypsinisation and agitation, and counted using the trypan blue exclusion assay.

Ochratoxin A Treatments

A stock solution of 200µM OTA was prepared in 60% dimethyl sulphoxide (DMSO). Cells were treated with a range (0.25–50µM) of concentrations to obtain a half maximal inhibitory concentration (IC_{50}) for 24h and 48h incubation times.

Methylthiazol Tetrazolium (MTT) Assay

Cells were seeded into a 96-well microtiter plate (15,000 cells/well) and allowed to attach overnight. Attached cells were treated with a range of OTA concentrations (0.25–50µM) for 24h and 48h. Treatment media was removed and replaced with CCM (100µl) and 20µl MTT salt solution (5mg/ml in PBS). Cells were incubated with the MTT salt solution for 4h, thereafter the MTT solution was discarded and DMSO (100µl) was added; the cells were incubated with DMSO for 1h. Absorbance was read using a Bio-Tek MQx200 spectrophotometer at 595 nm (reference wavelength 655 nm). An inhibitory concentration at which 50% of cell growth was inhibited (IC_{50}) was calculated using GraphPad Prism statistical software (version 5).

Resveratrol Treatments

Stock solutions of 10mM resveratrol were prepared in 100% DMSO. Preliminary assays were carried out to determine the optimal resveratrol concentrations for all subsequent treatments. Cells were treated with 25µM, 50µM, and 100µM resveratrol during pre-, post- and co-OTA treatments for 24h, respectively. Flow cytometry was used to observe which resveratrol concentration greatly decreased the percentage of intracellular ROS, whereas luminometry was used to detect the levels of intracellular glutathione (GSH), thus indicating which concentration provided a more robust defence against oxidative damage. Based on these results, 25µM resveratrol was used for all subsequent assays.

Cell Preparation for Assays

Cells were incubated with the treatments for 24h and 48h. Treatments included a control (CCM containing the solvent vehicle, DMSO), the IC_{50} values of OTA, a resveratrol control (CCM containing 25mM resveratrol), and a co-treatment (IC_{50} values of OTA and 25µM resveratrol in CCM). Cells were

removed by trypsinisation and agitation, the trypan blue method of cell counting was employed to determine cell viability and number as required per assay performed.
The control was used for statistical comparison to all other treatments.

Glutathione Assay

The GSH assay [per GSH-Glo™ glutathione assay protocol (Promega)] was used to determine the concentration of GSH in HEK293 cells. Cells (10^4cells/well) were seeded in a luminometry plate in triplicate. Standards (0-50µM) were added in triplicate to generate a standard curve of known GSH concentrations. The GSH- Glo™ reagent (50µl) was added to each well; the plate was agitated for 30s, and incubated in the dark (RT, 30min). The luciferin detection reagent (100µl) was added to each sample and incubated as previously described (RT, 15min). The plates were read on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA). The GSH concentrations were determined by extrapolation from the standard curve.

Single Cell Gel Electrophoresis (SCGE) Assay

Cells were incubated with the treatments for 24h and 48h, thereafter cells were removed by trypsinisation and agitation, then counted; 15,000 cells were removed and resuspended in 25µl PBS. Low melting point agarose (LMPA) was used to prepare the gels. The first gel layer (700µl 2% LMPA) was formed on a frosted microscope slide, covered with a coverslip, and incubated at 4°C (10min). Thereafter, the coverslip was removed, a second gel layer (including 1.5µl GR red dye, 25µl cell suspension (10^4 cells) in 175µl 1% LMPA) was added, coverslips were placed over slides, and incubated as above. Coverslips were removed, and a third layer was added (1% LMPA, 200µl) onto the second. Once the third layer solidified, coverslips were removed, slides were submerged in an ice-cold cell lysing solution [10mM Tris (pH 10), 1% Triton X-100, 2.5M NaCl, 100mMEDTA, and 10% DMSO), and incubated in the dark (4°C, 1h). The lysing solution was removed, slides were placed in an electrophoresis tank filled with electrophoresis buffer (300mM NaOH and 1mM Na_2EDTA), and allowed to equilibrate for 20min. The electrophoresis tank was sealed, and a constant voltage was applied (25 V, 35min). After electrophoresis, slides were washed thrice (5min each) with neutralization buffer (0.4M Tris, pH 7.4). Slides were viewed using an Olympus IX5I inverted fluorescent microscope (510–560nm excitation, 590nm emission filters) using analySIS Image Processing Software (Novell). Approximately 50 comets per treatment (three replicates) were counted and analysed by measuring tail length (µm).

RNA Extraction

RNA was isolated following an in-house protocol using Trizol. Trizol reagent (500µl) was added to each flask and incubated (4°C, 10min). Cells were removed from flasks, transferred to 1.5ml eppendorfs, and stored in Trizol at -80 °C overnight. Chloroform (100µl) was added to thawed samples

and incubated (RT, 3min). Thereafter, cell suspensions were centrifuged (12,000g, 4°C, 15min), the aqueous phase removed, 250µl isopropanol was added, and samples were left overnight at -80 °C. Samples were thawed and centrifuged (12,000g, 4°C, 20min). The supernatant was discarded, the pellet was retained and washed with 75% cold ethanol (500µl), and centrifuged (7,400g, 4°C, 15min). Ethanol was removed, and samples were allowed to air dry. The pellet was resuspended in nuclease-free water (15µl) and incubated (RT, 3min). The RNA was quantified using the Nanodrop2000 spectrophotometer (Thermo-Scientific) and the A260/A280 ratio was used to assess the RNA integrity. The concentration of RNA was standardized to 2,500 ng/µl and used to prepare cDNA using the iScript cDNA synthesis Kit (Bio-Rad) as per manufacturer's instructions (4µL 5X iScript reaction mix, 1µL iScript reverse transcriptase, 11µL nuclease-free water, and 4µL of each RNA sample).

Quantitative Polymerase Chain Reaction (qPCR)

Gene expression was analysed using the iScript SYBR Green PCR kit (Bio-Rad), according to the manufacturer's instructions. The final reaction volume totalled 25µl (12.5µl SYBR Green, 1µl forward primer, 1µl reverse primer, 9µl nuclease-free water, and 1.5µl cDNA sample). All primers were obtained from Inqaba Biotec. The mRNA expressions of oxoguanine glycosylase 1 (OGG1) (forward 5'-GCATCGTACTCTAGCCTCCAC-3'; reverse 5'-GGACTTTGCTCCCTCCAC-3') and Nrf2 (forward 5'-AGTGGATCTGCCAACTACTC-3'; reverse 5'-CATCTACAAACGGGAATGTCTG-3') were investigated. This assay was carried out using three replicates per treatment and b-actin (forward 5'-TGACGGGTCACCCACACTGTGCCCAT-3'; reverse 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3') as a house-keeping gene. The initial denaturation occurred at 95 °C (4min). This was followed by 37 cycles of denaturation (95 °C; 15s), annealing (40s; OGG1-60 °C; Nrf2-58 °C), and extension (72°C; 30s). The method described by Livak and Schmittgen was employed to determine the changes in relative mRNA expression, where $2^{-\Delta\Delta Ct}$ represents the fold change observed in mRNA expression (Livak and Schmittgen, 2001).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) And Western Blotting

Crude protein was isolated from cells using Cytobuster™ (Novagen) supplemented with protease inhibitors (Roche 05892791001) and phosphatase inhibitors (04906837001). Cytobuster reagent (250µl) was added to flasks following treatments; the cells were scraped and transferred to a 1.5 ml tube and incubated on ice (10min). The cell solution was centrifuged (10,000g, 4°C, 5min). The supernatant was used for protein quantification using the bicinchoninic acid (BCA) assay. Bovine serum albumin (BSA) standards (0–1mg/ml) were prepared. Samples and standards (25µl) were pipetted into a 96-well microtiter plate in duplicate. The BCA reagent was prepared (198µL BCA: 4µL $CuSO_4$ per reaction) and 200µL of this working solution was added to each well. After incubation (37°C, 30min), the absorbance was read on a Bio-Tek MQx200 spectrophotometer at 562nm. A standard curve was constructed, and proteins were standardized to 1mg/ml. Laemmli buffer [dH_2O, 0.5M Tris-HCl (pH

6.8), glycerol, 10% SDS, β-mercaptoethanol, 1% bromophenol blue] was added to each sample (1:1, total volume 200 µl), and heated to 100°C (5min).

Samples were separated on SDS polyacrylamide gel (7.5% resolving gel and 4% stacking gel) for 1h at 150V, and transferred onto nitrocellulose membranes using the Transblot® Turbo™ Transfer system (Bio-Rad). Membranes were blocked with 3% BSA in Tris-buffered saline [TTBS, 25mM Tris (pH 7.6) 150mM NaCl, 0.05% Tween 20] for 30 min, and incubated with a primary antibody (1:1000, 3% BSA In TTBS at 4°C overnight). Antibodies used were anti-Nrf2 (8882), anti-phospo SIRT1 (2314L), anti-SIRT3 (C73E7), and anti-LonP1 (HPA002192-100UL). Protein concentrations were standardized to anti-β-actin (Sigma–Aldrich A3854). After incubation, the primary antibody was removed, membranes were washed thrice with TTBS (10min), and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) in 3% BSA (1:10 000) for 1h. Following incubation, membranes were washed with TTBS. Clarity Western ECL Substrate (Bio-Rad) (400µl) was added to the membranes and images were captured using a gel documentation system (UviTech Alliance 2.7). The membranes were then quenched with hydrogen peroxide, incubated in a blocking solution (3% BSA, 1h, RT), rinsed twice in TTBS, and probed with HRP-conjugated anti-β actin (house-keeping protein) (Sigma). Densitometry analysis was performed using the UviTech Analysis software and protein expression was read as relative band intensity (Arbillaga et al., 2007). Protein expression was reported as RBI of the protein of interest divided by the RBI of the loading control.

Flow Cytometry – Intracellular Reactive Oxygen Species (ROS)

Treated cells were removed and counted, 500,000 cells were required per treatment. Cells were incubated in phenol red-free media, supplemented with 10% FCS and 10µM dichlorodihydrofluorescein diacetate (DCFDA), in the dark (45min, 37°C). Thereafter, cells were rinsed thrice with 0.1M PBS and centrifuged (400*g*, RT, 5min), then suspended in 150µl 0.1M PBS and analysed using a BD Accuri™ flow cytometer. Live cells were gated and analysed using CFlow Plus Software (BD Biosciences).

Statistical Analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) test and the Students' t-test, using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). All data are expressed as mean ± standard error. All assays were run in triplicate and differences were considered statistically significant at values of $p < 0.05$.

Results

MTT Assay

The MTT assay was used to determine the IC_{50} value of OTA during 24h incubation (1.5µM) and 48h incubation (9.4µM) in HEK293 cells (Fig. 3.1). The IC_{50} values were used for all subsequent assays.

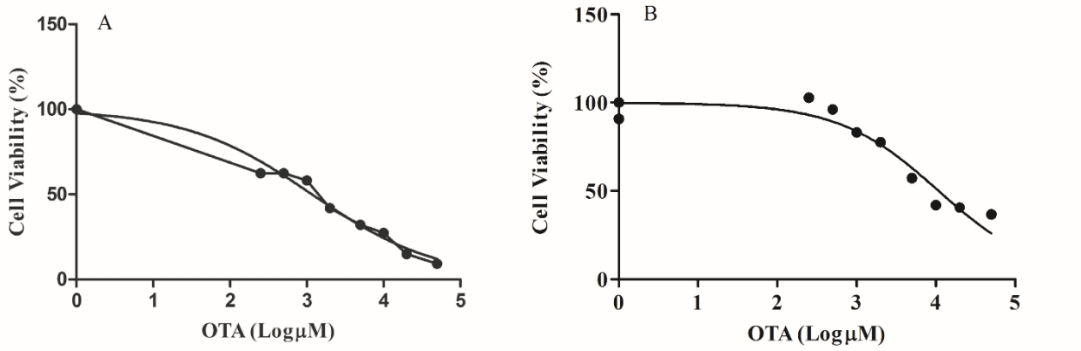

Figure 3.1: Percentage viability of cells exposed to OTA over (A) 24h and (B) 48h. An IC_{50} of 1.5µM (24h) and 9.4 (48h) was calculated from the dose-response curve.

Resveratrol Decreases Intracellular Ros Production During 24h Exposure.

Flow cytometry was used to measure intracellular ROS production in HEK293 cells. Resveratrol significantly reduced intracellular ROS in the kidney cells from 47.7% to 20.2% (Fig. 3.2; $p < 0.0001$). Both the OTA (25.3%) and OTA+resveratrol (28.9%) treatments significantly reduced ROS when compared to control cells (47.7%) (Fig. 3.2; $p = 0.0048$). The opposite was observed during the 48h exposure, where all treatments increased the intracellular ROS when compared to control cells. A significant increase was observed when cells were exposed to OTA+resveratrol over 48h (Fig. 3.2; $p = 0.0002$).

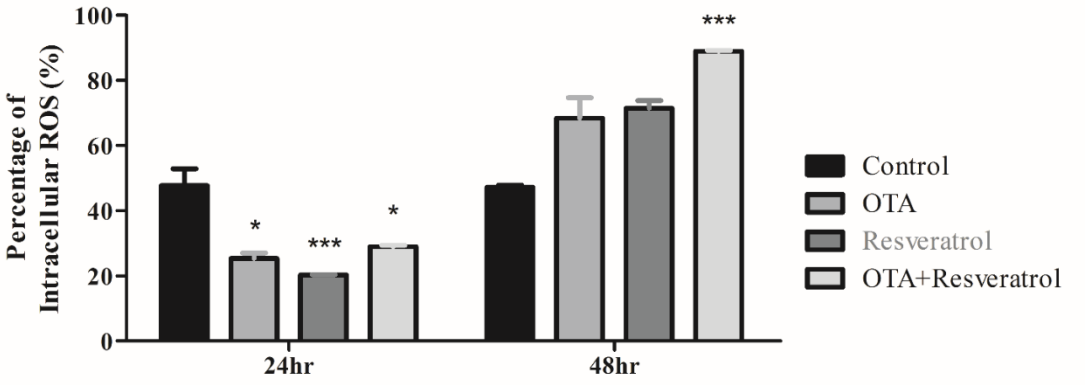

Figure 3.2: Assessment of the percentage of intracellular ROS in cells exposed to OTA and resveratrol over 24h and 48h. Resveratrol significantly decreased the percentage of intracellular ROS (***$p < 0.0001$), whereas OTA and OTA+resveratrol treatments decreased the values of ROS when compared to control cells during 24h exposure (*$p = 0.0048$). Upon 48h exposure, OTA+resveratrol significantly

increased the ROS values (***p = 0.0002), whereas the OTA and resveratrol treatments also increased intracellular ROS.

Assessment of DNA Damage and Repair

OTA-treated cells (24h) had significantly longer comet tails (44.82 ± 1.516µm; $p < 0.0001$) as compared to the control cells (Fig. 3.3B). In contrast, resveratrol significantly decreased comet tail lengths compared to all other treatments (11.69 ± 0.695µm; $p < 0.0001$). Interestingly, cells co-treated with OTA+resveratrol also showed significantly decreased comet tail lengths as compared to cells only exposed to OTA (Fig. 3.3B; 29.03 ± 1.110µm vs. 44.82 ± 1.516µm; $p < 0.0001$). These observations were similar for the 48h exposure period; however, the comet tails almost doubled in length. OTA-treated cells (48h) had significantly longer comet tails (86.67µm) as compared to the 24h treatment (44.82µm) (Fig. 3.3D). In addition, the comet tails were brighter and longer in OTA-treated cells, indicating that more DNA activity and increased DNA strand breaks occurred with increased exposure. The co-treatment (OTA+resveratrol) significantly decreased comet tail lengths compared to OTA-treated cells (Fig. 3.3D; 70.72 ± 1.030µm vs. 86.67 ± 1.694µm; $p < 0.0001$).

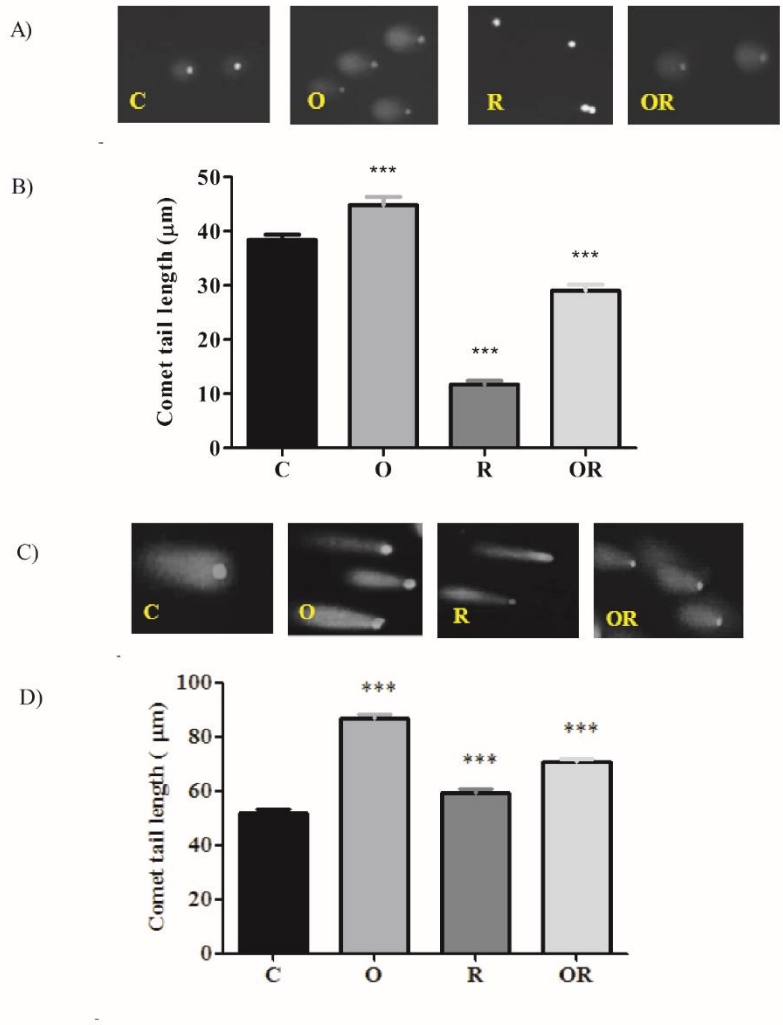

Figure 3.3: Assessment of DNA damage showing images of (A) comet tails during 24h exposure, (B) the measurement of comet tail lengths during 24h exposure, (C) comet tails during 48h exposure, and (D) the measurement of comet tail lengths during 48h exposure. OTA increased comet tail lengths during both exposure time periods (***$p < 0.0001$). Comet tail lengths doubled during the 48h exposure. The OTA+Resveratrol co-treatment significantly decreased lengths compared to cells only treated with OTA (***$p < 0.0001$).

Measurement of GSH Concentrations

Intracellular concentration of GSH was determined using luminometry. GSH concentrations in HEK293 cells were significantly increased by OTA+resveratrol treatment as compared to OTA-treated cells ($p < 0.05$); however, this concentration doubled after 48h exposure ($p < 0.001$) (Fig. 3.4). The co-treatment of cells with OTA+resveratrol yields increased concentrations of GSH than in cells exposed to OTA alone. Generally, the GSH concentrations are far higher over 48h exposure times than 24h exposures (Fig. 3.4). The concentration of GSH was seen to decrease in OTA-treated cells compared to controls, thus indicating that longer exposure to OTA could deplete the antioxidant defence of the cells, allowing ROS to overrun the cell.

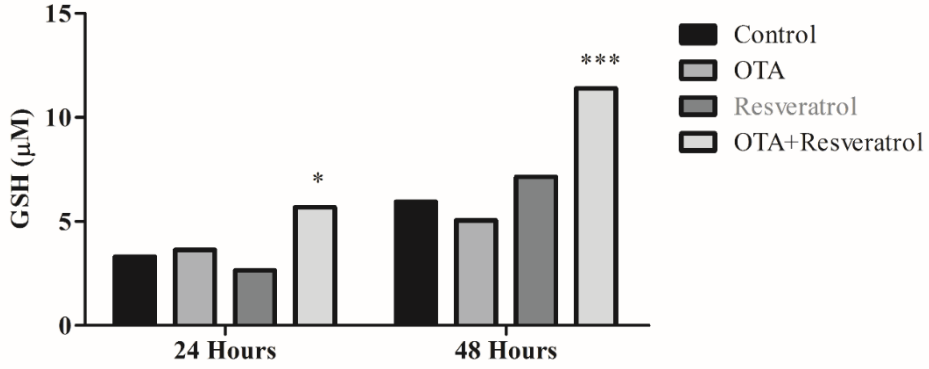

Figure 3.4: Concentrations of GSH in HEK293 cells after exposure to OTA and resveratrol. The OTA+resveratrol co-treatment increased the concentrations of GSH during the 24h exposure compared to GSH present in OTA-treated cells (*$p < 0.05$). The co-treatment also increased GSH during the 48h exposure (***$p < 0.001$).

qPCR Analysis of Antioxidant Response

The mRNA expression of Nrf2 and OGG1 was assessed by qPCR. The expression of Nrf2, an antioxidant defence regulator, was significantly decreased by OTA, resveratrol, and OTA+resveratrol after 24h exposure (Fig. 3.5A; $p < 0.05$); Nrf2 expression decreased further after 48h (Fig. 3.5B). Resveratrol increased the expression of OGG1 ($p < 0.05$), whereas the co-treatment significantly decreased its expression after 24h (Fig. 3.5C; $p < 0.05$). The co-treatment, however, significantly increased OGG1 expression when measured after 48h (Fig. 3.5D; $p < 0.05$).

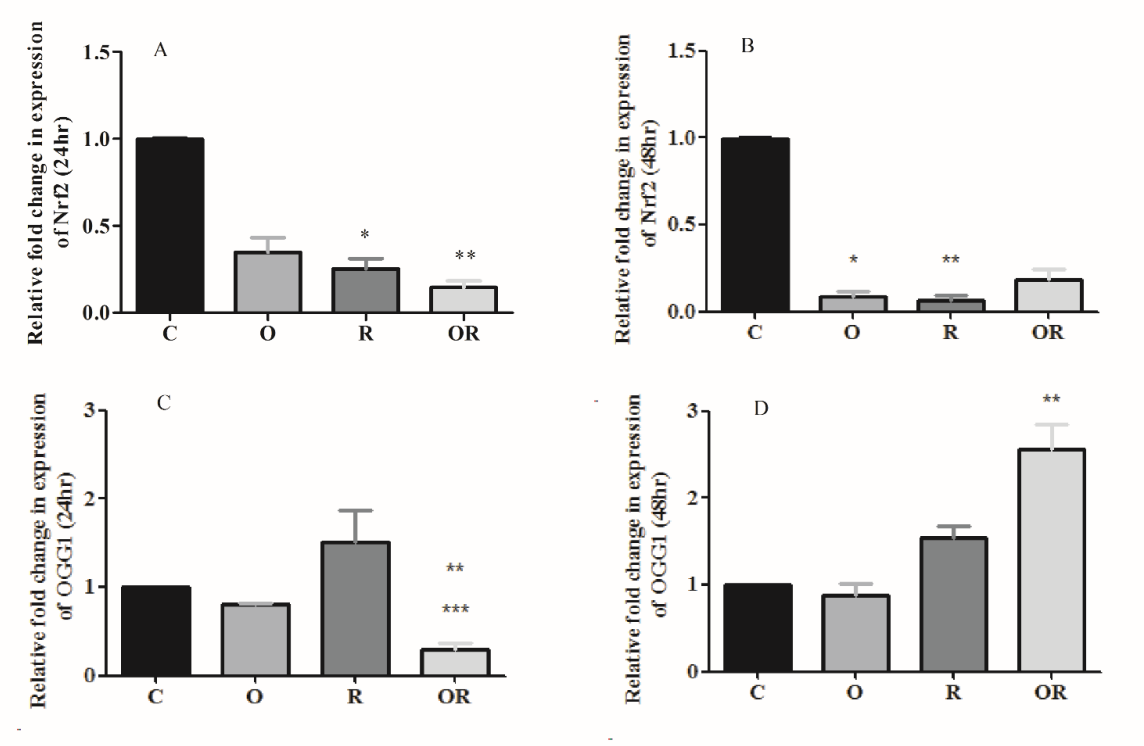

Figure 3.5: Analysis of the mRNA expression of genes associated with cellular protection. Nrf2 expression was seen to decrease upon exposure to OTA, resveratrol (*$p < 0.05$), and OTA+Resveratrol (**$p = 0.001$) for 24h. (B) The expression of Nrf2 was seen to decrease further when exposed to OTA (*$p < 0.05$) and resveratrol (**$p = 0.0021$) for 48h. The DNA glycosylase enzyme, OGG1, was observed to increase during 24h resveratrol exposure and significantly decrease during exposure to OTA+resveratrol (**$p < 0.05$) for 24h. (C) OGG1 expression was significantly decreased during OTA+resveratrol exposure compared to resveratrol-treated cells ($p = 0.0002$). (D) Recovery of OGG1 expression was observed during 48h exposure to OTA+resveratrol (**$p < 0.05$).

Western Blotting

The protein expression of Nrf2, LonP1, SIRT3, and pSIRT1 was determined using Western blot. Nrf2 protein expression was decreased by all treatments after 24h exposure, significantly by OTA+resveratrol ($p < 0.05$), but increased by OTA ($p < 0.05$) and resveratrol ($p < 0.0008$) after 48h. An inverse relationship was found to exist between SIRT3 and LonP1 after 24h, SIRT3 decreased and LonP1 increased. Resveratrol increased SIRT3 ($p < 0.05$) and LonP1 ($p = 0.0005$) expression (48h), and pSIRT1 at both time periods ($p = 0.0002$ and $p = 0.0054$, respectively) (Fig. 3.6).

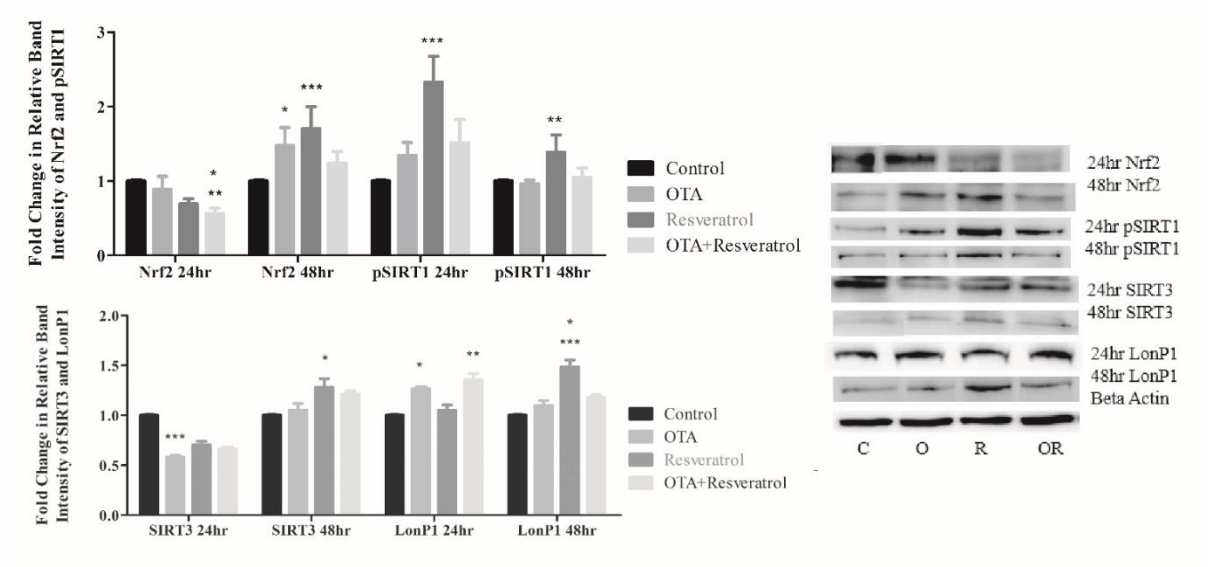

Figure 3.6: Western blot images and relative fold change in protein expression of Nrf2, pSIRT1, SIRT3, and LonP1 over 24h and 48h, in response to OTA and resveratrol exposure. Resveratrol decreases Nrf2 expression during 24h exposure, but increases Nrf2 during 48h exposure (***$p < 0.0008$). OTA significantly increased Nrf2 expression during 48h exposure (*$p < 0.05$). The OTA+resveratrol co-treatment significantly decreased Nrf2 expression during 24h exposure (*$p < 0.05$). Resveratrol significantly increased the expression of pSIRT1 during 24h exposure (***$p = 0.0002$) and 48h exposure (**$p = 0.0054$). SIRT3 expression decreased in all treatments during 24h exposure with a significant decrease during OTA exposure (***$p = 0.0003$). The opposite was observed during 48h exposure, all treatments increased SIRT3 expression with significance in the resveratrol treatment (*$p < 0.05$). LonP1 expression exhibits the inverse of SIRT3 expression, with an increase in LonP1 expression during 24h exposure to OTA (*$p < 0.05$) and OTA+Resveratrol (**$p = 0.0011$). LonP1 expression increased significantly upon 48h exposure to resveratrol (***$p = 0.0005$).

Discussion

OTA is a mycotoxin that frequently contaminates a wide range of foods. Once ingested, OTA exerts its toxic effects via several pathways. One such pathway in the kidney is increased oxidative stress, resulting in DNA damage and progressive renal failure in individuals who chronically ingest foods contaminated with high concentrations of OTA (Pfohl-Leszkowicz and Manderville, 2007). OTA poses a health risk to populations in developing countries, as they depend largely on agricultural crops often heavily contaminated with fungi, such as Aspergillus and Penicillium (Castegnaro et al., 2006). OTA has been identified as an etiological agent in BEN (Castegnaro et al., 2006). The exact mechanism of OTA toxicity is still relatively unknown, although many mechanisms have been proposed. The general trend of reduced antioxidant defence capacity has been documented in different cell lines, such as the normal rat kidney (NRK) epithelial cells (Cavin et al., 2007; Schilter et al., 2005). In this study, we provide insight into the biochemical changes induced by OTA in human embryonic kidney (HEK293)

cells. Resveratrol, a phytoalexin, possesses antioxidant characteristics, chemopreventive potential, and has been proposed as a treatment for illnesses, such as diabetes, cancer, and cardiovascular disease (Rossi et al., 2012). Our study determined whether resveratrol could reduce/prevent OTA-induced cytotoxicity in kidney cells. Our aim was to determine whether resveratrol could prevent cytotoxicity induced by OTA in kidney cells.

Under normal conditions, ROS regulates intracellular signalling; however, under conditions of excessive ROS production, intracellular signalling is compromised and will result in cellular damage (Wang et al., 2013). Excessive oxidative stress induces cytotoxicity and alters cellular functioning (Weydert and Cullen, 2010). Lipids, proteins, and DNA are susceptible to ROS attack and subsequent damage (Marin-Kuan et al., 2011). OTA decreased intracellular ROS (as determined by FACS), but still induced single strand DNA breaks (comet assay). Exposure to OTA (24h, 1.5µM) resulted in significant DNA strand breaks in HEK293 cells, suggesting that OTA is genotoxic. In comparison to control cells, OTA significantly increased the lengths of comet tails by 16.8%, whereas resveratrol significantly decreased comet tail lengths by 69.5% (Fig. 3.3). Resveratrol also significantly reduced OTA genotoxicity as the co-treatment decreased DNA tail lengths (35.2%). In addition, the intracellular ROS was the lowest in resveratrol-treated cells (20.2%).

When DNA damage occurs, the cell activates DNA repair enzymes, such as OGG1 – a DNA glycosylase enzyme, which removes 8-oxoguanine base lesions, that could result in mutations and carcinogenesis if left to accumulate (Cheng et al., 2013). OTA decreased expression of OGG1 and increased DNA tail lengths. This suggests that OGGI enzyme activity is compromised and as a consequence, base lesions persist. However, resveratrol increased OGG1 expression in kidney cells, resulting in significantly shorter DNA tail lengths, whereas the co-treatment, although inducing low expression of OGG1, significantly decreased DNA tail lengths. In the co-treatment, resveratrol afforded a protective function, due to its antioxidant properties, by decreasing intracellular ROS and increasing OGG1 expression. This may explain its cyto-protective and potential anti-cancer property. The 48h ROS results differ, in that all treatments show increased intracellular ROS (Fig. 3.2) and a concomitant significant increase in OGG1 expression and shorter comet tails. This suggests that resveratrol aided the cells to survive in response to OTA toxicity.

Palma et al. (2007) showed that OTA induced DNA damage by increasing the production of free radicals. Our data are in agreement with this study as demonstrated by the comet and flow cytometry ROS assays (Palma et al., 2007). GSH, a low molecular weight thiol found abundantly in cells, is a potent endogenous antioxidant (Wu et al., 2004). GSH depletion indicates an increase in ROS and oxidative stress. OTA severely depleted GSH concentrations (5.06µM) in kidney cells, whereas resveratrol increased GSH (2.65µM at 24h; 7.14µM at 48h). Further in the co-treated cells, resveratrol

significantly increased GSH from 5.68µM (24h) to 11.39µM (48h). These results indicate that resveratrol increases the antioxidant capacity of the cell over longer periods of time by increasing GSH synthesis. In addition, resveratrol clearly shows its potent antioxidant capacity by dampening the effects of OTA induced toxicity in kidney cells. At both 24h and 48h treatment periods, Nrf2 mRNA expression was significantly decreased. Nrf2 expression was lowest at 24h but higher at 48h in the co-treated cells. Although resveratrol induced low expression of Nrf2 compared to other treatments, the concentrations of GSH were significantly increased and DNA tail lengths were shorter.

Taken together, our data show that resveratrol is a potent antioxidant that can function independently in the cell without influence from Nrf2. OTA induced a non-significant increase in Nrf2 expression with increased DNA damage, high ROS levels, and lower GSH concentrations. Western blot protein analysis revealed changes in Nrf2 expression over the two treatment periods; at 24h, all treatments decreased Nrf2 expression, whereas at 48h, Nrf2 expression was increased. The decreased Nrf2 expression strongly suggests that resveratrol itself mediates the antioxidant defence response, in keeping with its antioxidant properties (Momchilova et al., 2014). The protein and mRNA expression of Nrf2 at 24h is in agreement; however, these results are different at 48h. The mRNA expression of Nrf2 is significantly decreased, but the protein expression is significantly increased. This result could be due to alterations in post-translational modifications or the increased expression of LonP1.

LonP1, a protease, is known for its regulatory function and could assist in degrading potentially compromised proteins (Bota et al., 2005). SIRT3 has been identified as a regulator of LonP1 expression; increased SIRT3 decreases LonP1 (Gibellini et al., 2014). This inverse relationship is clearly evident at 24h decreased SIRT3 and increased LonP1 expression. A significant increase in LonP1 and decreased SIRT3 expression was observed in OTA and cotreated kidney cells. The increased expression of LonP1 is triggered by increased oxidative stress to combat accumulation of defective proteins. This mitochondrial protease could have acted in a compensatory manner by degrading proteins damaged by oxidative stress (Zhang et al., 2014).

SIRT1, a NAD^+-dependent deacetylase, is activated by resveratrol. In our study, resveratrol increased SIRT1 expression in cells at both 24h and 48h. Activated SIRT1 by phosphorylation influences many processes, such as DNA repair and cellular metabolism, thereby impacting the cellular stress response. Phosphorylated SIRT1 increases nuclear localization of the protein, as well as increased enzymatic activity (Sasaki et al., 2008). The stress protection pathway is mediated by SIRT1, increased phosphorylation would influence this function, and induce possible deacetylation of stress proteins, such as p53 and PGC-1α, causing cellular protection and survival (Nasrin et al., 2009).

These data provide insight into the behaviour of OTA, as well as the therapeutic potential of resveratrol. We are proposing that short-term effects of OTA contribute to its toxicity. The cells experience an increase in the antioxidant response due to the recognition of a cytotoxic environment; however, it is not possible to sustain an increased protective response while DNA damage occurs. This is evidenced by the 48h data, as time progresses, so too does the extent of damage inflicted on the cell by the toxin. Our data suggest that OTA exhausts the cell's antioxidant reservoir during acute exposure, and thus the cell is left vulnerable as the exposure period continues. Taken together, these data suggest that resveratrol could be a potential treatment for OTA toxicity, as well as a possible therapy for other oxidative stress-inducing toxins. Regarding the information on DNA damage prevention, this research also attests to the chemopreventive potential of resveratrol.

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CHAPTER 4

Acute Ochratoxin A exposure induces inflammation and apoptosis in human embryonic kidney (HEK293) cells.

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Abstract

Ochratoxin A (OTA), a common contaminant of grain and fruit and known carcinogen, has been linked to impaired antioxidant response and cellular repair. The effect of OTA on inflammation in cells has not been explored. This study investigated OTA's influence on inflammatory mediators using a range of OTA concentrations (0.5µM (sub-IC_{50}), 1.2µm (IC_{50}) and 2µm (supra-IC_{50})) on human embryonic kidney (HEK293) cells over 24hr. The markers of inflammation in HEK293 cells were evaluated using the following techniques: western blotting (phosphorylated (p-)NFκB (Ser536), p-IKK (Ser176/180) and p-p53 (Ser392), total NFκB, IKK, IκBα and p53), luminometry (caspases 1, 3/7, 8, 9, ATP) and ELISA to determine IL-1β levels. The results indicate increased activation of the inflammatory pathway in the sub-IC_{50} concentration, evidenced by significant increases in p-NFκB (p = 0.0006). The IC_{50} concentration indicates decreased inflammatory induction supported by decreased levels of IL-1β and caspase 1 (p = 0.0186 and p = 0.0068 respectively) with decreased IKK and increased IκBα (p = 0.0046 and p = 0.0006 respectively). Furthermore, a decrease in inflammatory pathway activation was seen in O3 (increased IκBα, $p < 0.05$) coupled with increased apoptosis via elevated caspase 3/7 (p = 0.0002), 8 (p = 0.0011) and 9 activity (p = 0.0002); as well as decreased ATP levels. This data suggests a new mechanism of OTA toxicity and its involvement in inflammation, kidney disease and fibrosis.

Introduction

Toxic insult on the kidney promotes inflammatory processes as a means of cytoprotection, however, persistent inflammation in the kidney causes tubulointerstitial damage, diminished functionality and eventual fibrosis (Rodríguez-Iturbe et al., 2005). Ochratoxin A (OTA), a mycotoxin, is a known renal toxicant (Marin-Kuan et al., 2011), however the role of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), a key transcription factor in the inflammatory response, has been overlooked as a mechanism of OTA toxicity in the kidney.

OTA is present in *Penicillium* and *Aspergillus* fungi contaminated foods, such as grains and cereals, fruit, coffee, and wine (Boesch-Saadatmandi et al., 2008; Pfohl-Leszkowicz and Manderville, 2007). Known mechanisms of OTA toxicity include increased production of reactive oxygen species (ROS) leading to oxidative stress (Liu et al., 2012), mitochondrial dysfunction, and genotoxicity promoting cellular mutations (Marin-Kuan et al., 2011). This mycotoxin has been linked to several disease conditions, such as renal dysfunction, immunotoxicity and tumorigenesis (Darif et al., 2016). It has been identified as a causal factor in Balkan Endemic Nephropathy (BEN), a disease observed in the Balkan regions of Europe, which presents as damage to renal tubular epithelium and progressive renal failure with the potential development of urinary tract tumours (UTT) (Castegnaro et al., 2006). The kidney is highly susceptible to OTA-induced damage, as it plays a role in the removal of the toxin by excreting OTA into the urine; however OTA forms protein adducts, specifically with albumin, thus extending its half-life in humans and prevents excretion (Koszegi and Poor, 2016). This results in an accumulation of OTA and allows the toxin to exert its toxic effects on the kidney tubules, impairing kidney functioning (Koszegi and Poor, 2016).

A previous study demonstrated OTA-induced oxidative damage via stress pathways in human kidney cells and based on these observations it seemed likely that inflammatory pathways may be activated (Raghubeer et al., 2015a). This study proposes a mechanism of action for OTA induction of inflammation that can eventually lead to apoptosis in human embryonic kidney (HEK293) cells. The acute effects of a range of OTA concentrations on HEK293 cells was investigated over 24 hours (hr). It is well documented that persistent inflammation influences cellular proliferation and fibrosis (Kalluri and Weinberg, 2009), therefore our research question was whether OTA increased inflammatory activation in human cells. OTA's inflammatory properties (and chronic exposure) may have contributed to the aetiology of BEN and the formation of UTT. The NFκB pathway, its inhibitor (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha - IκBα), and its downstream effects, as well as apoptosis induction was examined. These results provide a novel insight into OTA-induced human renal toxicity.

Materials

Human embryonic kidney (HEK293) cells were obtained from Highveld Biological (Johannesburg, South Africa). Tissue culture consumables and cell culture medium (Dulbecco's Minimum Essential Media (DMEM)) were purchased from Corning (New York, USA) and Lonza (Basel, Switzerland) respectively. Phosphate buffered saline (PBS) tablets, Ochratoxin A (OTA), Bicinchoninic acid (BCA) assay kit, Tris (hydroxymethyl) aminomethane, bis acrylamide and ammonium persulfate (APS) were obtained from Sigma-Aldrich (Missouri, USA). Tetramethylethylenediamine (TEMED), salts and solvents were purchased from Merck (Darmstadt, Germany). Luminometry assay kits were purchased from Promega (Madison, USA) for the determination of intracellular ATP (CellTiter-Glo® Luminescent Cell Viability Assay), caspase 1 (Caspase-Glo® 1 Inflammasome Assay) and caspases, 8, 9, 3/7 (Caspase-Glo® 8, 9, 3/7 Assay). Cytobuster was obtained from Novagen (San Diego, CA, USA), with phosphatase (04906837001) and protease (05892791001) inhibitors from Roche (Basel, Switzerland) and nitrocellulose membrane from AEC Amersham (Johannesburg, South Africa). The IL-1β enzyme-linked immunosorbent assay (ELISA) was purchased from BD Biosciences (San Jose, CA, USA). All antibodies were purchased from Cell Signaling Technology (Massachusetts, USA).

Methods

Cell Culture

Human embryonic kidney (HEK293) cells were cultured in $25cm^3$ flasks, in a monolayer (10^6 cells per flask), using DMEM supplemented with 10% foetal calf serum (FCS), 1% Penstrepfungizone and 1% L-glutamine. The cells were cultured in a 37°C humidified incubator (5% CO_2). Once 80% confluent, cells were treated with concentrations of OTA, incubated for 24hr, thereafter removed using trypsin, counted, and adjusted for each subsequent assay using the trypan blue exclusion method of cell counting.

OTA Treatments

A 200µM stock solution of OTA was prepared in 60% dimethyl sulphoxide (DMSO). Concentrations of OTA treatments were based on an inhibitory concentration of 50% (IC_{50}) - 1.2µM; at 24hr in a previous study (Raghubeer et al, 2015). The range of OTA concentrations were conducted as O1: sub-IC_{50} (0.5µM), O2: IC_{50} (1.2µM) and O3: supra-IC_{50} (2µM).

Luminometry Assays

Luminometry assay kits were used for the determination of intracellular ATP, caspases- 1, 3/7, 8 and 9 as per the manufacturer's protocol. Briefly, post-treatment 20,000 cells in 0.1M PBS were seeded per well in triplicate in an opaque microtitre plate. The respective detection reagents were added to each well (20µL), plates were incubated at room temperature (RT) in the dark for 1hr and read on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA).

IL-1β ELISA

Detection of IL-1β was carried out as per the manufacturer's instructions. Briefly, 96-well microtitre plates were coated with capture antibody diluted in coating buffer and incubated at 4°C overnight. The wells were then washed thrice (300µL/well) and residual buffer was removed. Plates were blocked with assay diluent (200µL/well) and incubated at RT for 1hr, thereafter assay diluent was removed and plates were washed thrice. Standards and samples were added to each well (100µL) and incubated at RT for 2hr, thereafter plates were washed five times. Detection antibody was added (100µL) and incubated at RT for 1hr; the plates were then washed five times. Diluted enzyme reagent (horse-radish peroxidase (HRP)-conjugated Streptavidin (Sav-HRP)) was added (100µL), incubated at RT for 30min, then removed and washed seven times. Substrate solution was then added (100µL) and incubated at RT for 30min in the dark. Following this incubation, stop solution was added to each well (50µL) and the plate was read using a Bio-Tek µQuant MQx200 spectrophotometer after 30min at 450nm with a reference wavelength of 570nm.

Protein Isolation

Protein was isolated from cells using Cytobuster™ supplemented with protease and phosphatase inhibitors. Following treatment, cells were rinsed twice with 0.1M PBS. Cytobuster™ reagent was added to cell culture flasks (250µL), and cells were mechanically lysed by scraping. The cell lysate was transferred to microcentrifuge tubes and incubated on ice for 10min, then centrifuged (10,000*g*, 4°C, 10min). Crude protein (supernatant) was quantified using the Bicinchoninic acid (BCA) assay. Proteins were standardised to 1mg/ml and boiled in Laemmli buffer (0.5M Tris-HCl, 1% bromophenol blue, glycerol, 10% sodium dodecyl sulphate (SDS), β-mercaptoethanol and dH_2O).

Sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Prepared protein samples were separated on an SDS polyacrylamide gel (stacking: 4%; resolving: 7.5%) at 150V, then transferred onto nitrocellulose membrane using the Trans-Blot® Turbo™ system (Bio-Rad). Membranes were blocked in 5% BSA in Tween20 Tris-buffered saline (TTBS: 25mM Tris, 150mM NaCl, 0.05% Tween 20) for 2hr, at RT on a shaker, prior to immune-probing with primary antibodies: anti-phosphorylated NFκB p65 Ser536 (3033), anti-total NFκB p65 (8242), anti-phosphorylated IκB kinase (IKK) Ser176/180 (2697), anti-total IKK (2370), anti-IκBα (4814), anti-phosphorylated p53 Ser392 (9281), anti-total p53 (6243). Membranes were incubated with primary antibodies (1:5000 dilution in 5% BSA) at 4°C overnight. Thereafter membranes were washed with TTBS four times each (10min) and probed with HRP-conjugated secondary antibodies in 5% BSA for 1hr at RT on a shaker. Unbound antibody was removed by washing in TTBS (4 x 10min). Clarity western ECL chemiluminescent substrate (400µL) was added to each membrane and images were captured using the ChemiDoc™ XRS+ Imaging system (Bio-Rad). Membranes were then quenched

using hydrogen peroxide (37°C; 30min), blocked for 1hr (5% BSA in TTBS), washed twice using TTBS and probed with HRP-conjugated anti-β actin (Sigma-Aldrich). Densitometry analysis was performed using Image Lab software (version 5.1), relative band density (RBD) of each target antigen was measured and normalised against β-actin measurements for each respective sample.

Statistical Analysis

All assays were performed as 3 independent experiments with 3 technical replicates each. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad software, San Diego, CA) and employing a one-way analysis of variance (ANOVA) test. Data is expressed as mean ± standard deviation with data considered statistically significant at $p < 0.05$.

Results

The effect of OTA on inflammatory and cell death pathways in HEK293 cells was evaluated at sub-IC_{50} (O1): 0.5µM ; IC_{50} (O2): 1.2µM and supra-IC_{50} (O3): 2µM concentrations; derived from the MTT assay reported previously (Raghubeer et al., 2015a).

OTA activates the canonical NFκB pathway

The protein expression of phosphorylated (p-)NFκB, total NFκB, p-IKK, total IKK, IκBα, p-p53 and total p53 was determined using SDS-PAGE and western blotting. A significant increase in p-NFκB was observed in treatment O1 ($3.1x10^6 \pm 436357$ RBD) as compared to the control ($1.8x10^6 \pm 216168$ RBD) (Fig 4.1; $p = 0.0006$). Both p-IKK (305736 ± 49040 vs 355933 ± 53887 RBD) and total IKK ($5.3x10^6 \pm 1.2x10^6$ vs $5.9x10^6 \pm 391511$ RBD) were significantly decreased in O3 (Fig 4.2; $p = 0.0143$, $p < 0.05$ respectively) while IκBα ($1.4x10^7 \pm 1.3x10^6$ vs $9.7x10^6 \pm 2.9x10^6$ RBD) was significantly increased (Fig 4.2; $p < 0.05$). A significant increase in IκBα ($1.6x10^7 \pm 1.1x10^6$ vs $9.7x10^6 \pm 2.9x10^6$ RBD) was observed in O2 (Fig 4.2; $p = 0.0006$), while total IKK ($5.6x10^6 \pm 432107$ vs $5.9x10^6 \pm 391511$) was significantly decreased (Fig 4.2; $p = 0.0046$). Analysis indicates activation of the inflammatory pathway in O1, with decreased inflammatory signalling in O2 and O3 compared to O1.

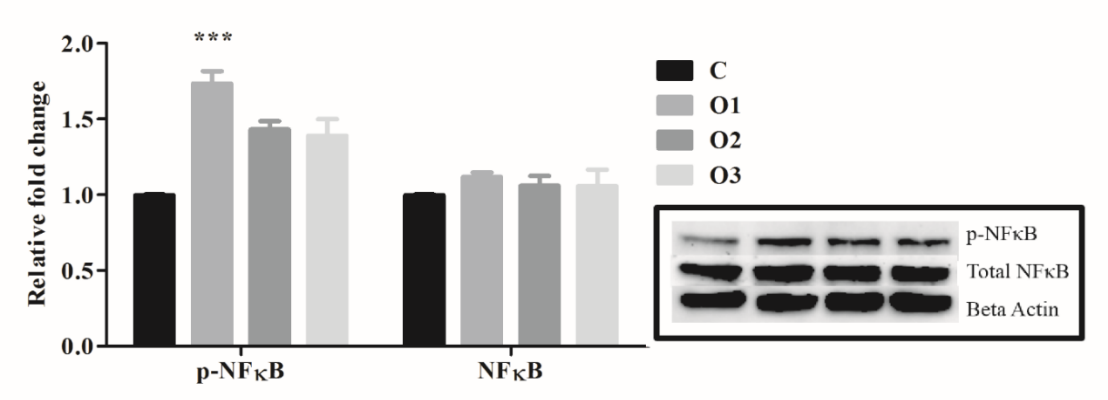

Figure 4.1: Densitometric analysis and western blot images showing relative fold change in inflammatory protein expression at 24hr exposure to OTA with a significant increase in p-NFκB in O1 treated HEK293 cells. ***$p < 0.001$ relative to untreated control.

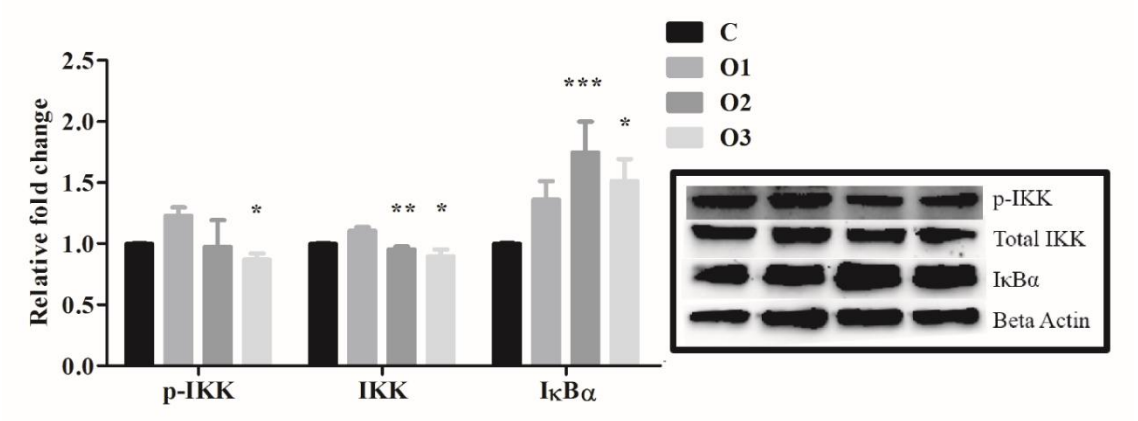

Figure 4.2: Densitometric analysis and western blot images showing relative fold change in inflammatory protein expression over 24hr exposure to OTA. Significant increases were observed in IκBα in O2 and O3 treated HEK293 cells. Significant decreases were observed in p-IKK and total IKK in O3. *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$ relative to untreated control.

OTA significantly increases the phosphorylation of p53 (Ser392) in O1 and O2.

Figure 4.3 indicates increased expression of p-p53 in O1 ($6.4x10^6 \pm 412979$ vs $3.8x10^6 \pm 547685$) and O2 ($6.4x10^6 \pm 433139$ vs $3.8x10^6 \pm 547685$), with a simultaneous increase in total p53 in O1 ($7.8x10^6 \pm 324906$ vs $5.9x10^6 \pm 597597$) and O2 ($8x10^6 \pm 508071$ vs $5.9x10^6 \pm 597597$).

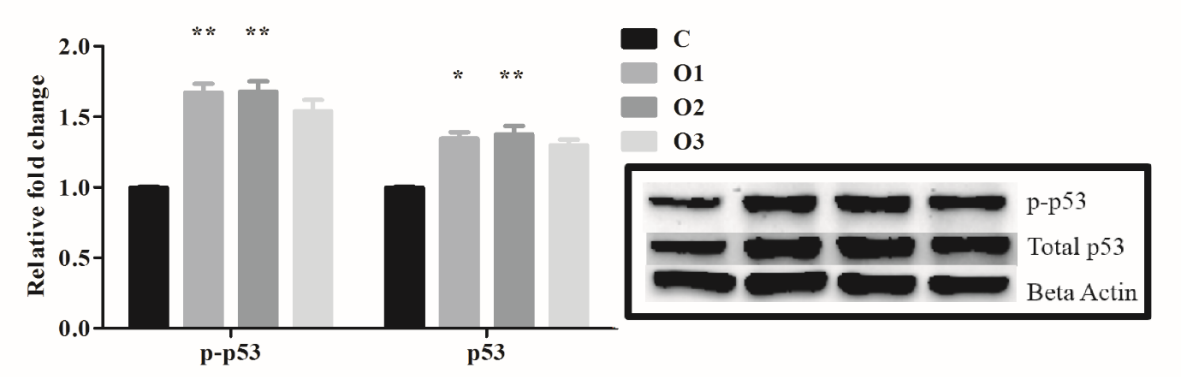

Figure 4.3: Densitometric analysis and western blot images showing relative fold change in protein expression of tumour suppressor p53. Significant increases were observed in p-p53 (Ser392) in O1 and O2, as well as total p53 in O1 and O2. *$p < 0.05$; **$p < 0.005$ relative to untreated control.

Decreased IL-1β and Caspase 1 indicate decreased inflammatory signalling in O2.

Caspase 1 activates IL-1β, which in turn activates NFκB. Significantly decreased IL-1β levels were observed in O2 ($p = 0.0186$) coupled with decreased caspase 1 levels when compared to O1 ($p = 0.0068$). The decrease observed in O2 indicates that pro-inflammatory signalling has been dampened.

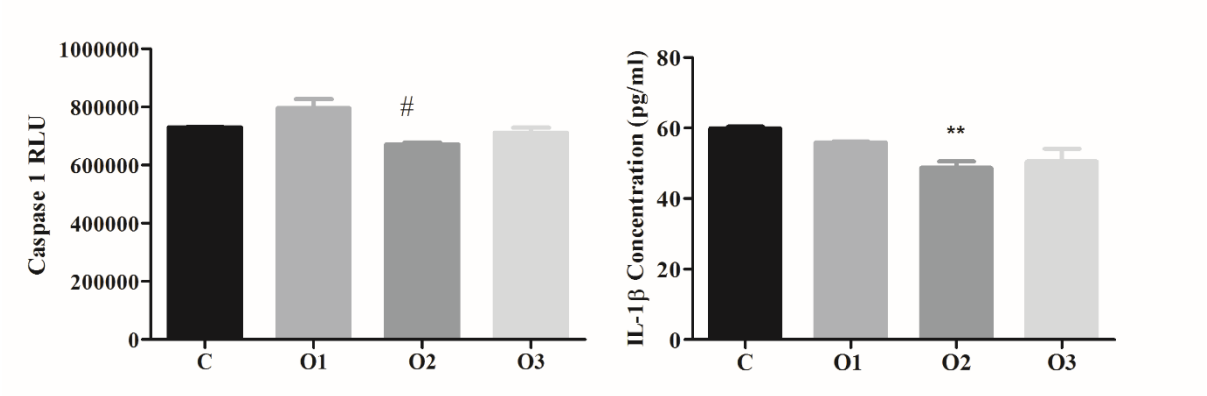

Figure 4.4: Luminometric quantification of caspase 1 and ELISA results for IL-1β measurement (pg/ml). IL-1β and caspase 1 was significantly decreased in O2. $^{\#}p < 0.005$ relative to treatment O1; $^{**}p < 0.05$ relative to untreated control cells.

Increased apoptotic activity in O3.

Caspases 3/7, 8, and 9 were all significantly increased in O3 ($p = 0.0002$, $p = 0.0011$ and $p = 0.0002$ and respectively) with no changes in O1 and O2 ($p > 0.05$) (Fig. 4.5). This data indicates increased caspase activity in both the extrinsic and intrinsic pathways.

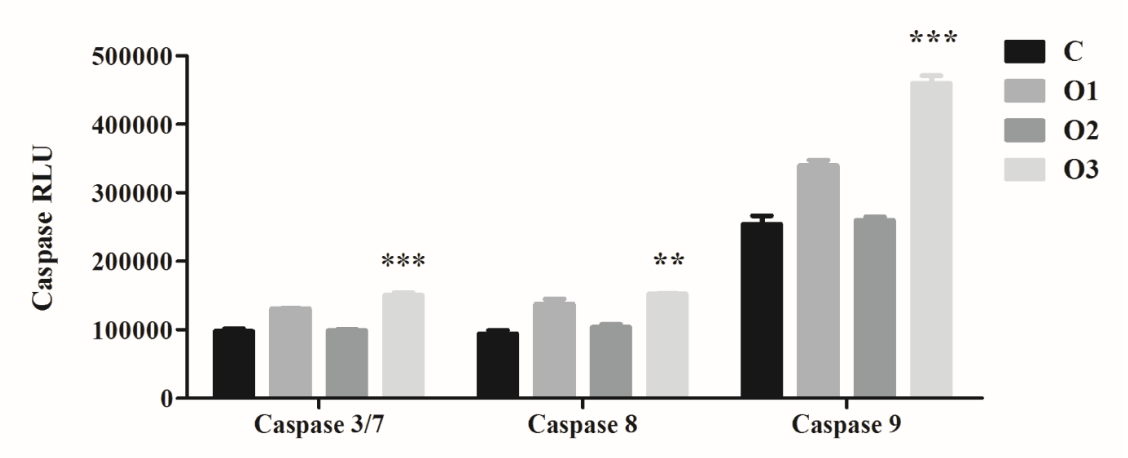

Figure 4.5: Luminometric assessment of caspase 3/7, 8 and 9 activity in HEK293 cells. Significantly increased caspase 3/7, 8, and 9 activity was observed in O3. $^{**}p < 0.05$, $^{***}p < 0.001$ relative to untreated control.

Increased ATP levels in lower OTA concentrations.

Intracellular ATP levels were significantly increased in O1 and O2 ($p < 0.05$ and $p = 0.0001$ respectively; Fig 4.6). The highest OTA concentration (O3) also elevated ATP levels relative to the control but this result was lower than that observed in O1 and O2.

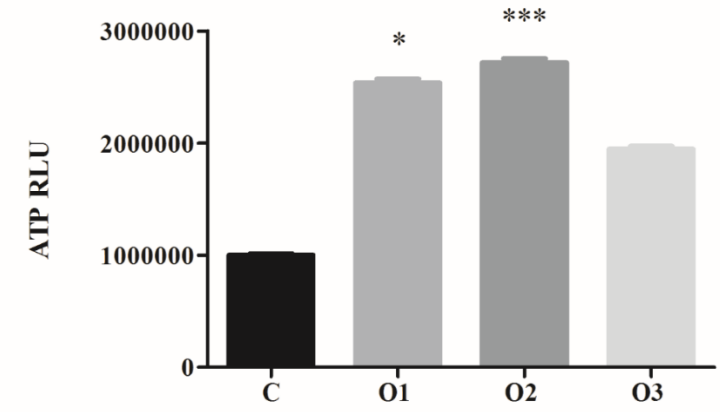

Figure 4.6: Intracellular ATP levels of HEK293 cells following acute exposure to OTA. Intracellular levels were significantly increased in O1 and O2 ($p < 0.05$ and $p = 0.0001$ respectively). $^{*}p < 0.05$ and $^{***}p < 0.001$ relative to untreated control.

Discussion

OTA, a mycotoxin contaminant of grains, animal feed and fruit, is classified as a group 2B carcinogen by the International Agency for Research on Cancer (IARC), indicating that the toxin is a possible human carcinogen (Koszegi and Poor, 2016). OTA has been implicated in BEN, a chronic kidney disease (CKD) resulting in tubulointerstitial nephritis and possible tumour formation (Pfohl-Leszkowicz and Manderville, 2007). Tubulointerstitial nephritis and CKD results in renal dysfunction

with the formation of tumours in some cases (Gagliano et al., 2005). OTA participates in several adverse reactions once ingested by animals and humans. The toxin is known to promote pro-oxidant conditions - thus inducing oxidative stress, protein synthesis disruptions and DNA damage (Schaaf et al., 2002); all of which provide molecular mechanisms to clinical manifestations of OTA toxicity.

Chronic inflammation aggravates kidney disease; however, OTA induced inflammation has not been assessed in renal toxicity. The transcription factor, NFκB, controls the transcription of genes associated with inflammation and apoptosis, determining the fate of the cell. This study reports, for the first time, the influence of OTA on NFκB in kidney cells. Under normal circumstances NFκB is rendered inactive in the cytosol by IκBα, however upon stimulation of appropriate extracellular signals IKK is activated and subsequently phosphorylates IκBα for ubiquitination (Lawrence, 2009). Once phosphorylated, IκBα dissociates from NFκB and is degraded by proteases while NFκB is free to translocate to the nucleus (Chaves et al., 2009). Nuclear translocation allows binding to a response element, allowing transcription of genes relating to inflammatory responses and even apoptosis. Activators of NFκB include ROS and IL-1β (Oeckinghaus and Ghosh, 2009).

The sub-IC_{50} concentration is highly relevant in determining the toxicity of OTA, as it approximates plasma levels (0.5μM) in endemic populations. Our data indicates that the lowest concentration of OTA (O1) induced a strong pro-inflammatory response by increasing p-NFκB and IKK, with a concomitant decline in IκBα (Fig 4.2). Down-regulation of IκBα promotes NFκB translocation to the nucleus. Activation of NFκB induces an inflammatory response with the possible induction or inhibition of apoptosis, dependent on the extracellular signals received (Barkett and Gilmore, 1999). An inflammatory environment exacerbates the progression of kidney disease as it promotes the accumulation of pro-inflammatory cytokines and adhesion molecules, prompting fibrosis of kidney epithelia and progressive dysfunction (Silverstein, 2009). No significant apoptosis induction is seen in treatments O1 and O2 based on the caspase activity assays (Fig 4.5).

As the concentration of OTA increases (O2), however, a decrease in inflammation is noted possibly due to decreased IL-1β and caspase 1 levels (Fig 4.4). Caspase 1 and IL-1β play a significant role in inflammation induction. Caspase 1, also known as interleukin-1 converting enzyme (ICE), has been found to process pro-IL-1β to the active IL-1β form, which influences NFκB-mediated inflammation (Denes et al., 2012). Decreased caspase 1 and IL-1β indicate decreased NFκB activation in O2. We also observe increased IκBα and decreased IKK levels (Fig 4.2), suggesting that NFκB is sequestered in the cytosol and IκBα is not targeted for degradation. The data indicates a shift from an anti-apoptotic, pro-inflammatory environment to a pro-apoptotic environment as OTA concentration increases. This is evident by the increased activity of caspases 8, 9, 3 and 7 at the highest OTA concentration O3,

suggesting that both the extrinsic and intrinsic pathways of apoptosis are being stimulated. Furthermore, a significant decrease in IKK levels coupled with increased IκBα was observed (Fig 4.2).

Apoptosis induction by OTA in varying concentrations has been reported in several studies (Essid et al., 2012; Petrik, 2005; Ringot et al., 2006; Sorrenti et al., 2013), with accompanying DNA damage in some cases (Hibi et al., 2013a; Kuroda et al., 2015). Petrik et al. (2005) proved (using MDCK, LLC-PK1 and RK 13 cells) that as the concentration of OTA increases, so too does the induction of apoptosis (Petrik, 2005). This change in inflammatory environment as a result of increased OTA concentration could be attributed to the increased toxic insult to the cells. The adaptive cellular stress response preferentially selects apoptosis, over persistent inflammatory activation in response to high concentrations of OTA. Apoptosis is a viable means of restoring homeostasis by removing irreversibly compromised cells in a highly toxic environment.

NFκB influences apoptosis by promoting the transcription of both pro and anti-apoptotic proteins (Kaltschmidt et al., 2000). The tumour suppressor, p53, is a pivotal regulator of cell cycle and apoptosis; it reacts to DNA damage or oncogene expression to bring about repair and initiate cell cycle checkpoints (Fridman and Lowe, 2003). Protein expression of both phosphorylated and total p53 were significantly increased in HEK293 cells in treatments O1 and O2 (Fig 4.3). Phosphorylation of p53 is an important posttranslational modification to regulate the tumour suppressor function (Matsumoto et al., 2004). Phosphorylation of p53 at serine residue 392 is reportedly increased in tumours; which correlates with the theory of eventual tumorigenesis as a result of persistent inflammation (Ullrich et al., 1993). Kuroda et al. (2015) reported that p53 was activated in response to DNA damage by OTA (Kuroda et al., 2015). This information suggests that OTA causes DNA damage and induces an appropriate repair response. Our previous study also showed increased DNA damage, evidenced by increased comet tail lengths in HEK293 cells induced by OTA, as well as increased transcription of OGG1 (a DNA repair enzyme) (Raghubeer et al., 2015a). The present results indicate that OTA-induced DNA damage could eventually result in mutations, providing a purpose for phosphorylation of p53 at Ser392.

Apoptosis is an energy consuming biological process. The ATP levels correlate with caspase activity, as its levels increased in O1 and O2, but not in O3, indicating that the process of apoptosis depletes ATP (Fig 4.6). Schwerdt et al. (2003) reported a similar increase in ATP levels upon acute exposure to OTA in kidney cells (Schwerdt et al., 2003). This research provides an alternative mechanism of OTA toxicity centred on the mycotoxin's ability to induce inflammation via the canonical NFκB pathway. Endemic populations display up to a thousand times higher OTA plasma levels than healthy populations. Koszegi et al. (2016) reported that OTA plasma levels could reach hundreds of nmol/L in endemic populations , thus O1 provides a plausible model for kidney toxicity as the kidney accumulates OTA during nephron reabsorption (Koszegi and Poor, 2016).

We propose that OTA's effect on the inflammatory pathway could be an influencing factor in the progression of kidney disease. Persistent inflammation could play a role in epithelial-mesenchymal transition and eventual tumorigenesis as a result of uncontrolled cell proliferation (Kalluri and Neilson, 2003). Further research with chronic exposure is warranted, as well as to ascertain the extent of OTA's influence on the inflammatory and proliferative pathways. This research could serve as a foundation for investigation into the inflammatory effects of OTA using an *in vivo* model, such as rats or mice. This could provide important answers to OTA's mechanism of toxicity and involvement in the propagation of renal tumours.

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CHAPTER 5

Varying Concentrations of Ochratoxin A promote Endoplasmic Reticulum stress-induced survival in human kidney and liver cells.

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Abstract

Protein misfolding and aggregation caused by cellular stress activates the endoplasmic reticulum (ER)-mediated unfolded protein response (UPR). The mycotoxin Ochratoxin A (OTA), a food contaminant, has been reported to disrupt redox status and protein homeostasis. We evaluated the effect of various OTA concentrations (0.5µM – 0.125µM) on ER stress and UPR markers in HEK293 and HepG2 cells over 24hr and 48hr. We employed western blotting, luminometry, qPCR and protein carbonyl quantification. OTA increased protein carbonyl concentrations in kidney (HEK293) cells, and simultaneously increased protein expression of LonP1, HSP70, PERK and p-eIF2α ($p < 0.05$) in both kidney and liver (HepG2) cells over both 24hr and 48hr time periods. Further, OTA increased gene expression of *ATF4* ($p < 0.05$), with decreased *CHOP* expression and caspase 3/7 activity in both cell lines. Thus, OTA toxicity induces ER stress response in cell survival, and could provide new insights into the mechanisms of OTA toxicity in human kidney and liver cells.

Introduction

Mycotoxins are secondary metabolites of fungal origin and are commonly found in human food products and animal feed - ensuring its presence in the food chain. Ochratoxin A (OTA) is a mycotoxin produced by species of *Aspergillus* and *Penicillium* fungi (Cabañes et al., 2010), and is a frequent contaminant of coffee, alcoholic beverages, animal products (pork), spices and fruit (Ostry et al., 2013). OTA-associated renal toxicity is well established, with secondary effects observed in the liver and immune system (Malir et al., 2016). Oxidative stress, DNA adducts, and protein damage all present molecular effects of OTA which manifest as clinical outcomes, such as tumour induction, renal failure, and immune dysfunction (Boesch-Saadatmandi et al., 2008; Mally, 2012). OTA has been suspected as an aetiological agent in nephropathies around the world, diseases associated with progressive kidney dysfunction, destruction of the renal tubular epithelium and possible tumour formation (Castegnaro et al., 2006; Wafa et al., 1998). Selective reabsorption of OTA exacerbates kidney toxicity, increasing the presence and effects of an already persistent toxin – the half-life in humans has been recorded at 35 days (Reddy and Bhoola, 2010).

Ochratoxin A has been reported to increase oxidative stress and induce DNA damage in both the kidney and liver (Gayathri et al., 2015; Pfohl-Leszkowicz and Manderville, 2007). Considering that oxidative stress leads to oxidative protein modifications, and OTA binds to plasma proteins; the protein maintenance network mediated by the ER may provide novel insight into OTA toxicity as knowledge of its effects on endoplasmic reticulum (ER) stress is limited. The ER is responsible for the cellular response to protein misfolding – a result of disruptions in protein homeostasis (Chaudhari et al., 2014). Under normal conditions protein aggregates are proteolytically cleared, however, an accumulation of misfolded proteins activates the unfolded protein response (UPR) via the ER stress pathway. The UPR aims to restore protein homeostasis by degrading damaged proteins, inhibiting further translation, and upregulating chaperone proteins to assist with protein folding efforts. Should this response fail, the UPR directs the cell towards apoptosis (Araki and Nagata, 2011).

The protein kinase RNA-like endoplasmic reticulum kinase (PERK), eukaryotic initiation factor 2 alpha (eiF2α) and activating transcription factor 4 (ATF4) axis of the UPR was investigated in both HEK293 and HepG2 cells. During ER stress the PERK-eIF2α pathway is activated immediately (Fribley et al., 2009); activated PERK phosphorylates the alpha subunit of eIF2α at serine residue 51, thus inhibiting eIF2α and reducing global protein synthesis, to assist in reducing overall protein load (Fribley et al., 2009). Selected proteins such as the transcription factor ATF4 are translated during this time; ATF4 regulates expression of genes pertaining to amino acid metabolism, redox homeostasis and the ER stress response (Ye et al., 2010). Research shows that ATF4 is overexpressed in some human tumours, and could possibly allow tumour cell survival and cancer progression (Ye et al., 2010). ATF4 is a member of the cAMP response element binding (cREB) family and is central in regulating genes involved with

cell survival; it can upregulate the expression of CHOP, a pro-apoptotic factor in times of persistent ER stress. Overexpression of CHOP promotes apoptosis (Rozpędek et al., 2016).

Recent research confirmed that OTA induced ER stress in mouse and rat mesangial cells (Sheu et al., 2016). A previous study by our own research group showed the induction of oxidative stress and activation of Lon protease (LonP1) by OTA at IC_{50} concentrations in human kidney cells (Raghubeer et al., 2015). These findings prompted an investigation into the effects of OTA on protein and ER stress responses in both human kidney and liver cells. Our study used a range of OTA concentrations based on upper levels (serum/urine) recorded in high OTA exposure regions (Jonsyn-Ellis, 2001; Wafa et al., 1998). We also investigated protein oxidation, LonP1 expression, and executioner caspase activity. It is hoped that the data can be used to better understand the stress responses induced by OTA in humans and possibly lead to future therapeutic interventions.

Materials

Vendor authenticated human embryonic kidney (HEK293) and liver hepatocellular carcinoma (HepG2) cells were purchased from Highveld Biologicals (Johannesburg, South Africa). Dulbeccos' minimum essential media (DMEM), Eagle's minimum essential media (EMEM)), HEPES buffer and trypsin was purchased from Lonza (Basel, Switzerland). OTA (O1877) was sourced from Sigma-Aldrich (Missouri, USA). Luminometry activity kits were purchased from Promega (Madison, USA). All solvents and salts were purchased from Merck (Darmstadt, Germany) unless otherwise stated.

Methods

Cell Culture

Human embryonic kidney (HEK293) cells and liver hepatocellular carcinoma (HepG2) cells were cultured in $25cm^3$ flasks, in monolayer (10^6 cells per flask), using DMEM and EMEM respectively (both supplemented with 10% foetal calf serum (FCS), 1% Penstrepfungizone, HEPES buffer and 1% L-glutamine). The cells were cultured in a 37°C humidified incubator (5% CO_2). At 80% confluence, cells were treated with varying concentrations of OTA (0.125µM – 0.5µM) for 24hr and 48hr. Following treatment, cells were detached by incubation with trypsin (5min; 37°C), counted (Invitrogen Countess™ automated cell counter), and adjusted for each subsequent assay.

Ochratoxin A Treatments

A 5mM stock solution of OTA was prepared in 60% dimethyl sulphoxide (DMSO). Concentrations used for treatment were based on high levels recorded in serum and urine (Jonsyn-Ellis, 2001; Wafa et al., 1998). The OTA range was delivered in increasing concentrations – $OTA_{0.125}$: 0.125µM, $OTA_{0.25}$: 0.25µM and $OTA_{0.5}$: 0.5µM.

Methylthiazol Tetrazolium (MTT) Assay

Cells were seeded into a microtitre plate (15 000 cells/well) and attached overnight. Thereafter, cells were exposed to OTA (0.25µM – 50µM) over 24hr and 48hr. Culture media was removed and replaced with 20 µL MTT solution (5mg/ml in PBS) and CCM (100µL). After a 4hr incubation, the solution was removed and DMSO was added (100µL, 1hr incubation). A Bio-Tek MQx200 spectrophotometer was used to read absorbance at 595nm (reference wavelength 655nm). The 50% inhibitory concentration (IC_{50}) was calculated.

Luminometric assessment of Caspase activity

The Caspase-Glo® 3/7 (G8098) and 6 (G0970) assay systems were used to assess caspase activity in HEK293 and HepG2 cells as per the manufacturer's protocol. Briefly, 20, 000 cells (in 0.1M PBS) were seeded per well (triplicate) in an opaque microtitre plate. The respective detection reagents were added to each well (20µL) and plates were incubated at room temperature (RT) in the dark for 1hr and read on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA). Results were reported as relative light units (RLU), directly proportional to caspase activity.

ER stress protein expression

Protein isolation

Protein was isolated from HEK293 and HepG2 cells using Cytobuster™ (Novagen (San Diego, USA)), with supplementary phosphatase (004906837001) and protease (05892791001) inhibitors as previously described (Sheik Abdul et al., 2016). Crude protein was quantified using the Bicinchoninic acid (BCA) assay, thereafter proteins were standardised to 1mg/ml and boiled (5min) in Laemmli buffer (0.5M Tris-HCl, 1% bromophenol blue, glycerol, 10% sodium dodecyl sulphate (SDS), β-mercaptoethanol and dH_2O).

Sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Protein samples were separated on SDS polyacrylamide gel (stacking: 4%; resolving: 7.5%) at 150V (1hr) and subsequently electro-transferred to nitrocellulose membranes using a pre-programmed mixed MW protocol on the Trans-Blot® Turbo™ system (Bio-Rad). All membranes were blocked in 5% BSA in Tween20 Tris-buffered saline (TTBS: 25mM Tris, 150mM NaCl, 0.05% Tween 20) for 2hr, at RT on a shaker, prior to immune-probing with primary antibodies (1:1000) at 4°C overnight – anti-PERK (C33E10), anti-p-eIF2α (119A11) from Cell Signaling Technology (Massachusetts, USA), anti-LonP1 (HPA002192) from Sigma-Aldrich (Missouri, USA) with anti-HSP70 (610607) and anti-HSP90 (610418) antibodies from BD Biosciences (San Jose, CA, USA). Membranes were washed (TTBS, 4 x 10min) and probed with horse-radish peroxidase (HRP)-conjugated anti-mouse (7076S) or HRP-conjugated anti-rabbit (7074P2) secondary antibodies in 5% BSA (1hr, RT). Membranes were washed

(4 x 10min). Clarity™ Western ECL chemiluminescent substrate (Bio-Rad) was added to each membrane and images were captured using the ChemiDoc™ XRS+ Imaging system (Bio-Rad). Membranes were quenched using hydrogen peroxide (37°C; 30min), blocked for 1hr (5% BSA in TTBS), washed twice, and probed with HRP-conjugated anti-β actin (Sigma-Aldrich). Densitometry analysis was performed using Image Lab software (version 5.1) and was quantified as relative band intensity (RBI) converted to fold change. All results were normalized against β-actin prior to statistical analysis.

Messenger RNA (mRNA) quantification of ER stress regulators

RNA Isolation and cDNA Synthesis

RNA was isolated using an in-house protocol (Pillay et al., 2015) with QIAzol extraction reagent from Qiagen (Hilden, Germany). Once isolated, RNA was quantified using the NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and standardised to 1000ng/µL. Standardised RNA was used to synthesise cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, California, USA) as per the manufacturer's protocol. Thermocycler conditions were 25°C(5min), 42°C (30min), 85°C (5min) and a final hold of 4°C.

Quantitative Polymerase Chain Reaction (qPCR)

Transcript levels of *ATF4* (sense: 5'-GTTCTCCAGCGACAAGGCTA-3', antisense: 5'-ATCCTCCTTGCTGTTGTTGG-3') and *CHOP* (sense: 5'-GGAAACAGAGTGGTCATTCCC-3', antisense: 5'-CTGCTTGAGCCGTTCATTCTC-3') was evaluated by qPCR. Primer sequences were obtained from Inqaba Biotechnical Industries (Pretoria, South Africa). A reaction mix of 5.5µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 1µL cDNA, 1µL sense and antisense primers, and nuclease free H_2O was made up to 10µL. The assays were carried out using 6 replicates per treatment with glyceraldehyde 3-phosphate dehydrogenase; *GAPDH* (sense: 5'-TCCACCACCCTGTTGCTGTA-3', antisense: 5'-ACCACAGTCCATGCCATCAC-3') and β-actin (sense: 5'-TGACGGGTCACCCACACTGTGCCCAT-3', antisense: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3') as housekeeping genes. Reactions were carried out on the CFX Touch™ Real Time PCR detection system (Bio-Rad) as follows: Initial denaturation at 95°C (4min), followed by 37 cycles of denaturation (95°C, 15s), annealing (40sec; *CHOP* - 55°C, *ATF4* - 65°C) and extension (72°C, 30sec). Analysis was performed as per the method described by Livak and Schmittgen, where $2^{-\Delta\Delta Ct}$ represents the fold change relative to the housekeeping gene (Livak and Schmittgen, 2001).

Protein Carbonyl Assay

Protein was isolated from treated cells and standardised to 5mg/ml for protein carbonyl quantification. Dinitrophenylhydrazine (DNPH – 10mM in 2.5M hydrochloric acid) was added to each sample and

incubated (1hr, RT). Samples were then incubated on ice with 20% trichloroacetic acid (TCA) solution, protein precipitates were collected and washed with 10% TCA solution. Pellets were washed with ethanol-ethyl acetate (2 washes) and dissolved in guanidine hydrochloride (6M) during incubation (37°C, dark, 10min). The supernatant was collected, aliquoted into a 96-well microtiter plate (triplicate) and absorbance was measured using a Bio-Tek MQx200 spectrophotometer at 370nm. Optical density measurements were used to calculate protein carbonyl concentrations in each sample.

Statistical Analysis

Analysis was performed using GraphPad Prism version 5.0 (GraphPad software, San Diego, CA) and employing one-way analysis of variance (ANOVA) tests. Data is expressed as mean ± standard deviation with statistical significance considered at $p < 0.05$.

Results

Previously we showed that OTA induced oxidative stress and increased LonP1 expression at an IC_{50} value in HEK293 cells (Raghubeer et al., 2015). In the present study we used a range of OTA concentrations (0.125µM – 0.5µM) to determine the effects on protein stress, ER stress and cell death in HEK293 and HepG2 cells. An increase in oxidative protein damage was observed, with the canonical PERK-eIF2α-ATF4 pathway being activated.

HepG2 cytotoxicity test using MTT assay

Acute exposure of HepG2 cells to OTA showed resistance to cell death induction by the toxin, producing an IC_{50} value of 47µM. However, at 48hr the cells responded similarly to previous cytotoxicity tests using HEK293 cells (Raghubeer et al., 2015), indicating an IC_{50} of 9µM.

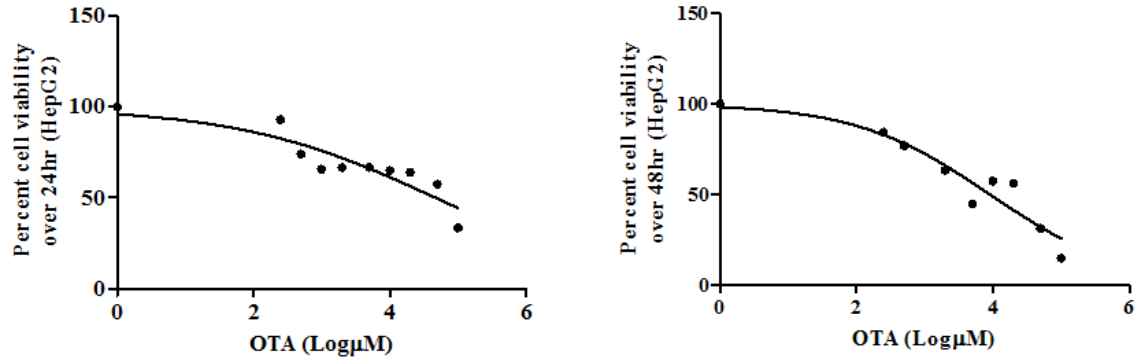

Figure 5.1: Cellular viability (%) post-exposure to a range of OTA concentrations over 24hr and 48hr. An IC_{50} of 47µM (24hr) and 9µM (48hr) was calculated from the dose-response curves.

LonP1 and HSP70 protein expressions were increased post-exposure to a range of OTA concentrations over 24hr and 48hr in HEK293 and HepG2 cells.

The lowest concentration of OTA ($OTA_{0.125}$ – 0.125µM) induced the highest LonP1 expression (0.34±0.082RBI vs control 0.013±0.005RBI; $p = 0.0002$), and increased HSP70 protein expression (2.028±0.4RBI vs control 0.82±0.07RBI, $p = 0.0002$) in HEK293 cells at 24hr (Fig 5.2). Although not as significant as $OTA_{0.125}$, the higher doses of OTA also significantly increased LonP1 expression (9.5- and 8.3-fold respectively, 24hr). These observations differ with the 48hr results: $OTA_{0.5}$ (0.5µM) significantly increased expression of LonP1 (0.42±0.02RBI vs control 0.26±0.02RBI; $p < 0.005$), while increases in HSP70 are observed in $OTA_{0.25}$ (1.057±0.04RBI; $p = 0.001$) and $OTA_{0.125}$ (1±0.18RBI; $p < 0.05$) compared to controls (0.67±0.005RBI) (Fig 5.2). In HepG2 cells exposed to OTA for 24hr we observed increased LonP1 protein levels at all concentrations with the most significant increase in $OTA_{0.5}$ (0.64±0.1RBI vs control 0.12±0.04RBI; $p = 0.0002$) (Fig 5.3). At 48hr the lowest OTA concentration exhibited increased LonP1 (0.57±0.12RBI vs control 0.19±0.08RBI; $p = 0.0003$) in HepG2 cells (Fig 5.3).

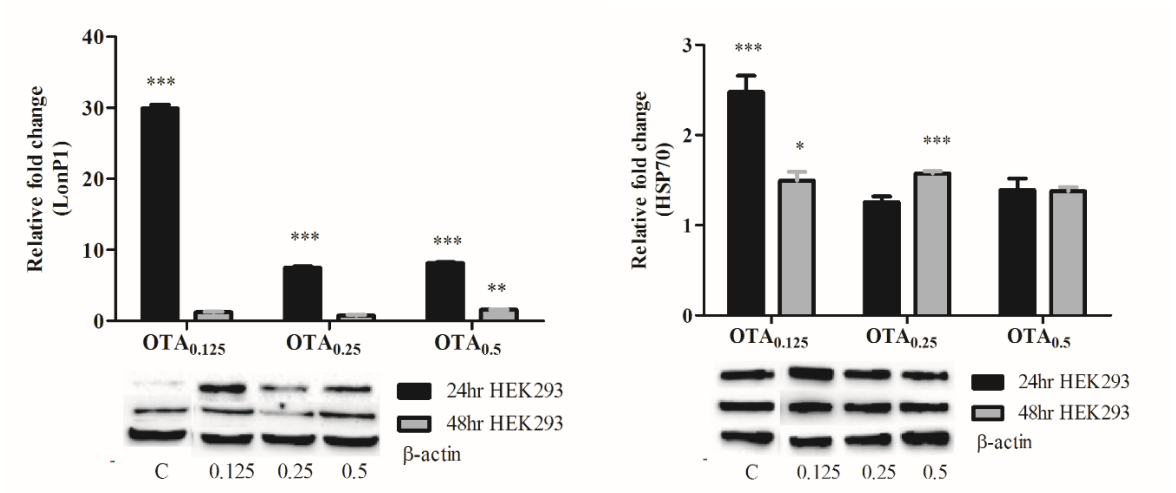

Figure 5.2: Densitometric analysis and western blot images showing relative fold change in LonP1 and HSP70 in HEK293 cells over 24hr and 48hr exposure to OTA – $OTA_{0.125}$ (0.125µM), $OTA_{0.25}$ (0.25µM) and $OTA_{0.5}$ (0.5µM). *$p < 0.05$, **$p < 0.005$ and ***$p < 0.0005$ relative to untreated control.

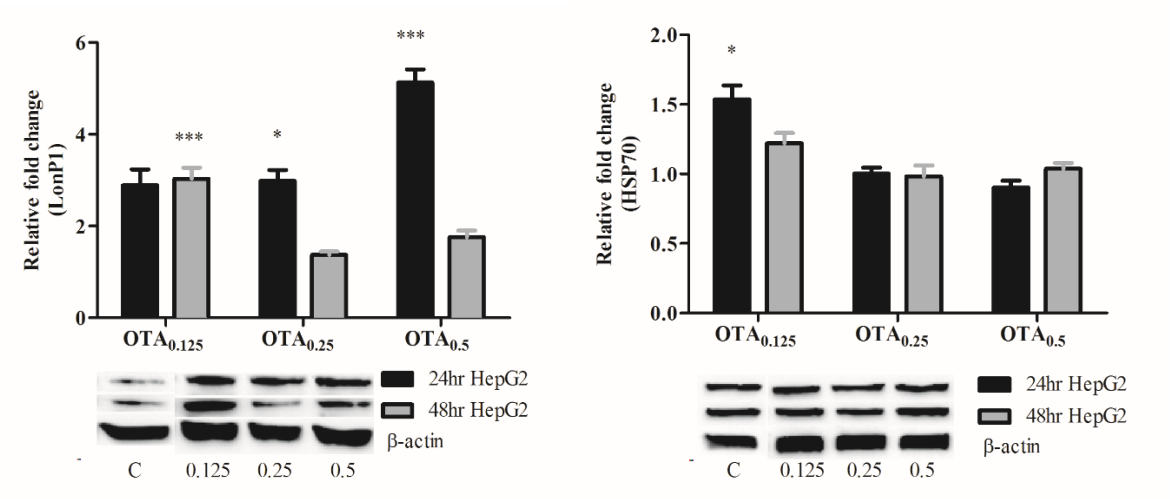

Figure 5.3: Densitometric analysis and western blot images showing relative fold change in LonP1 and HSP70 in HepG2 cells over 24hr and 48hr exposure to OTA – $OTA_{0.125}$ (0.125µM), $OTA_{0.25}$ (0.25µM) and $OTA_{0.5}$ (0.5µM). *$p < 0.05$ and ***$p < 0.0005$ relative to controls.

Lower concentrations of OTA induce protein oxidation in HEK293.

OTA significantly increased protein oxidation in $OTA_{0.25}$ compared to $OTA_{0.5}$ (1.5-fold, $p < 0.0005$) in HEK293 cells; whereas protein oxidation in $OTA_{0.125}$ was significantly greater than both the control and $OTA_{0.5}$ (1.2-fold, $p < 0.05$, Fig 5.4).

48hr exposure to OTA induces a robust effect on protein oxidation in HepG2 cells.

The opposite was true for HepG2 cells exposed to $OTA_{0.125}$ over 24hr. $OTA_{0.125}$ exhibits significantly lower protein carbonyl production than both $OTA_{0.5}$ and $OTA_{0.25}$, while $OTA_{0.25}$ is significantly higher than both treatments and controls ($p < 0.05$, Fig 5.4). At 48hr, the lowest OTA concentration induces a greater increase in protein carbonyl formation. Both $OTA_{0.25}$ and $OTA_{0.125}$ show significantly greater protein oxidation than $OTA_{0.5}$ ($p < 0.05$, Fig 5.4). Further to LonP1 expression and protein damage, we then evaluated the effects of OTA on ER stress.

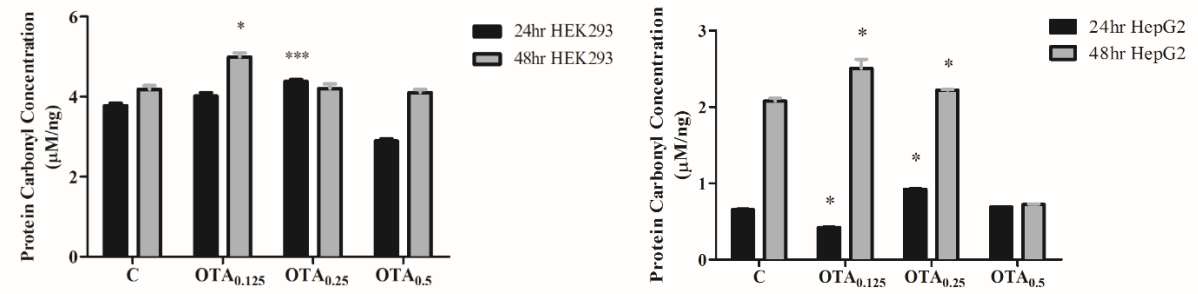

Figure 5.4: Analysis of protein carbonyl concentrations during exposure to a range of OTA in HEK293 and HepG2 cells over 24hr and 48hr. *$p < 0.05$ and ***$p < 0.0005$ relative to respective controls.

OTA activates the ER stress pathway over 24hr and 48hr in HEK293 cells.

$OTA_{0.5}$ significantly increased the expression of PERK over 24hr (0.4±0.08RBI vs control 0.25±0.03RBI, $p = 0.013$), with no change in eIF2α phosphorylation, however $OTA_{0.125}$ significantly increased p-eIF2α (0.78±0.09RBI vs control 0.57±0.13RBI, $p = 0.0002$) (Fig 5.5). At 48hr $OTA_{0.5}$ increased both PERK (0.89±0.04RBI vs control 0.41±0.03RBI, $p = 0.0005$) and p-eIF2α (1.58±0.06RBI vs control 1.17±0.05RBI, $p = 0.0001$) significantly. $OTA_{0.25}$ increased expression of p-eIF2α (1.49±0.07RBI), while $OTA_{0.125}$ increased PERK expression (0.75±0.15RBI) at 48hr (Fig 5.5). Overall, this data indicates activation of the ER stress response across a range of OTA concentrations.

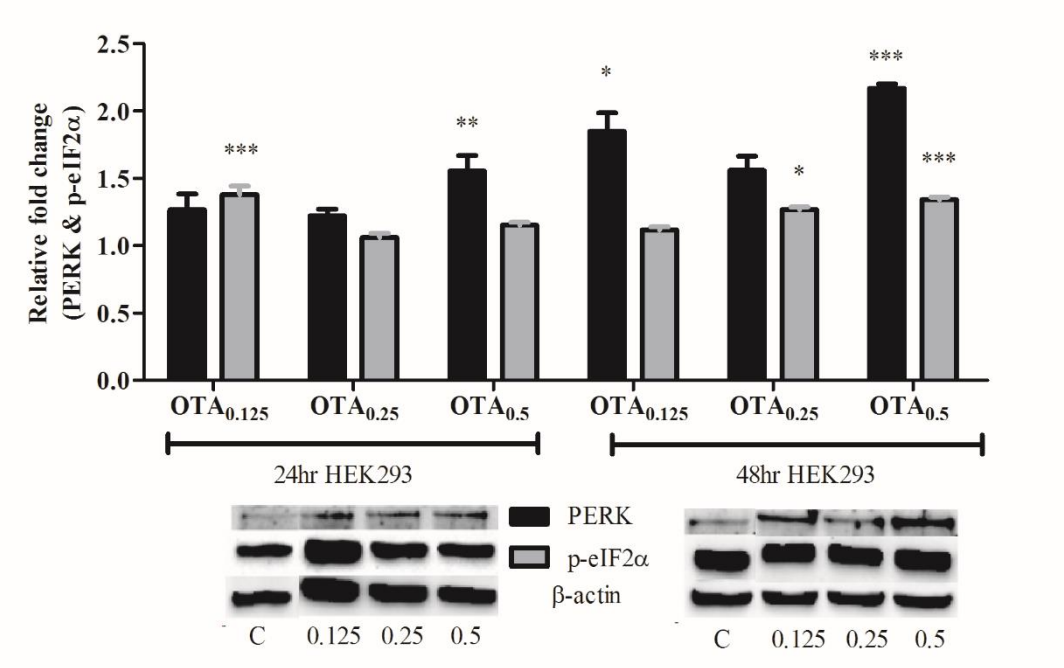

Figure 5.5: Densitometric analysis and western blot images showing relative fold change in PERK and p-eIF2α following 24hr and 48hr exposure of HEK293 cells to a range of OTA concentrations. *$p <$ 0.05, **$p < 0.005$ and ***$p < 0.0005$ relative to respective controls.

PERK expression increases during acute, but not prolonged exposure to OTA; p-eIF2α expression increases during prolonged, but not acute exposure to OTA concentrations in HepG2 cells.

PERK and p-eIF2α display an inverse relationship in expression profiles over 24hr and 48hr exposure to OTA in HepG2 cells. At 24hr PERK protein expression is markedly increased at $OTA_{0.5}$ (0.1±0.01RBI, $p = 0.0005$), $OTA_{0.25}$ (0.08±0.03RBI) and $OTA_{0.125}$ (0.13±0.09RBI) compared to controls (0.04±0.0007RBI), with increased p-eIF2α only evident at $OTA_{0.5}$ (1.35±0.3RBI vs control 0.93±0.33RBI, $p = 0.04$) (Fig 5.6). At 48hr $OTA_{0.25}$ (0.44±0.12RBI) and $OTA_{0.125}$ (0.4±0.09RBI) significantly decreased PERK expression when compared to $OTA_{0.5}$ (0.66±0.14RBI), while significant increases in p-eIF2α was observed during $OTA_{0.5}$ (1.06±0.17RBI, $p = 0.0002$), $OTA_{0.25}$ (0.81±0.23RBI) and $OTA_{0.125}$ (0.64±0.21RBI) exposure, compared to controls (0.41±0.02RBI).

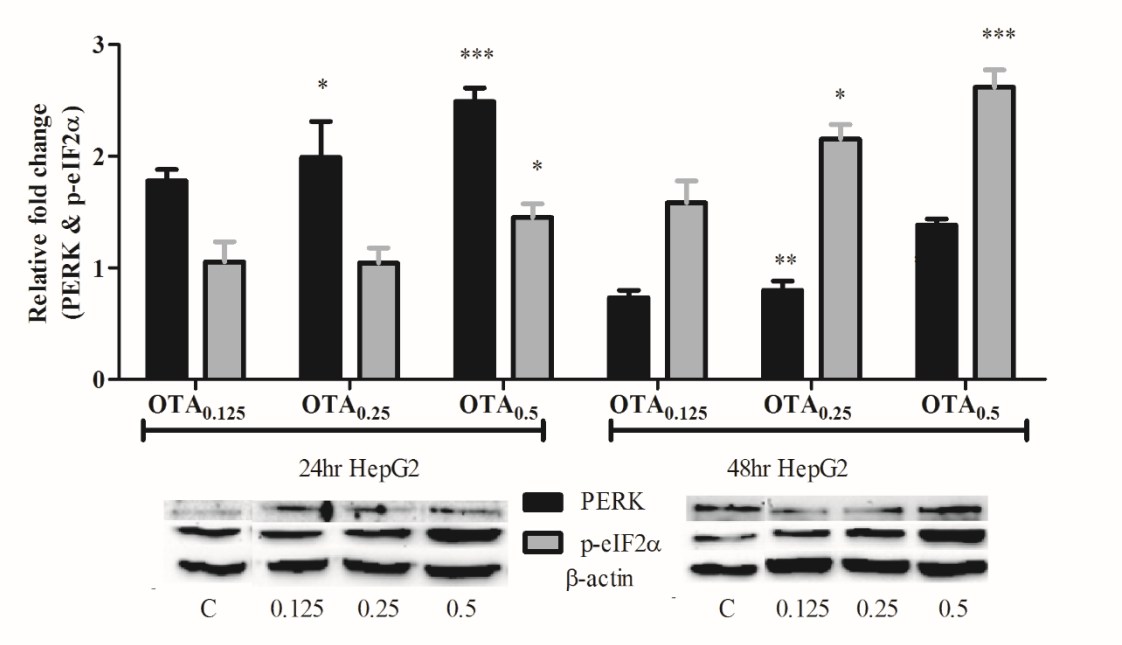

Figure 5.6: Densitometric analysis and western blot images showing relative fold change in PERK and p-eIF2α following HepG2 cell exposure to a range of OTA concentrations over 24hr and 48hr. *p < 0.05, **p < 0.005 and ***p < 0.0005 relative to controls.

The ATF4 axis is stimulated at prolonged and not acute exposure to OTA in HEK293 cells.

Increases in p-eIF2α correlate with *ATF4* increases observed during 48hr exposure to OTA. $OTA_{0.25}$ significantly decreased mRNA levels of *ATF4* and *CHOP* at 24hr (p < 0.0005), whilst $OTA_{0.125}$ significantly decreased *ATF4* expression only (p < 0.0005) in HEK293 cells (Fig 5.7). *ATF4* expression was significantly increased by $OTA_{0.5}$ (3-fold, p < 0.0005), non-significantly by $OTA_{0.25}$ (1.4-fold) and $OTA_{0.125}$ (2.6-fold, Fig 5.7). A decrease in *CHOP* expression suggests the promotion of cell survival.

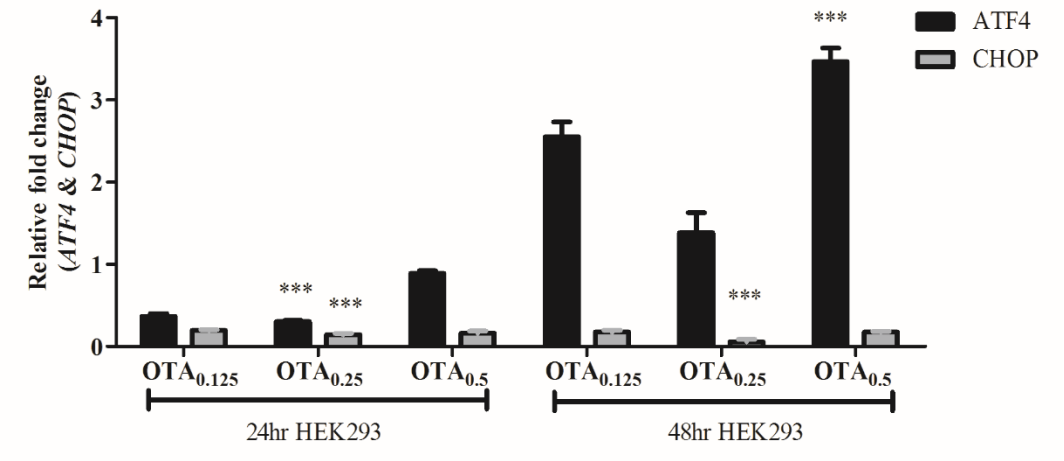

Figure 5.7: mRNA analysis of *ATF4* and *CHOP* in HEK293 cells after treatment with a range of OTA concentrations over 24hr and 48hr. **p < 0.005 and ***p < 0.0005 relative to control.

OTA decreased the activity of Caspases 3/7 and 6 during both 24hr and 48hr exposure in HEK293 cells.

$OTA_{0.125}$ significantly decreased caspase 3/7 activity in HEK293 cells over 24hr ($p < 0.005$; Table 1), however after 48hr $OTA_{0.5}$ also significantly decreased caspase 3/7 and 6 activities ($p < 0.05$ and $p < 0.005$ respectively; Table 1). In addition, $OTA_{0.125}$ significantly decreased caspase 3/7 activity at 48hr ($p < 0.05$).

Table 1: Caspase 3/7 and 6 activities assessed by luminometry in HEK293 cells over 24hr and 48hr post OTA exposure.

24hr HEK293	Caspase 3/7	Caspase 6
Control	100089±22656	230788±20509
$O_{0,125}$	72944±5495**	195533±9598
$O_{0,25}$	104016±1447	240231±25981
$O_{0,5}$	96429±3588	218417±15058
48hr HEK293		
Control	274809±52901	333573±12434
$O_{0,125}$	192683±38448*	293875±37132
$O_{0,25}$	211130±8147	331035±15422
$O_{0,5}$	201828±16593 *	247386±16070**

ATF4 gene expression was increased by the highest OTA concentration at both 24hr and 48hr in HepG2 cells.

$OTA_{0.5}$ induced a significant increase in *ATF4* gene expression at both 24hr (3.9-fold, $p < 0.05$, Fig 5.8) and 48hr (2.6-fold, $p < 0.0005$), whilst $OTA_{0.125}$ significantly decreased *ATF4* expression at 24hr ($p < 0.05$) and significantly increased its expression at 48hr ($p < 0.05$). *CHOP* expression was significantly decreased by $OTA_{0.25}$ ($p < 0.005$) and $OTA_{0.125}$ ($p < 0.0005$) exposure over 24hr (Fig 5.8). These results indicate ATF4-induced cell survival.

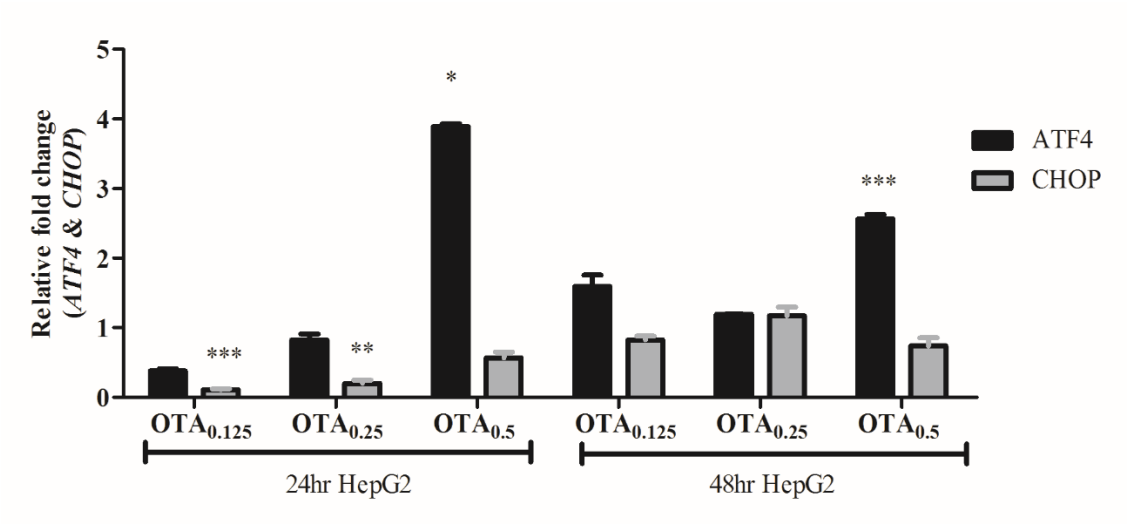

Figure 5.8: Analysis of *ATF4* and *CHOP* mRNA levels in HepG2 cells following treatment with a range of OTA concentration over 24hr and 48hr. *$p < 0.05$ and **$p < 0.005$ and ***$p < 0.0005$ relative to untreated controls.

Executioner caspases were not significantly affected across OTA treatments during 48hr exposure of HepG2 cells.

Caspase 6 activity was significantly increased by $OTA_{0.5}$ exposure at 24hr. All other treatments did not significantly increase activity of executioner caspases.

Table 2: Quantification of caspase 3/7 and 6 activity in OTA treated HepG2 cells at 24hr and 48hr.

24hr HepG2	Caspase 3/7	Caspase 6
Control	72992±1975	83294±3504
$O_{0,125}$	61874±8953	72454±3272
$O_{0,25}$	70711±8294	103603±5990
$O_{0,5}$	123340±6642	123255±12401*
48hr HepG2		
Control	90066±21323	129587±16861
$O_{0,125}$	86861±7270	141528±6195
$O_{0,25}$	112046±14333	151011±33071
$O_{0,5}$	70031±3009	123372±11254

*$p < 0.05$ comparable to controls

Discussion

Ochratoxin A is a frequent contaminant of foods consumed by humans. OTA is one of the most persistent mycotoxins in humans, with a half-life of 35 days and plasma detectability up to 280 days

(Petzinger and Ziegler, 2000), up to 99% of the ingested toxin binds to plasma proteins (Hagelberg et al., 1989). While OTA is best known as a renal toxicant; however, it can also participate in enterohepatic circulation, increasing interaction with hepatocytes and contributing to hepatotoxicity (Roth et al., 1988). Current literature shows induction of ER stress in rat and mouse mesangial cells (Sheu et al., 2016) and in the kidney and spleen of pigs (Gan et al., 2017). This mode of toxicity has yet to be investigated in human stress responses. Using both renal (HEK293) and hepatic (HepG2) *in vitro* models, we investigated the effects of OTA toxicity related to protein damage and ER stress.

Exogenous stress inducers, such as toxins or drugs, often alter protein homeostasis and disrupt the proper folding and transportation of proteins, resulting in protein aggregation and occlusions (Oyadomari and Mori, 2003). LonP1 is a protease that regulates mitochondrial quality control by degrading damaged proteins and maintaining mitochondrial DNA turnover (Quiros et al., 2014). LonP1 is a pro-survival response initiated by ER stress and hypoxia, however, it has also been linked to tumour promotion when overexpressed (Quiros et al., 2014). Heat shock protein (HSP) 70 interacts with LonP1 as a chaperone protein (Kao et al., 2015) and assists in the folding of misfolded proteins, thereby promoting cell survival (Bukau and Horwich, 1998). Together with HSP60, LonP1 and HSP70 provide a mitochondrial response to ER stress.

LonP1 expression was increased at 24hr in human kidney cells (Fig 2), with the most significant increase occurring at the lowest OTA concentration ($OTA_{0.125}$). However, expression of LonP1 was markedly reduced after 48hr, suggesting that OTA induced acute stress to mitochondria (Fig 2). In cancerous liver cells, all OTA concentrations increased expression of LonP1 over 24hr, with an increase observed at 48hr by the lowest concentration ($OTA_{0.125}$; Fig 3). The transient increase in LonP1 expression at 24hr in kidney and liver cells suggests the induction of cellular repair responses. Differences in expression over 48hr could be attributed to the HepG2 cancer cell as opposed to the normal HEK293 cells. Normal kidney cells might respond to the repair processes more effectively than cancerous liver cells. The HepG2 MTT assay also demonstrated that the liver cells were more resistant to OTA-induced cell death; kidney cells proved far more sensitive to OTA toxic effects. For the first time, we report on OTA-induced protein carbonylation in HEK293 cells, with increased concentrations of protein carbonyls at the lower concentrations of OTA over 24hr and 48hr (Fig 4). HEK293 cells appeared more sensitive to protein oxidation than HepG2 cells. The data shows that low OTA concentrations generate higher protein carbonyl concentrations.

During toxic insult or intracellular disruptions, proteins destabilise and revert to non-native forms, these proteins become misfolded and aggregate in the ER, which then activates the ER stress response and the UPR (Bravo et al., 2013). The UPR attempts to restore homeostasis by preventing further accumulation of dysfunctional proteins in the ER lumen. It does this by altering protein synthesis,

increasing protein folding capacity and degrading misfolded proteins that cannot be repaired (Fribley et al., 2009). PERK protein expression levels were increased only during $OTA_{0.5}$ exposure at 24hr, and during $OTA_{0.5}$ and $OTA_{0.125}$ exposure at 48hr in HEK293 cells (Fig 5). In addition, increased phosphorylation of eIF2α was observed, this strongly suggests attenuation of protein synthesis due to misfolded protein accumulation. In liver cells, a similar pattern of protein synthesis inhibition was observed (Fig 6). PERK protein expression increased at 24hr, but decreased at 48hr, however, eIF2α activation increased significantly suggesting that greater levels of PERK had been activated to phosphorylate eIF2α.

Phosphorylation of eIF2α would inhibit global protein synthesis and favour *ATF4* upregulation. Prolonged exposure of kidney cells to OTA increased transcription of *ATF4* (Fig 7), which correlated with increased p-eIF2α over 48hr in kidney cells. All 3 concentrations of OTA decreased *CHOP* gene expression in HEK293 cells during acute and prolonged exposure, implying promotion of cell survival. Contrary to results observed in kidney cells, we see increased *ATF4* expression in liver cells exposed to $OTA_{0.5}$ (24hr and 48hr, Fig 8). Increased *ATF4* expression correlates with observed increases in PERK and p-eIF2α. Furthermore, expression of *CHOP* is decreased in HepG2 cells (24hr), while we observe a slight increase at $OTA_{0.25}$ (48hr, Fig 8). All OTA concentrations decreased caspase 3/7 activity in kidney cells, indicating the stimulation of cell survival efforts (Table 1). In liver cells we observed no change in caspase 3/7 expression (Table 2).

Overall, this data indicates that OTA can induce toxicity by inducing ER stress, modulating UPR pathways, and preventing apoptosis. Feng (2014) shows that the PERK-eIF2α-ATF4 pathway is activated during epithelial-mesenchymal transition (EMT) and this pathway promotes cancer progression (Feng et al., 2014). The research indicates that the PERK pathway could promote malignant behaviour and allow for cell survival (Feng et al., 2014). It is known that OTA induces inflammation (González-Arias et al., 2015; Raghubeer et al., 2017); this suggests that OTA could increase inflammation and ER stress, while promoting EMT and tumourigenesis. Our research corresponds with this theory as ER stress and protein damage was observed, without apoptosis induction.

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CHAPTER 6

Ochratoxin A upregulates acute hypoxia-induced protein response, with increased VEGF and TGFβ expression during prolonged exposure in human kidney and liver cells.

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Abstract

Cellular adaptation to hypoxia is controlled by hypoxia-inducible factor 1α (HIF1α); a transcription factor activated in response to oxygen tension, reactive oxygen species (ROS) and inflammation. Overexpression of HIF1α and HSP90 has been associated with cancer induction. Ochratoxin A (OTA), a mycotoxin often contaminating food and beverages, has been linked to renal tumours and progressive nephropathies, and displays inflammatory and pro-oxidant effects. The aim of our study was to determine the effects of OTA on hypoxic stress response proteins and the resulting metabolic alterations. We evaluated the protein expression of phosphorylated (p)NFκB, pNrf2, HIF1α, HSP90 and PDK1 (western blotting), gene expression of *HIF1α*, *VEGF* and *TGFβ* (qPCR), and ATP production (luminometry) in HEK293 and HepG2 cells exposed to a range of OTA concentrations (0.125μM-0.5μM) at 24hr and 48hr. Acute (24hr) OTA exposure decreased pNrf2 expression in both HEK293 and HepG2 cells, with increased inflammatory proteins in HEK293 cells (48hr) and HepG2 cells (24hr) ($p < 0.05$). OTA-induced hypoxia (HIF1α protein) was recorded at 24hr in kidney and liver cells ($p < 0.005$). *VEGF* and *TGFβ* was significantly increased in kidney cells at 48hr, while only *TGFβ* was increased in HepG2 cells at 48hr. We also observed alterations in PDK1 expression, which correlates with ATP presence. We conclude that OTA induces acute hypoxia and modulates growth signalling (*VEGF*, *TGFβ*), which may lead to tumourigenesis.

Introduction

Ochratoxin A (OTA) is a mycotoxin produced by various species of *Penicillium* and *Aspergillus* fungi (Pfohl-Leszkowicz and Manderville, 2007). It contaminates foods frequently ingested by humans (grains, fruit, wine, coffee), as well as animal feed – thereby contaminating animal products (Malir et al., 2016). A known renal toxicant, OTA has been linked to nephropathies around the world (Castegnaro et al., 2006; Abid et al., 2003). These diseases present as renal dysfunction often progressing into renal failure, and may lead to tumour initiation (Castegnaro et al., 2006). Toxic effects of OTA on the liver and immune system have previously been reported to a lesser extent as that observed in the kidney (Darif et al., 2016; Gayathri et al., 2015). Research has shown the oxidative stress induction following OTA exposure (Arbillaga et al., 2007; El Golli Bennour et al., 2009), as well as inflammation (Bernardini et al., 2014). The effects of OTA on hypoxic response, however, have not been elucidated in human cells. Considering the redox imbalance and inflammatory environment induced by OTA, understanding the biochemical response to hypoxia would provide important information on OTA toxicity and its possible role in tumourigenesis.

The tumour microenvironment contains areas of hypoxia, low pH levels, and nutrient deprivation (Vaupel, 2004). Hypoxia is characteristic of cancer cells, O_2 deficiency provides a highly mutagenic environment and allows for genetic instability (Yuan and Glazer, 1998). Cellular hypoxic response is regulated by hypoxia inducing factor 1 (HIF1α) – a heterodimer consisting of two subunits, HIF1α and HIF1β (Wang et al., 1995a). The cytoplasmic HIF1α is dependent on cellular oxygen (O_2) tension, while nuclear HIF1β is O_2 independent (Semenza, 2001a). In well-oxygenated cells O_2-dependent enzymes hydroxylate prolyl residues on HIF1α, allowing the binding of von Hippel-Lindau protein (pVHL), inducing proteasomal degradation of HIF1α (Vaupel, 2004). In hypoxic cells HIF1α accumulates and translocates to the nucleus, where it binds with HIF1β to transcribe genes relating to the hypoxic response, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF) and glycolytic enzymes (Vaupel, 2004). HIF1α stabilisation not only depends on O_2 tension, it can also be influenced by ROS (Chun et al., 2002) – a product of OTA toxicity. Research has shown that HIF1 activity is not only induced in hypoxic cancers; HIF1 protein levels are often increased in some cancers without measurable hypoxia (Kuschel et al., 2012).

Heat shock protein (HSP) 90, a chaperone protein, has been found to modulate the expression of HIF1α, as well as stabilise or degrade damaged proteins (Buchner, 1999). HSP90 can stabilise mutant proteins, alter growth signals and induce VEGF expression (Fontana et al., 2002), enabling a pro-survival phenotype. HIF1α modulates the expression of pyruvate dehydrogenase kinase 1 (PDK1), which inactivates pyruvate dehydrogenase (PDH) – an enzyme that converts pyruvate into acetyl-CoA (used in the Krebs cycle for ATP production) (Liu and Yin, 2017). This process, called the "Pasteur Effect", promotes cytosolic lactate formation and reduces pyruvate oxidation (Prigione et al., 2014). Together,

HIF1α and PDK1 induce a glycolytic shift. In this way OTA would influence metabolic efficiency (ATP production) and ROS generation.

Nuclear factor kappa light-chain-enhancer of activated B-cells (NFκB) phosphorylation leads to its translocation to the nucleus and transcription of genes relating to the inflammatory response and cell cycle, such as growth factors, apoptosis regulators and stress response genes (Pahl, 1999). Recent research has shown OTA induction of inflammation via NFκB activation (Raghubeer et al., 2017). An inflammatory environment can heighten the effects of transforming growth factor beta (TGFβ) – a cytokine with angiogenic, apoptotic and proliferative abilities – leading to fibrotic or malignant conditions (Massagué et al., 2000). TGFβ can be activated by oxidative stress and inflammation, and its overexpression has been reported in several human cancers (dos Reis et al., 2011; Higashi et al., 2001). There are six hallmarks of cancer, they are defined as the ability to continuously proliferate, bypass apoptosis, evade the immune system, ignore growth suppressive signals, induce angiogenesis and foster metastatic characteristics (Hanahan and Weinberg, 2011). TGFβ and VEGF represent hallmarks of the cancer phenotype. Therefore, OTA-induced reactive oxygen species (ROS) and inflammation could promote transformation and carcinogenesis. NFκB has also been reported to activate HIF1α in conjunction with ROS (Bonello et al., 2007). Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcription factor responsible for the response to redox imbalance. Phosphorylation of Nrf2 allows it to translocate to the nucleus, bind to the antioxidant response element (ARE) in the promoter regions of antioxidant enzyme genes, and transcribe genes functional in neutralising ROS and restoring homeostasis (Bryan et al., 2013). OTA has been widely reported to decrease Nrf2 activity, thus dampening cellular response to ROS, rendering the cell vulnerable to oxidative damage (Boesch-Saadatmandi et al., 2008; Raghubeer et al., 2015b).

Previous studies showed that OTA induced oxidative stress at IC_{50} concentrations (24hr: 1.5µM, 48hr: 9.4µM) (Raghubeer et al., 2015b) and inflammation at a sub-IC_{50} concentration (0.5µM) in kidney cells (Raghubeer et al., 2017). The lack of information on OTA-hypoxia research relating to inflammation and oxidative stress in human cells prompted our investigation of these pathways. This study employed a range concentrations based on plasma levels reported in regions with high OTA exposure (Koszegi and Poor, 2016). We exposed human embryonic kidney (HEK293) and hepatocellular carcinoma (HepG2) cells to three different OTA concentrations (0.125µM, 0.25µM and 0.5µM) over 24 hours (hr) and 48hr, thereafter, we examined the effects on biomarkers of inflammation, oxidative stress, and hypoxia response.

Materials

Vendor authenticated human embryonic kidney (HEK293) and liver hepatocellular carcinoma (HepG2) cells were purchased from Highveld Biologicals (Johannesburg, South Africa). Dulbeccos' minimum essential media (DMEM), Eagle's minimum essential media (EMEM), HEPES buffer and trypsin was purchased from Lonza (Basel, Switzerland). Ochratoxin A (O1877) was sourced from Sigma-Aldrich (Missouri, USA). Solvents and salts were purchased from Merck (Darmstadt, Germany). All quantitative PCR (qPCR) consumables and reagents were obtained from Bio-Rad (Hercules, USA) with primer sequences synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa).

Methods

Cell Culture

HEK293 cells and HepG2 cells were cultured in 25cm^3 flasks, in monolayer (10^6 cells per flask), using DMEM and EMEM respectively (both supplemented with 10% foetal calf serum (FCS), 1% Penstrepfungizone, HEPES buffer and 1% L-glutamine). The cells were cultured in a humidified incubator (37°C, 5% CO_2). At 80% confluence, cells were exposed to OTA (0.125µM; 0.25 µM; 0.5µM) for 24hr and 48hr. Following treatment, cells were detached by incubation with trypsin (5min; 37°C), counted (Invitrogen Countess™ automated cell counter), and adjusted for subsequent assays. Experiments were performed under non-hypoxic conditions.

Ochratoxin A Treatments

A 5mM stock solution of OTA was prepared in 60% dimethyl sulphoxide (DMSO). Concentrations used for treatment were based on high levels recorded in serum and urine (Jonsyn-Ellis, 2001; Wafa et al., 1998). The OTA range was delivered in concentrations – $O_{0.5}$: 0.5µM, $O_{0.25}$: 0.25µM and $O_{0.125}$: 0.125µM.

Luminometric assessment of Adenosine Triphosphate (ATP)

The CellTiter-Glo® Luminescent Cell Viability assay (G7570, Promega, Madison, USA) was used, as per the manufacturer's protocol, to measure ATP in cells exposed to OTA. Briefly, 20, 000 cells (in 0.1M PBS) were seeded in an opaque microtitre plate (in triplicate). The detection reagent was added to each well (20µL), plates were incubated (room temperature (RT), dark, 1hr), and read on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA). Results were reported as relative light units (RLU), directly proportional to ATP content.

Quantification of Protein Expression

Protein isolation

Cytobuster™ (Novagen, San Diego, USA), with supplementary phosphatase (004906837001) and protease (05892791001) inhibitors (Roche, Basel, Switzerland), was used to isolate protein from HEK293 and HepG2 cells as previously described (Ghazi et al., 2017). The Bicinchoninic acid (BCA) assay was used to quantify crude protein, thereafter samples were standardised to 1mg/ml and boiled (5min) in Laemmli buffer (0.5M Tris-HCl, 1% bromophenol blue, glycerol, 10% sodium dodecyl sulphate (SDS), β-mercaptoethanol and dH_2O).

Sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Protein samples were separated on SDS polyacrylamide gel (stacking: 4%; resolving: 7.5%) at 150V (1hr) and electro-transferred to nitrocellulose membranes using the pre-programmed Trans-Blot® Turbo™ system (Bio-Rad). Membranes were blocked in 5% BSA in Tween20 Tris-buffered saline (TTBS: 25mM Tris, 150mM NaCl, 0.05% Tween20) on a shaker (1hr, RT) prior to immune-probing with primary antibodies (1:1000) at 4°C overnight – anti-PDK1 (ab207450) and anti-phosphorylated (p)Nrf2 (ser40, ab76026) from Abcam (Cambridge, UK), anti-pNFκB (ser536, 3033T), anti-NFκB (8242T), and anti-Nrf2 (12721S) from Cell Signaling Technology (Massachusetts, USA), anti-HIF1α (PLA0081) from Sigma-Aldrich (Missouri, USA), and anti-HSP90 (610418) from BD Biosciences (San Jose, CA, USA). Membranes were washed post-primary antibody incubation (TTBS, 4 x 10min), probed with horse-radish peroxidase (HRP)-conjugated anti-mouse (7076S) or HRP-conjugated anti-rabbit (7074P2) secondary antibodies (5% BSA, 1hr, RT) and washed post-secondary antibody incubation (4 x 10min). Clarity™ Western ECL chemiluminescent substrate (Bio-Rad) was added to each membrane and images were captured using the ChemiDoc™ XRS+ Imaging system (Bio-Rad). Membranes were quenched using hydrogen peroxide (37°C; 30min), blocked, (5% BSA in TTBS, 1hr, RT), washed twice, and probed with HRP-conjugated anti-β actin (Sigma-Aldrich). Image Lab software (version 5.1) was used for densitometry analysis, and quantified as relative band intensity (RBI) converted to fold change. All results were normalized against β-actin prior to statistical analysis.

Messenger RNA (mRNA) quantification of hypoxic response

RNA Isolation and cDNA Synthesis

An in-house protocol using QIAzol extraction reagent (Qiagen, Hilden, Germany) was employed to isolate RNA (Pillay et al., 2015). Isolated RNA was quantified using the NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and standardised to 1000ng/µL. cDNA was synthesised using iScript cDNA synthesis kit (Bio-Rad) as per manufacturer's protocol. Thermocycler conditions were 25°C (5min), 42°C (30min), 85°C (5min) and a final hold of 4°C.

Quantitative Polymerase Chain Reaction (qPCR)

Transcript levels of *HIF1α* (sense: 5′-GAACGTCGAAAAGAAAAGTCTCG-3′, antisense: 5′-CCTTATCAAGATGCGAACTCACA-3′), *VEGF* (sense: 5′-AGGGCAGAATCATCACGAAGT-3′, antisense: 5′-AGGGTCTCGATTGGATGGCA-3′), and *TGFβ1* (sense: 5′-CTAATGGTGGAAACCCACAACG-3′, antisense: 5′-TATCGCCAGGAATTGTTGCTG-3′), were evaluated by qPCR. A reaction mix of 5.5µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 1µL cDNA, 1µL sense and antisense primers, and nuclease-free H_2O was made up to 10µL. Three independent experiments were carried out using six replicates per treatment with glyceraldehyde 3-phosphate dehydrogenase *GAPDH* (sense: 5'-TCCACCACCCTGTTGCTGTA-3', antisense: 5'-ACCACAGTCCATGCCATCAC-3') and β-actin (sense: 5'-TGACGGGTCACCCACACTGTGCCCAT-3', antisense: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3') as housekeeping genes. Reactions were performed on the CFX Touch™ Real Time PCR detection system (Bio-Rad) as follows: Initial denaturation (95°C, 4min), denaturation (37 cycles, 95°C, 15s), annealing (*HIF1α, VEGF* and *TGFβ* – 55°C, 40sec) and extension (72°C, 30sec). Analysis was performed using the method described by Livak and Schmittgen, where $2^{-\Delta\Delta Ct}$ represents fold change relative to housekeeping gene (Livak and Schmittgen, 2001).

Statistical Analysis

GraphPad Prism version 5.0 (GraphPad software, San Diego, CA) was used for all analysis, employing one-way analysis of variance (ANOVA) tests. Data is expressed as mean ± standard deviation with statistical significance considered at $p < 0.05$.

Results

Previous work showed that OTA induced oxidative stress and inflammation in human kidney cells (Raghubeer et al., 2017; Raghubeer et al., 2015b). The current study investigated the effect of OTA on cellular hypoxia and related signalling in HEK293 and HepG2 cells exposed to OTA.

Protein expression of pNFκB and pNrf2 is decreased at 24hr, but increased at 48hr in HEK293 cells, suggesting acute oxidative stress with OTA-induced inflammation at prolonged exposure periods.

Phosphorylation of both NFκB and Nrf2 was downregulated at 24hr by OTA in HEK293 cells (Fig 6.1), with significant decreases in pNFκB and pNrf2 observed in $O_{0.25}$ compared to controls (0.36 ± 0.11 vs 0.18 ± 0.03RBI, $p = 0.0018$ and 17.12 ± 2.06 vs 5.23 ± 0.66RBI, $p < 0.05$ respectively). pNrf2 was also significantly decreased at $O_{0.125}$ (24hr; 2.99 ± 0.37RBI, $p < 0.0001$). This trend was reversed during 48hr exposure with significant upregulation of pNFκB at $O_{0.25}$ (0.34 ± 0.03RBI, $p < 0.005$) and $O_{0.125}$ (0.36 ± 0.13RBI, $p = 0.0003$) compared to controls (0.24 ± 0.03RBI; Fig 6.1). pNrf2 is significantly

increased at the same OTA concentrations – $O_{0.25}$ (9.14 ± 1.69RBI, $p < 0.05$) and $O_{0.125}$ (12.71 ± 1.55RBI, $p = 0.0001$).

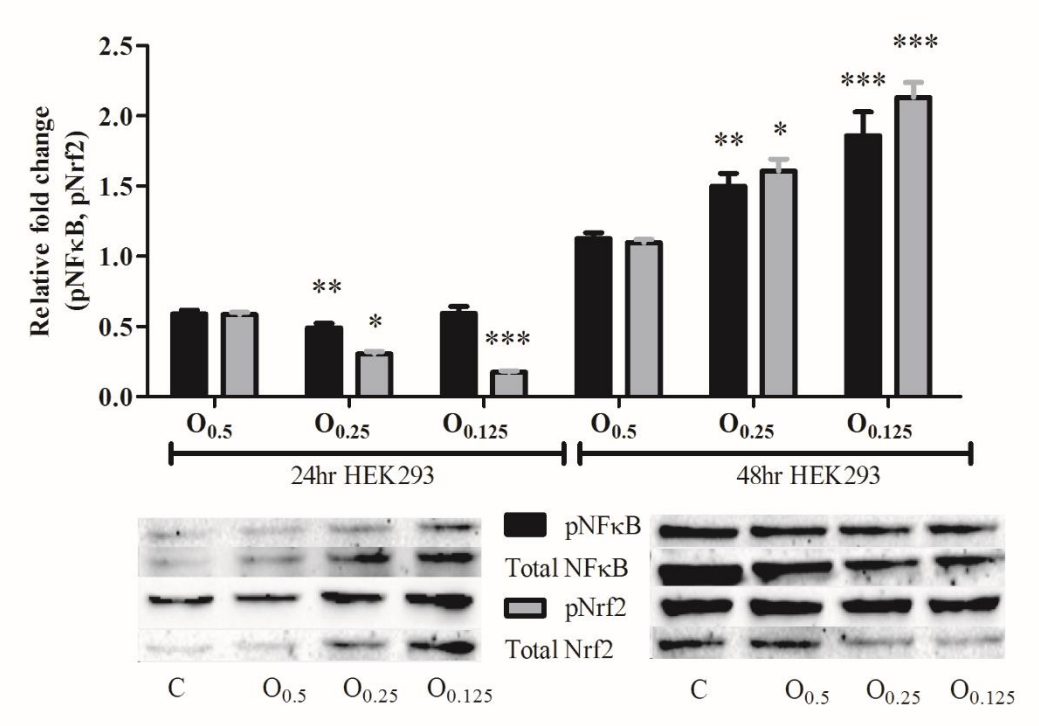

Figure 6.1: Densitometric analysis and western blot images showing relative fold change in pNFκB and pNrf2 in HEK293 cells over 24hr and 48hr exposure to OTA – $O_{0.5}$ (0.5µM), $O_{0.25}$ (0.25µM) and $O_{0.125}$ (0.125µM). *$p < 0.05$, **$p < 0.005$ and ***$p < 0.0005$ relative to untreated control.

OTA induces inflammation at 24hr by increasing phosphorylation of NFκB in liver cells, while decreasing activation of Nrf2. Expression of pNFκB is decreased at $O_{0.5}$ exposure and pNrf2 expression is decreased at $O_{0.125}$ exposure in HepG2 cells over 48hr.

Significant increases in pNFκB were recorded at $O_{0.5}$ (0.45 ± 0.09RBI, $p < 0.05$) and $O_{0.125}$ (0.39 ± 0.10RBI, $p = 0.0023$) at 24hr compared to controls in HepG2 cells (0.27 ± 0.04RBI) (Fig 6.2). Phosphorylation of NFκB was decreased at 48hr during $O_{0.5}$ exposure (1.27 ± 0.08 vs 0.86 ± 0.14RBI, $p < 0.05$). Contrary to NFκB phosphorylation, we observed decreased expression of pNrf2 across treatments in HepG2 cells (Fig 6.2). Significant pNrf2 decreases were observed at $O_{0.25}$ (8.99 ± 3.09RBI, $p < 0.05$) and $O_{0.125}$ (7.6 ± 2.63RBI, $p = 0.0013$) compared to controls (18.72 ± 3.9RBI) at 24hr, and at $O_{0.125}$ (13.98 ± 0.94 vs 7.19 ± 1.13RBI, $p < 0.05$) at 48hr in HepG2 cells. Activation of transcription factors relating to oxidative stress and inflammation led to interest in the expression of proteins involved in hypoxia responses.

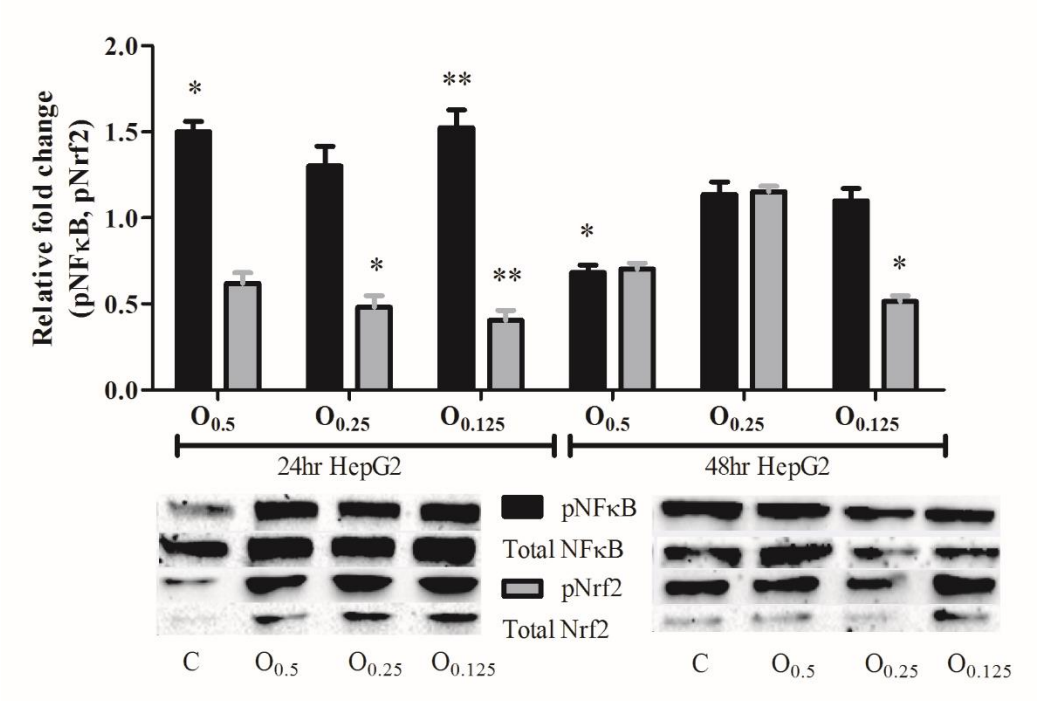

Figure 6.2: Densitometric analysis and western blot images showing relative fold change in pNFκB and pNrf2 in HepG2 cells over 24hr and 48hr exposure to OTA. *$p < 0.05$ and **$p < 0.005$ relative to untreated control.

OTA upregulates hypoxic responses in kidney cells at 24hr, but decreases these responses at 48hr exposure.

The protein expression of HIF1α was upregulated 3.25-fold at the lowest OTA concentration ($O_{0.125}$) at 24hr exposure (0.11 ± 0.01 vs 0.35 ± 0.04RBI, $p < 0.0001$), however, this expression is dampened at 48hr (Fig 6.3). HIF1α expression was decreased significantly at $O_{0.25}$ (0.26 ± 0.07RBI, $p < 0.005$) and $O_{0.125}$ (0.27 ± 0.12RBI, $p = 0.0002$) compared to controls (1.01 ± 0.04RBI) at 48hr in HEK293 cells. Expression of the chaperone protein, HSP90, was significantly increased across all OTA treatments at 24hr ($p = 0.0044$) compared to controls (0.83 ± 0.14RBI) – $O_{0.5}$ (1.02 ± 0.15RBI), $O_{0.25}$ (1.07 ± 0.13RBI) and $O_{0.125}$ (1.05 ± 0.2RBI). HSP90 was also significantly increased at $O_{0.125}$ during 48hr in HEK293 cells (1.05 ± 0.11 vs 1.5 ± 0.2RBI, $p = 0.0037$). HIF1α and PDK1 function in tandem to modulate energy usage and ATP production during hypoxic stress. Expression of PDK1 was significantly increased at $O_{0.5}$ (0.75 ± 0.08RBI) and $O_{0.25}$ (0.76 ± 0.09RBI) compared to controls (0.44 ± 0.09RBI) during 24hr ($p = 0.0018$), however, PDK1 expression was decreased at 48hr OTA treatments with a significant decline observed at $O_{0.25}$ compared to controls (0.78 ± 0.13 vs 0.6 ± 0.08RBI, $p = 0.022$).

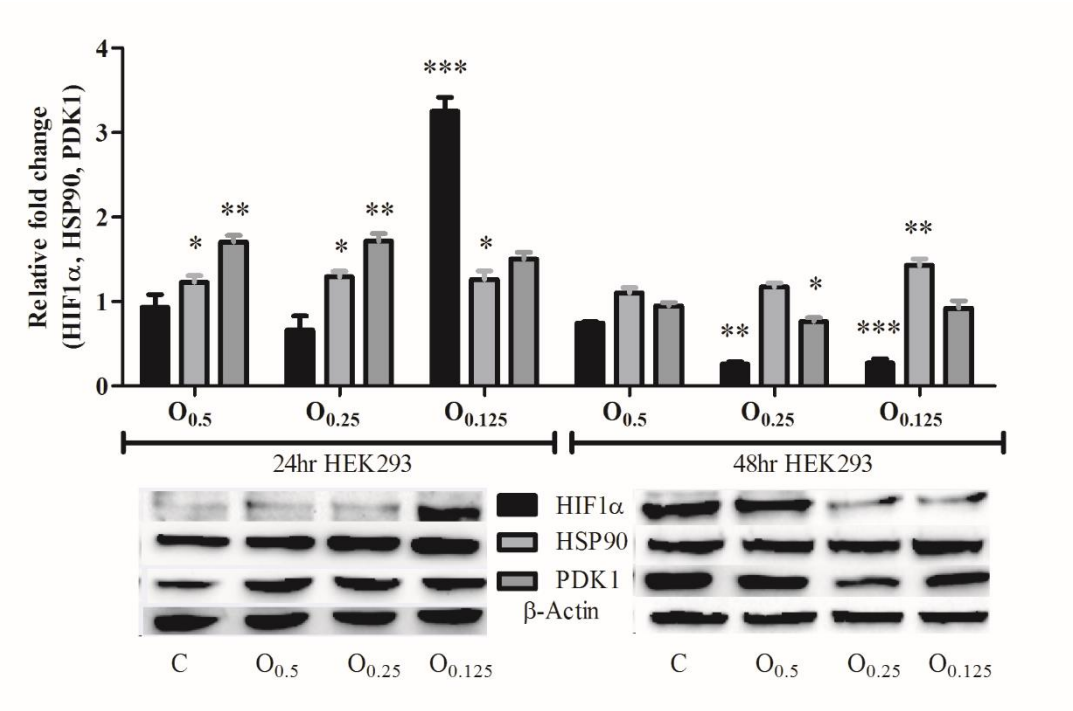

Figure 6.3: Densitometric analysis and western blot images showing relative fold change in proteins related to hypoxic response. Expression of HIF1α, HSP90 and PDK1 in HEK293 cells over 24hr and 48hr exposure to OTA. *$p < 0.05$, **$p < 0.005$ and ***$p < 0.0005$ relative to untreated control.

Liver cells exhibit increased protein expression of HIF1α and HSP90 at 24hr during $O_{0.125}$ exposure, with decreased overall hypoxic responses at 48hr.

Hypoxic stabilisation and accumulation of HIF1α was observed during $O_{0.125}$ exposure to HepG2 cells at 24hr (0.40 ± 0.05 vs 0.72 ± 0.11RBI, $p = 0.0012$), this was accompanied with a non-significant increase in HSP90 expression (Fig 6.4). Reduced protein expression of PDK1 was noted at $O_{0.25}$ (24hr; 0.11 ± 0.02 vs 0.07 ± 0.01RBI, $p = 0.0017$) and at $O_{0.125}$ (48hr; 0.16 ± 0.01 vs 0.09 ± 0.02RBI, $p < 0.05$) exposure. We then investigated the gene expression of *HIF1α* as well as the downstream targets.

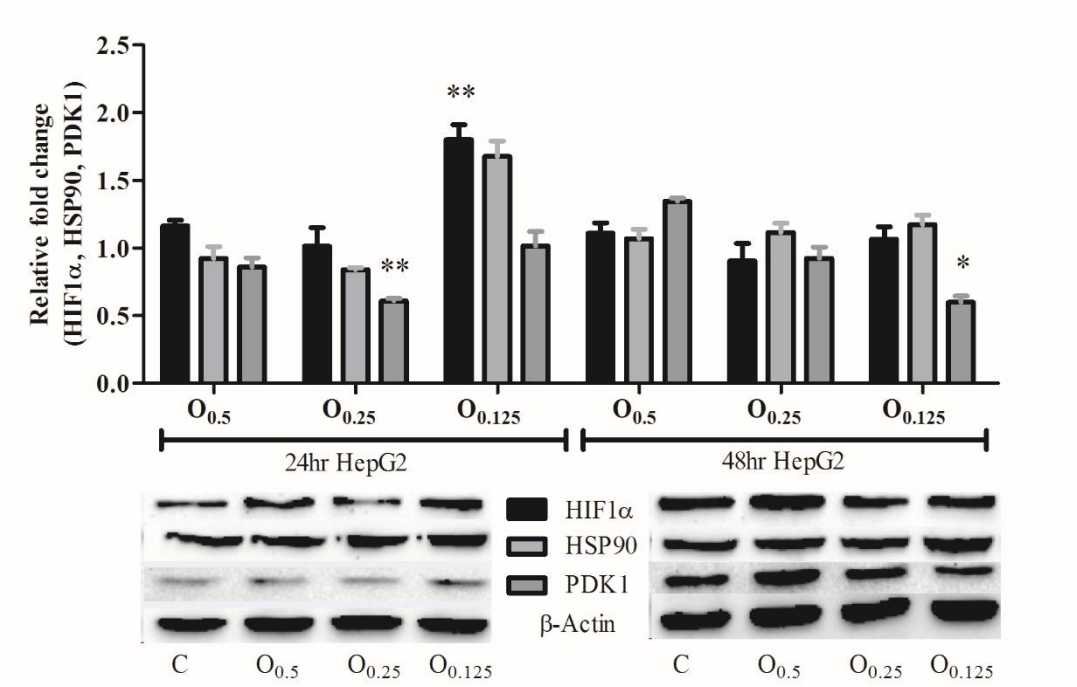

Figure 6.4: Densitometric analysis and western blot images showing proteins related to hypoxic response. Relative fold change of HIF1α, HSP90 and PDK1 in HepG2 cells over 24hr and 48hr exposure to OTA. *$p < 0.05$ and **$p < 0.005$ relative to controls.

Gene expression of HIF1α is decreased at 24hr and 48hr in HEK293 cells. VEGF and TGFβ is decreased in HEK293 cells at 24hr, while increased at 48hr.

The mRNA levels of *HIF1α* do not correlate with the protein expression at $O_{0.125}$ at 24hr in HEK293 cells, as a decreased gene expression of *HIF1α* ($p < 0.05$) was noted. Decreased *HIF1α* mRNA at $O_{0.25}$ (Fig 6.5) in kidney cells (48hr) correlates with decreased HIF1α protein expression (Fig 6.3). Gene expression of *VEGF* was significantly decreased at $O_{0.5}$ ($p < 0.05$) and $O_{0.25}$ ($p = 0.0005$) during 24hr exposure, however, the opposite was observed at 48hr with a 2.7-fold increase in *VEGF* mRNA content at both $O_{0.5}$ ($p = 0.0002$) and $O_{0.125}$ ($p < 0.005$) in kidney cells (Fig 6.5). A similar pattern was observed with *TGFβ* mRNA levels – significant decreases were recorded at $O_{0.5}$ ($p < 0.05$) and $O_{0.25}$ ($p = 0.0003$), while 48hr exposure to $O_{0.125}$ yielded a 2-fold increase in *TGFβ* expression ($p < 0.0001$) in HEK293 cells (Fig 6.5).

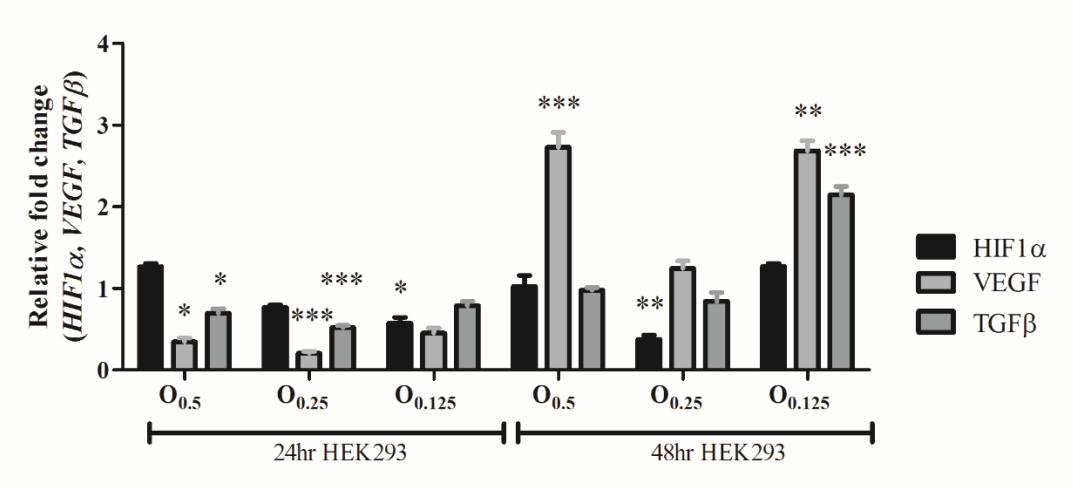

Figure 6.5: mRNA analysis of *HIF1α, VEGF and TGFβ* in HEK293 cells after treatment with a range of OTA concentrations over 24hr and 48hr. *$p < 0.05$, **$p < 0.005$ and ***$p < 0.0005$ relative to control.

HIF1α gene expression was downregulated at 24hr and 48hr in liver cells. VEGF was upregulated at 24hr only, with TGFβ decreased at 24hr and increased at 48hr post OTA-exposure in HepG2 cells. Gene expression of *HIF1α* was significantly decreased at $O_{0.25}$ ($p < 0.05$) and $O_{0.125}$ ($p < 0.0001$) in HepG2 cells (24hr); expression was also decreased at 48hr by $O_{0.5}$ ($p < 0.05$) and $O_{0.25}$ ($p = 0.0005$) (Fig 6.6). As previously observed in HEK293 cells, the mRNA and protein expressions of HIF1α at $O_{0.125}$ does not correlate. mRNA transcripts of *VEGF* significantly increased at 24hr during $O_{0.5}$ and $O_{0.25}$ exposure ($p = 0.0004$ and $p < 0.05$ respectively). Gene expression of *TGFβ* was significantly decreased at $O_{0.25}$ ($p < 0.05$) during 24hr exposure, however, the opposite was observed during 48hr exposure of HepG2 cells to $O_{0.25}$ – *TGFβ* expression increased 4.7-fold ($p = 0.0002$) (Fig 6.6).

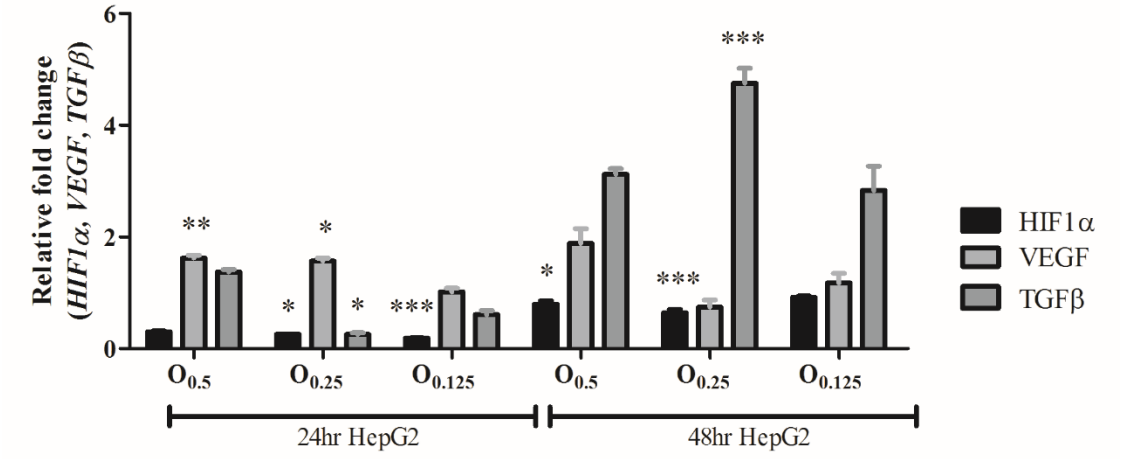

Figure 6.6: Analysis of *HIF1α, VEGF and TGFβ* mRNA levels in HepG2 cells following treatment with a range of OTA concentrations over 24hr and 48hr. *$p < 0.05$ and **$p < 0.005$ and ***$p < 0.0005$ relative to untreated controls.

ATP production was increased at 24hr and 48hr in both HEK293 and HepG2 cells exposed to a range of OTA concentrations.

Cellular ATP content was measured to determine the energy status of hypoxic cells, and the influence of PDK1 on its production. ATP was significantly increased by $O_{0.125}$ during 24hr ($p < 0.0001$) and $O_{0.25}$ during 48hr exposure ($p = 0.0002$) in HEK293 cells. The presence of ATP was significantly increased during 24hr exposure to $O_{0.5}$ ($p < 0.0001$) and $O_{0.25}$ ($p < 0.05$), as well as 48hr exposure to $O_{0.125}$ ($p = 0.0023$) in HepG2 cells. Decreases recorded in PDK1 expression correlate with increased ATP levels.

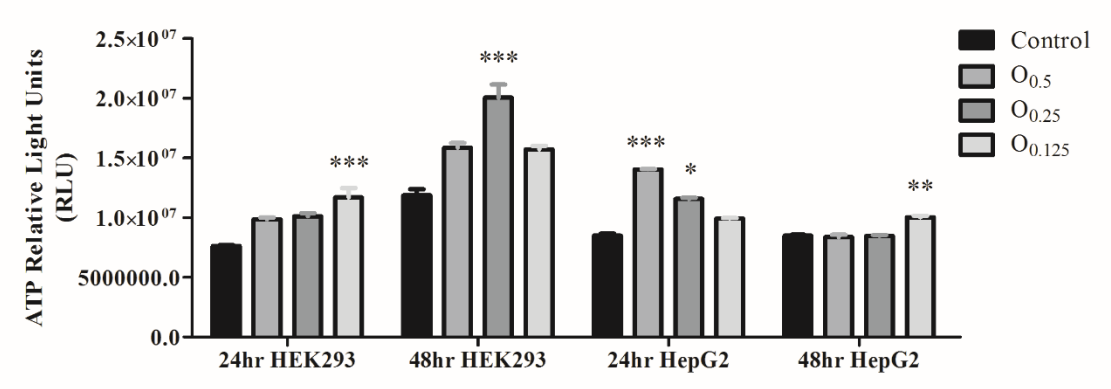

Figure 6.7: Luminometric quantification of ATP in HEK293 and HepG2 cells over 24hr and 48hr post-exposure to a range of OTA concentrations. *$p < 0.05$ and **$p < 0.005$ compared to control measurements.

Discussion

In general, a hypoxic environment is often characteristic of a cancer phenotype (Eales et al., 2016). These conditions can promote mutagenesis and cell survival; it is therefore fundamental to determine hypoxic effects of mycotoxins in relation to carcinogenesis. OTA targets the kidney and liver, and has been linked to immune dysfunction and cancer initiation (Castegnaro et al., 2006). It is well documented that OTA induces oxidative stress and modulates cellular antioxidant responses, however, there is a dearth of research regarding its hypoxia-inducing abilities in human kidney and liver cells. Stachurska et al. (2011) demonstrated differential regulation of hypoxia inducing factors (HIFs) and VEGF in porcine kidney cells (Stachurska et al., 2011), this research prompted an investigation into the effects of plausible plasma range concentrations of OTA on human kidney and liver cells. Using renal (HEK293) and hepatic (HepG2) *in vitro* models, we investigated the biochemical responses to OTA toxicity related to inflammation and hypoxia.

OTA disrupts the antioxidant response by downregulating Nrf2 activity, thus dampening the transcription of antioxidant enzymes (Boesch-Saadatmandi et al., 2008; Ramyaa and Padma, 2013). At 24hr exposure to a range of OTA concentrations, we observed significantly decreased pNrf2 in HEK293 cells at $O_{0.25}$ and $O_{0.125}$, indicating a decreased antioxidant response. At 48hr, significantly higher levels

of pNrf2 was recorded in $O_{0.25}$ and $O_{0.125}$, coupled with increased phosphorylation of NFκB, a transcription factor responsible for inflammatory induction (Fig 6.1). This suggests OTA induces acute oxidative stress without inflammation, but over prolonged exposure the toxin induces an inflammatory environment while cellular antioxidant responses increase.

In the liver, a different trend is observed. OTA induces inflammation during acute exposure to $O_{0.5}$ and $O_{0.125}$, with similar dampening of Nrf2 activity at $O_{0.25}$ and $O_{0.125}$ (Fig 6.2). The inflammatory environment is not sustained at 48hr, and pNrf2 does not increase in HEK293 cells, but significantly decreased during $O_{0.125}$ exposure in liver cells. This indicates a compromised antioxidant response. In both cell lines we observe inflammatory activation with increased oxidative stress, these conditions allow for genomic instability and cell cycle disruptions.

HIF1α stabilisation occurs during periods of low oxygen, allowing transcription of genes that would increase oxygen delivery (EPO) and angiogenesis (VEGF) (Vaupel, 2004). HIF1α functions closely with HSP90, which aids in stabilising protein conformation, apoptosis signalling, and plays a role in VEGF induction (Calderwood et al., 2006). Significantly increased expression of HIF1α was recorded at the lowest OTA concentration ($O_{0.125}$), with increased HSP90 at all three OTA concentrations ($O_{0.5}$, $O_{0.25}$, $O_{0.125}$) at 24hr in kidney cells (Fig 6.3). Chandel et al (1998) demonstrated that increased ROS further stabilises and activates HIF1α (Chandel et al., 1998). This can be seen in the downregulation of pNrf2 at $O_{0.125}$ in HEK293 cells at 24hr (Fig 6.1), with increased HIF1α expression (Fig 6.3), as well as decreased pNrf2 in HepG2 cells (Fig 6.2) accompanied with increased HIF1α (Fig 6.4). At 48hr, HIF1α protein expression was significantly decreased, with increased HSP90 at $O_{0.125}$. HSP90 also participates in oncogenic processes as it can alter growth receptor activity and angiogenesis; these capabilities allow it to promote proliferation of mutated cells and induce angiogenesis, as such HSP90 is often overexpressed in tumours (Whitesell and Lindquist, 2005). Overexpression of HSP90 and HIF1α has been linked to carcinogenic tendencies by promoting cancer cell survival, proliferation and adaptation to hostile environments (Graeber et al., 1996). Yasuda et al. (2004) showed that HIF1α can upregulate VEGF and hexokinase expression in hepatocellular carcinoma (HCC), thereby activating a metabolic switch in cancer cells (Yasuda et al., 2004). Increased levels of HIF1α and VEGF have been reported in metastatic breast cancer (Kallergi et al., 2009).

PDK1 alters the mechanism of energy production; it prevents the conversion of pyruvate into acetyl-CoA and instead promotes lactate formation in the cytosol (Liu and Yin, 2017). In HEK293 cells, we observed significantly increased PDK1 expression at $O_{0.5}$ and $O_{0.25}$ (24hr; Fig 6.3), but significantly decreased PDK1 at $O_{0.25}$ (48hr). The expression of PDK1 correlates with ATP production in HEK293 cells; we see lower ATP levels at 24hr and greater levels at 48hr (Fig 6.7), indicating that increased PDK1 resulted in lower ATP production.

The mRNA levels of *HIF1α* did not correlate with protein expression at $O_{0.125}$ (24hr) in HEK293 cells; we observed significantly decreased gene expression (Fig 6.5), but significantly increased protein expression (Fig 6.3). It is relevant to note that HSP90 was also upregulated during 24hr at $O_{0.125}$. This discrepancy in protein expression could be due to post-translational modifications or HSP90 stabilising HIF1α protein. HIF1α expression can be affected by several post-translational mechanisms – acetylation, hydroxylation and phosphorylation (Kuschel et al., 2012). At 48hr HIF1α gene expression was significantly decreased at $O_{0.25}$ (Fig 6.5) which corresponds with the observed protein expression.

A main target of HIF1α is VEGF, a potent inducer of angiogenesis. The gene expression of *VEGF* was decreased across all three OTA treatments at 24hr in kidney cells, with significance reported in $O_{0.5}$ and $O_{0.25}$ (Fig 6.5). This was coupled with significant decreases in *TGFβ* expression at $O_{0.5}$ and $O_{0.25}$. The opposite trend was observed at 48hr in kidney cells – a 2.7-fold increase in *VEGF* was recorded at $O_{0.5}$ and $O_{0.125}$, with a 2-fold increase in *TGFβ* during $O_{0.125}$ exposure. *VEGF* and *TGFβ* levels indicate a robust increase in gene expression during prolonged exposure to OTA as opposed to acute exposure periods. This scenario is very likely to occur as reabsorption coupled with the extended half-life of OTA results in toxin persistence in the kidney (Ringot et al., 2006).

Protein expression of HIF1α, PDK1 and HSP90 did not vary as significantly in liver cells as compared to kidney cells. The HepG2 cell line represents an *in vitro* liver model, however it is a cancerous cell line, and thus behaves differently to normal cells (HEK293). The kidney is more sensitive to effects of OTA, and the toxin would therefore induce a more robust effect in HEK293 than HepG2 cells. The kidney and liver exhibit vastly different metabolic activities, which could be another reason for differences between cell lines. PDK1 expression was significantly decreased during $O_{0.25}$ exposure and HIF1α was significantly increased (1.8-fold) during $O_{0.125}$ exposure at 24hr in HepG2 cells (Fig 6.4). At 48hr the only significant observed change was a decrease in PDK1 expression at $O_{0.125}$ in HepG2 cells (Fig 6.4). The mRNA transcript of *HIF1α* in HepG2 cells exhibited a similar trend to that seen in HEK293 cells. *HIF1α* was significantly decreased at $O_{0.25}$ and $O_{0.125}$ at 24hr, and at $O_{0.5}$ and $O_{0.25}$ at 48hr exposure in HepG2 cells (Fig 6.6). The mRNA expression conflicts with protein expression of HIF1α observed at $O_{0.125}$ (24hr). HSP90 protein was increased, and could possibly affect HIF1α stability, or post-translational modifications could have altered the protein expression.

Interestingly, the gene expression of *VEGF* was significantly increased at $O_{0.5}$ and $O_{0.25}$ at 24hr, with a non-significant increase (1.8-fold) at $O_{0.5}$ during 48hr in HepG2 cells (Fig 6.6). Suggesting acute induction of *VEGF*, but a less potent response during prolonged exposure to OTA. The expression of *TGFβ* was increased across all three OTA concentrations – $O_{0.5}$ (3-fold), $O_{0.125}$ (2.8-fold), with a significant increase at $O_{0.25}$ (4.7-fold) (Fig 6.6). This data indicates that prolonged exposure of OTA to liver cells could disrupt normal growth signals, possibly contributing to malignant transformation.

Increased ATP production was recorded during exposure to $O_{0.5}$ and $O_{0.25}$ over 24hr, and $O_{0.125}$ over 48hr in liver cells (Fig 6.7). This parallels the decline in PDK1 expression during exposure to $O_{0.25}$ at 24hr, and $O_{0.125}$ at 48hr (Fig 6.4).

Our data shows novel interactions between OTA and the hypoxic response in human kidney and liver cells. We observed acute inflammatory induction, coupled with increased hypoxia (HIF1α protein expression) and *VEGF* expression in HepG2 cells, while HEK293 cells exhibited decreased inflammation, increased oxidative and metabolic stress with hypoxia at 24hr. At prolonged exposure to OTA, kidney cells upregulated *VEGF* and *TGFβ* gene expression, while HepG2 cells demonstrated increased *TGFβ* expression. We report, for the first time, the effects of OTA on HIF1α, HSP90, *VEGF* and *TGFβ* in HEK293 and HepG2 cells. This data is crucial in providing a molecular basis for the mechanism of OTA toxicity and carcinogenesis, especially regarding the tumour promoting characteristics of HIF1α and HSP90.

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CHAPTER 7
CONCLUSION

OTA is a known renal toxicant, with proven carcinogenicity in rodents (Cavin et al., 2009; Gautier et al., 2001). It is classified as a group 2B carcinogen – possibly carcinogenic to humans – and has been linked to the onset and progression of several nephropathies and tumour formation (Castegnaro et al., 2006; Zaied et al., 2011). The mechanism of renal fibrosis and tumour development has not been conclusively elucidated, instead the toxin is reported to act on several biochemical pathways that promote cancer induction. OTA is also hepatotoxic and immunotoxic (Gayathri et al., 2015; Thuvander et al., 1995). OTA was shown to induce renal carcinomas in male and hepatic carcinomas in female B6C3F1 mice (Bendele et al., 1985). DNA fragmentation was observed in Sprague-Dawley rat kidney cells and cultured primary human kidney cells (Robbiano et al., 2004), indicating OTA-induced DNA damage.

The oxidative capacity of OTA has been extensively reported (Arbillaga et al., 2007; Ferrante et al., 2006; Kamp et al., 2005b), with research suggesting that disruption of the antioxidant defence response filters through multiple downstream pathways, pre-disposing malignant transformation. The Nrf2 pathway is the first line of defence in cellular antioxidant response; OTA involvement in Nrf2 downregulation has been reported in several *in vitro* (Boesch-Saadatmandi et al., 2009; Stachurska et al., 2013) and *in vivo* (Cavin et al., 2007; Loboda et al., 2017) models. Resveratrol possesses antioxidant and anti-cancer properties (Fremont, 2000; Leonard et al., 2003). Studies have shown that resveratrol is able to upregulate Nrf2 expression, augmenting the antioxidant response (Bishayee et al., 2010; Chen et al., 2005). Thus, natural compounds could be promising in minimising OTA-induced oxidative stress.

Sheu et al. (2016) demonstrated OTA-induced oxidative and ER stress, with resultant apoptosis (Sheu et al., 2016); indicating toxicity induction by multiple interconnected pathways. An imbalance between cell proliferation and apoptosis is critical in cancer initiation and progression. Bouaziz et al. showed OTA-induced modulation of proteins involved in apoptosis control – Bcl-2 family proteins (Bouaziz et al., 2011), suggesting that OTA can affect cell cycle check points and induce cell death. Prolonged inflammation and hypoxia can contribute to cell death and cancer formation. OTA upregulates inflammatory genes via NFκB activity (Hennemeier et al., 2012; Ramyaa and Padma, 2013), and the activity of HIFs; ultimately promoting genetic instability and compromising cell function (Stachurska et al., 2011). OTA has been proven to disrupt several pathways involved in cancer initiation, however few studies chronicle these events in human cells, with little information available on how the toxin integrates manipulation of different pathways.

This study showed that OTA affects biochemical pathways in human kidney (HEK293) and liver (HepG2) cells. The data shows that OTA at 48hr (IC_{50} = 9.4µM) induced oxidative stress and depleted GSH; mRNA expression of *Nrf2* was significantly downregulated while protein expression increased. OTA at 24hr (IC_{50} = 1.5µM) and 48hr exposure induced DNA fragmentation evidenced by increased comet tail length. The addition of resveratrol (25µM) produced significant reductions in OTA toxicity: decreased intracellular ROS, increased GSH (24hr), decreased DNA fragmentation, and increased DNA repair via base excision repair enzyme *OGG1* (48hr) relative to the OTA only treatment. Resveratrol increased the protein expression of pSIRT1 (Ser47), and LonP1, a mitochondrial repair protein, at acute and prolonged time periods. SIRT1 is involved in the regulation of cellular stress responses, DNA repair and metabolism (Nasrin et al., 2009). This data suggests that OTA is involved in the downregulation of antioxidant repair responses, while resveratrol is able to rescue the cell from oxidative damage and promote repair processes. Cellular redox imbalance promotes an inflammatory environment, governed by NFκB. This transcription factor is responsible for pro- and anti-inflammatory proteins, as well as apoptosis induction.

The canonical NFκB pathway was activated at a sub-IC_{50} (0.5µM) concentration of OTA. Protein expression of pNFκB (Ser536) and pIKK (Ser176/180) was increased, indicating NFκB translocation into the nucleus, initiating transcriptional activity. Furthermore, ATP content and phospho-p53 (Ser392) was increased (0.5µM and 1.2µM). Interestingly, 1.2µM OTA decreased inflammation markers – caspase 1 and IL-1β was reduced, while IκBα (inhibitor of NFκB translocation) protein expression was significantly increased, suggesting sequestration of NFκB in the cytoplasm. Initiator caspases 8 and 9, and executioner caspases 3/7 were significantly increased at the highest OTA concentration (2µM), coupled with decreased ATP (compared to other OTA concentrations). This indicates apoptosis induction and immunosuppression at high concentrations of OTA. Low concentrations of OTA, however, promote inflammation and cell survival, possibly leading to an environment which favours transformation (epithelial-mesenchymal transition), which would encourage renal cell fibrosis. Oxidative stress and inflammation increase the incidence of protein damage and misfolding; as such, the ER response is important in regaining homeostasis.

Plasma range concentrations of OTA showed increased protein damage and ER-stress induced cellular survival in human kidney and liver cells. LonP1 was significantly increased in HEK293 and HepG2 cells over 24hr and 48hr. Protein carbonyl concentrations were quantified in HEK293 cells exposed to OTA, with results showing increased sensitivity to protein oxidation by OTA compared to HepG2 cells. OTA increased expression of PERK, with subsequent increased peIF2α (Ser51) in both kidney and liver cells. Increased *ATF4* mRNA expression was observed, however, *CHOP* (apoptosis mediator) expression was significantly decreased; indicating the promotion of cell survival by ER-stress

pathways. This finding was further substantiated by decreased expression of executioner caspases 6 and 3/7.

Disturbances in antioxidant responses was observed in both kidney and liver cells over 24hr and 48hr, by determining levels of pNrf2 (Ser40). Liver and kidney cells exhibited inflammation (increased pNFκB) at 24hr and 48hr respectively. The lowest OTA concentration (0.125µM) increased protein expression of HIF1α in both cell lines at 24hr, indicating alterations in oxygen tension and metabolism. HSP90 expression was increased in HEK293 cells, with PDK1 increased at 24hr, but decreased at 48hr. HepG2 exhibited decreased PDK1 expression. PDK1 expression correlates with ATP presence in both cell lines. Interestingly, HEK293 cells exhibited decreased mRNA expression of *VEGF* and *TGFβ* at 24hr, with increased levels at 48hr. Similarly, HepG2 cells exhibited increased mRNA expression of *TGFβ* at 48hr, but increased *VEGF* at 24hr.

This data suggests that OTA affects antioxidant responses, inflammatory pathways and hypoxia responses simultaneously. OTA can exert toxicity at low concentration and is able to promote conditions favouring cancer formation (inflammation, hypoxia) while upregulating transformative biomarkers, such as VEGF, TGFβ and HSP90. High concentrations of OTA induce cell death; conversely, low concentrations promote cell survival accompanied with increased cellular stress. Endemic populations often ingest low concentrations of OTA over prolonged time periods – this data provides novel insight to nephropathies involving tumour formation. The cellular conditions induced by OTA would promote genetic instability and mutations, leading to organ dysfunction and possible renal or hepatic tumours.

This study provides valuable insight to molecular mechanisms of toxicity in human kidney and liver cells exposed to varying concentrations of OTA; however, there are shortcomings to *in vitro* studies. The kidney and liver are detoxification organs, as such organ function is determined by the efficiency of the entire system. *In vitro* models cannot account for explanations of toxicity in a multi-organ system, therefore the next step would be to carry out the study in an *in vivo* (animal) model, and eventually collect blood and tissue samples from individuals exposed to high concentrations of OTA. These results can be used to further investigate the carcinogenic effects of OTA.

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APPENDIX A

Preliminary intracellular ROS data

Data measured using DCFDA assay, intracellular ROS was measured in cells exposed to OTA (IC_{50} concentration – 1.5µM) and resveratrol. Multiple controls (untreated and DMSO vehicle controls) were observed to determine if DMSO significantly affected the assay results. Results from DMSO treated cells (vehicle controls ranging from 0.01% - 0.98% DMSO) did not significantly vary from untreated controls, as such untreated control cells were used for the length of the study.

Table 1: Measurements of intracellular ROS in cells exposed to OTA and resveratrol over 24hr.

	DCF positive						
A01 SR DCF C1	0,4249	0,3967	0,4249	0,3967	0,4249	0,3967	0,4249
A02 SR DCF C1	0,5087	0,4792	0,5087	0,4792	0,5087	0,4792	0,5087
A03 SR DCF C1	0,5379	0,5032	0,5379	0,5032	0,5379	0,5032	0,5379
A04 SR DCF C2	0,6238	0,5932	0,6239	0,5932	0,6239	0,5932	0,6239
A05 SR DCF C2	0,6133	0,5818	0,6133	0,5818	0,6133	0,5818	0,6133
A06 SR DCF C2	0,6368	0,6023	0,6368	0,6023	0,6368	0,6023	0,6368
A07 SR DCF V1	0,4448	0,419	0,4448	0,419	0,4448	0,419	0,4448
A08 SR DCF V1	0,5055	0,4737	0,5055	0,4737	0,5055	0,4737	0,5055
A09 SR DCF V1	0,5078	0,4764	0,5078	0,4764	0,5078	0,4764	0,5078
A10 SR DCF V2	0,3117	0,2912	0,3117	0,2912	0,3117	0,2912	0,3117
A11 SR DCF V2	0,3158	0,2954	0,3158	0,2954	0,3158	0,2954	0,3158
A12 SR DCF V2	0,322	0,3013	0,322	0,3013	0,322	0,3013	0,322
B01 SR DCF O1	0,239	0,2235	0,239	0,2235	0,239	0,2235	0,239
B02 SR DCF O1	0,2291	0,2137	0,2291	0,2137	0,2291	0,2137	0,2291
B03 SR DCF O1	0,2272	0,2109	0,2272	0,2109	0,2272	0,2109	0,2272
B04 SR DCF O2	0,1761	0,1613	0,1761	0,1613	0,1761	0,1613	0,1761
B05 SR DCF O2	0,2788	0,2559	0,2788	0,2559	0,2788	0,2559	0,2788
B06 SR DCF O2	0,27	0,2462	0,27	0,2462	0,27	0,2462	0,27
B07 SR DCF R1	0,2107	0,1949	0,2107	0,1949	0,2107	0,1949	0,2107
B08 SR DCF R1	0,2089	0,1925	0,2089	0,1925	0,2089	0,1925	0,2089
B09 SR DCF R1	0,2084	0,1925	0,2084	0,1925	0,2084	0,1925	0,2084
B10 SR DCF R2	0,4185	0,3935	0,4185	0,3935	0,4185	0,3935	0,4185
B11 SR DCF R2	0,4255	0,4002	0,4255	0,4002	0,4255	0,4002	0,4255
B12 SR DCF R2	0,4201	0,3969	0,4201	0,3969	0,4201	0,3969	0,4201
C01 SR DCF OR1	0,3341	0,3156	0,3341	0,3156	0,3341	0,3156	0,3341
C02 SR DCF OR1	0,3273	0,3073	0,3273	0,3073	0,3273	0,3073	0,3273
C03 SR DCF OR1	0,3021	0,2851	0,3021	0,2851	0,3021	0,2851	0,3021

C04 SR DCF OR2	0,2981	0,28	0,2981	0,28	0,2981	0,28	0,2981
C05 SR DCF OR2	0,3015	0,2828	0,3015	0,2828	0,3015	0,2828	0,3015
C06 SR DCF OR2	0,2915	0,2732	0,2915	0,2732	0,2915	0,2732	0,2915
C07 SR DCF Pre-treatment 1	0,1979	0,1847	0,1979	0,1847	0,1979	0,1847	0,1979
C08 SR DCF Pre-treatment 1	0,1955	0,1837	0,1955	0,1837	0,1955	0,1837	0,1955
C09 SR DCF Pre-treatment 1	0,1936	0,181	0,1936	0,181	0,1936	0,181	0,1936
C10 SR DCF Pre-treatment 2	0,4509	0,4239	0,4509	0,4239	0,4509	0,4239	0,4509
C11 SR DCF Pre-treatment 2	0,4345	0,4066	0,4345	0,4066	0,4345	0,4066	0,4345
C12 SR DCF Pre-treatment 2	0,4621	0,4328	0,4621	0,4328	0,4621	0,4328	0,4621
D01 SR Post treatment 1	0,305	0,2868	0,305	0,2868	0,305	0,2868	0,305
D02 SR Post treatment 1	0,311	0,2909	0,311	0,2909	0,311	0,2909	0,311
D03 SR Post treatment 1	0,2977	0,2795	0,2977	0,2795	0,2977	0,2795	0,2977
D04 SR Post treatment 2	0,6737	0,6355	0,6737	0,6355	0,6737	0,6355	0,6737
D05 SR Post treatment 2	0,6963	0,6562	0,6963	0,6562	0,6963	0,6562	0,6963
D06 SR Post treatment 2	0,7141	0,6743	0,7142	0,6743	0,7142	0,6743	0,7142

Fluorescence Peaks

Preliminary data for intracellular ROS assessment, using untreated control cells and OTA (24hr, 1.5µM).

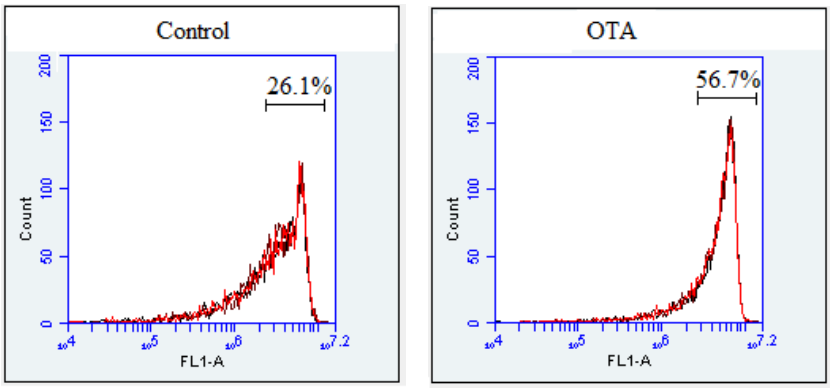

Figure 1: Fluorescence peaks for DCFDA intracellular ROS assay – determined using BD Accuri flow cytometer.

APPENDIX B

qPCR analysis of oxidative stress parameters

Gene expression of Nrf2, GPx, catalase, SOD1 and OGG1 was determined using qPCR. All treatments were compared to untreated controls and expressed as fold change

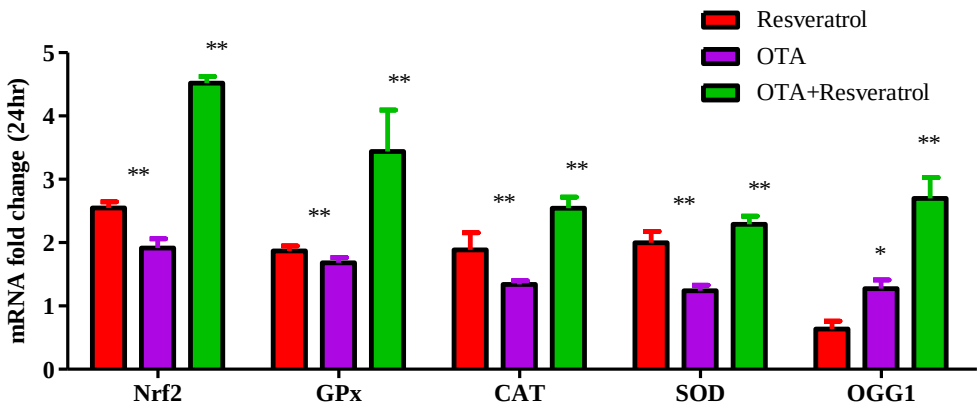

Figure 1: mRNA expression of genes associated with cellular protection and the antioxidant response determined post-exposure to OTA and resveratrol at 24hr in HEK293 cells. *$p < 0.05$, **$p < 0.005$

Mitochondrial DNA damage

Damage to mitochondrial DNA was measured using qPCR and isolated DNA.

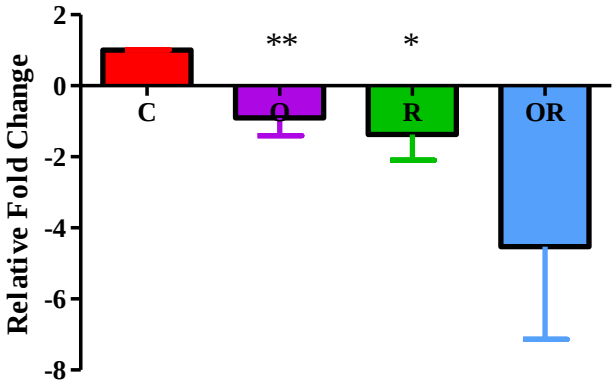

Figure 2: mtDNA damage induced in kidney cells exposed to OTA and resveratrol at 24hr. *$p < 0.05$ and **$p < 0.005$.

APPENDIX C

Lactate dehydrogenase measurement

Lactate dehydrogenase (LDH) catalyses the conversion of lactate to pyruvic acid, it also reverses the reaction. LDH often leaks out of cells with compromised membrane integrity, it can be used as a marker of necrosis. Measurements were obtained using the LDH cytotoxicity detection kit (Roche, Mannheim, Germany) by incubating 100µL of treatment supernatant (collected media post-treatment incubation) with 100µL substrate solution (diaphorase/NAD^+; INT/sodium lactate) in a 96-well microtitre plate (30min, RT, dark). Thereafter, the optical density was read at 500nm using a spectrophotometric plate reader.

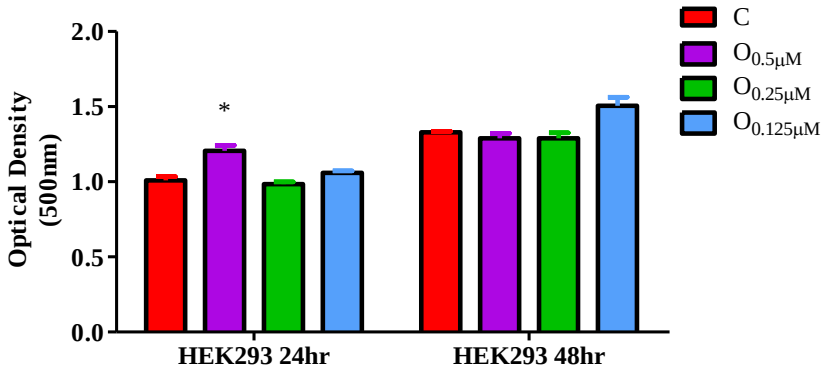

Figure 1: Extracellular LDH levels used as a biomarker for necrosis and membrane damage to HEK293 cells over 24hr and 48hr post-exposure to varying concentrations of OTA (*$p < 0.05$).

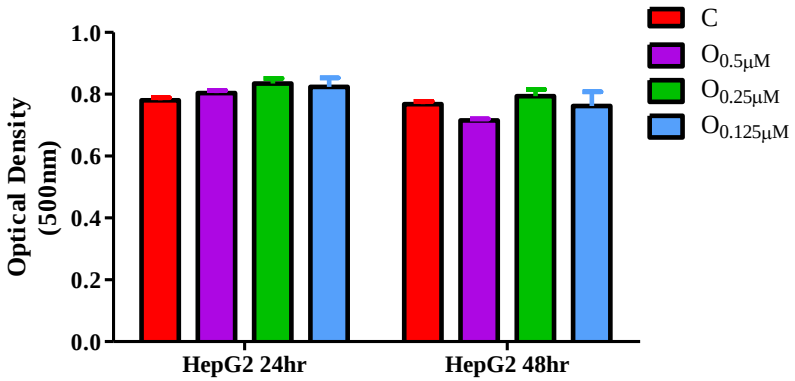

Figure 2: Extracellular LDH levels used as a biomarker for necrosis and membrane damage to HepG2 cells over 24hr and 48hr in response to OTA exposure.

APPENDIX D

Caspase activity in HEK293 cells

The activity of caspase 8 and 9 in HEK293 cells was measured using Caspase-Glo® 8 and 9 assay kits from Promega, as per the manufacturer's protocol. Caspase 8 represents the initiator of the extrinsic apoptotic pathway, whereas caspase 9 initiates the intrinsic pathway. OTA promoted activity of caspase 8 over 24hr and 48hr. Caspase 9 activity was increased at 24hr, but decreased at 48hr.

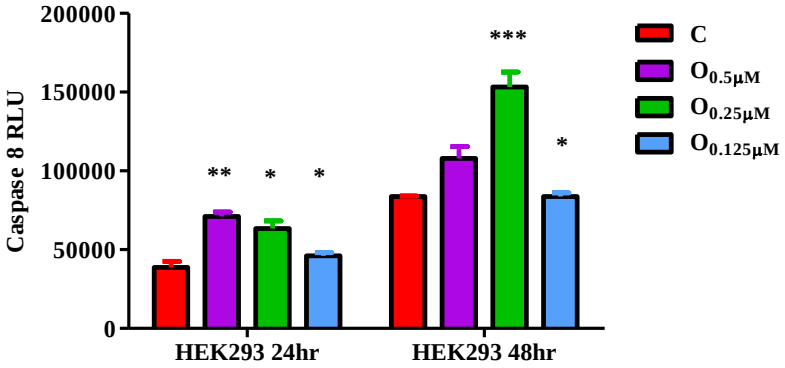

Figure 1: Quantification of caspase 8 activity in OTA treated HEK293 cells at 24hr and 48hr. *p < 0.05, **p < 0.005 and ***p < 0.0005 relative to untreated controls.

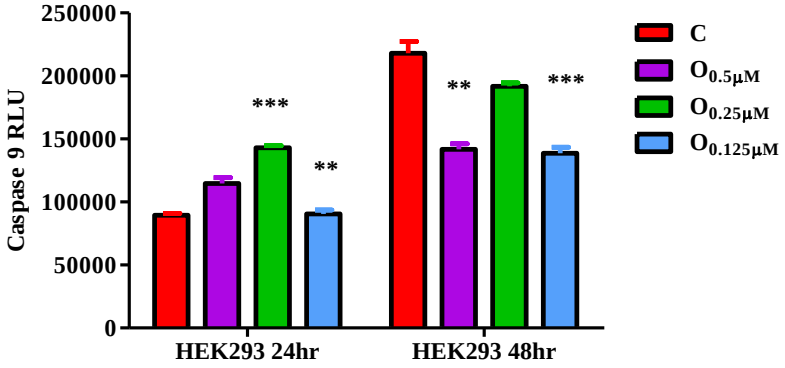

Figure 2: Quantification of caspase 9 activity in OTA treated HEK293 cells at 24hr and 48hr. *p < 0.05, **p < 0.005 and ***p < 0.0005 relative to untreated controls.

Caspase activity in HepG2 cells

The activity of caspase 8 and 9 in HepG2 cells was measured using Caspase-Glo® 8 and 9 assay kits from Promega, as per the manufacturer's protocol. OTA increased acute activity of caspase 8 in all 3 concentrations (0.5µM, 0.25µM, 0.125µM), but increased activity in only 0.125µM at 48hr. The same

pattern was observed with caspase 9 activity – increased at 24hr in all concentrations, but only increased at 0.125µM at 48hr.

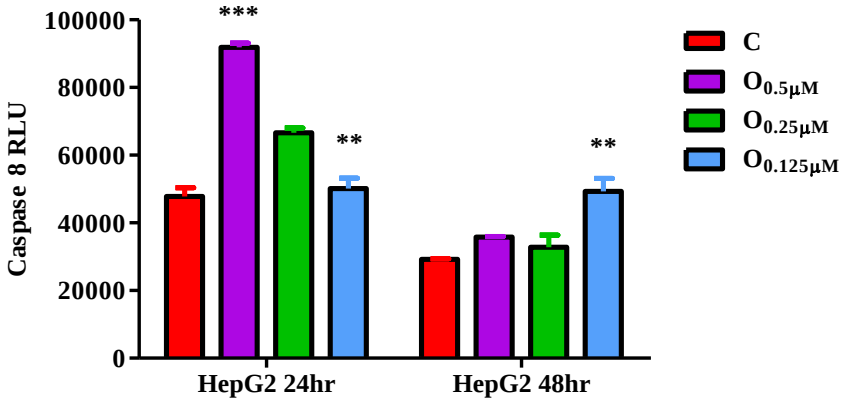

Figure 3: Quantification of caspase 8 activity in HepG2 cells exposed to varying concentrations of OTA over 24hr and 48hr. $^{**}p < 0.005$ and $^{***}p < 0.0005$ relative to untreated controls.

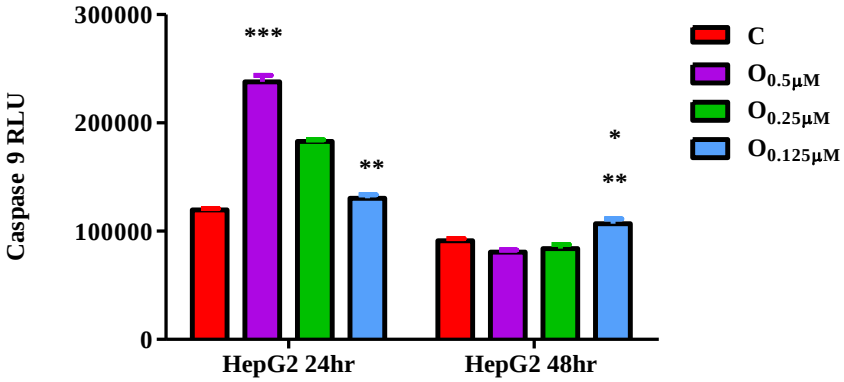

Figure 4: Quantification of caspase 9 activity in HepG2 cells exposed to varying concentrations of OTA over 24hr and 48hr. $^{*}p < 0.05$, $^{**}p < 0.005$ and $^{***}p < 0.0005$ relative to untreated controls.