T2 TOXIN ABROGATES LPS-INDUCED PROINFLAMMATORY RESPONSE IN HEPG2 LIVER CELLS: EVIDENCE FOR A NOVEL MECHANISM INVOLVING MITOCHONDRIAL STRESS RESPONSES

[1]NAEEM SHEIK ABDUL, [2]MAMONENE NKOANA, [3]SAVANIA NAGIAH, [4]ANIL CHUTURGOON

Discipline of Medical Biochemistry, School of Laboratory Medicine and Medical Sciences,
College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
E-mail: naeemsheik11@gmail.com, nkoana.mamonene@gmail.com, nagiah.savania@gmail.com, Chutur@ukzn.ac.za

Abstract— The type A tricothecene T2 toxin is a principal mycotoxin known to inhibit protein synthesis. Its toxicity is mediated by mitochondrial dysfunction and oxidative stress. Mitochondrial stress and generation of reactive oxygen species are involved in the signalling events controlling the activation of the inflammatory transcription factor NF-κB. The aim was to assess if these pathways are involved in T2 toxin-mediated effects on LPS-induced inflammatory responses. HepG2 cells were stimulated with Escherichia coli LPS in the presence and absence of T2 toxin. T2 toxin inhibited the activation of NF-κB and up-regulated mitochondrial stress responses. In conclusion, T2 toxin potently inhibits expression of LPS-induced inflammatory responses via mechanisms that involve modulation and activity of mitochondrial stress proteins.

Index Terms— LPS, Mitochondria, NFκB, T2 toxin

I. INTRODUCTION

Trichothecene mycotoxins are highly toxic secondary metabolites produced by fusarium fungal species. Controlling their accumulation in small grains remains a huge challenge. T-2 toxin (T-2) is the most toxic member of the trichothecenes and is known to inhibit protein synthesis as well as inducing mitochondrial dysfunction and oxidative stress [1]. T-2 has been shown to modulate the expression and activity of NFκB [2, 3].

Among the intracellular pathways triggered by mitochondrial dysfunction and oxidative stress is the activation of the transcription factor NFκB [4, 5], a critical factor modulating the innate immune response in mammals [6]. Caspase-1 is a member of a family of caspases with large pro-domains and its activation is involved in inflammation. Caspase-1 activation induces inflammation via the maturation of the pro-inflammatory cytokine, IL-1β [7]. The expression of both caspase 1 and IL-1β is regulated by an NFκB mechanism involving the release of damage associated molecular patterns (DAMPs) from mitochondria [8]. Alleviation of mitochondrial dysfunction through up-regulation of mitochondrial stress responses has been shown to reduce the hyperinflammation. Such defence mechanisms are associated with the up-regulation of the cytoprotective battery of genes regulated by NRF2.

Despite the importance of trichothecenes in food safety and chronic environmental exposure, the molecular mechanism of their toxicity is not well-understood and there is a critical

gap in our knowledge about the mechanisms that can protect cells against trichothecenes.

While numerous in vitro studies have reported the anti-inflammatory effects of T-2, particularly on NF-kB activation, this is the first study to evaluate the effect of T-2 on the modulation of the mitochondrial stress response and activation of NFκB in HepG2 liver cells stimulated by LPS and the effect of this modulation on expression and activation of caspase 1.

II. RESULTS

2.1. Mitochondrial Output

To determine the effect of T-2 on mitochondrial output, cell viability and intracellular ATP levels were assessed.

A dose response curve was obtained using serially diluted concentrations of T-2 in HepG2 cells over 24h. The curve showed that T-2 decreased metabolic activity in a dose dependent manner; 0.2 μM T-2 caused a 50% inhibition of metabolic activity and this concentration was used in all subsequent assays (Figure 1A).

Intracellular ATP level was measured using a luminometric assay. Figure 1B shows that T-2 caused a significant decrease in ATP levels when compared to the control.

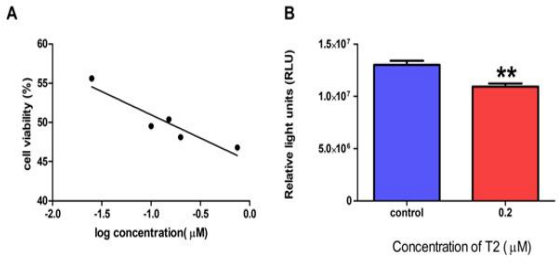


Fig 1: A dose dependent decline in metabolic activity following treatment with varying concentrations of T-2 (A). Intracellular ATP levels were significantly depleted (p=0.0062) in T-2 treated cells. Results are presented as mean ± standard deviation.**

2.2. T-2 induced mitochondrial depolarization

ΔΨM was assessed by JC-1 staining of HepG2 cells. T-2 toxin significantly increased the percentage of depolarised mitochondria at 6h relative to the control (Figure 2).

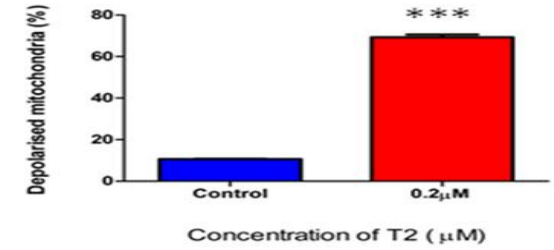


Fig 2: ΔΨM in HepG2 cells treated with T-2 toxin at 6h (*p < 0.0001 relative to control). This gives a general indication of mitochondrial health. Results are presented as mean ± standard deviation.**

2.3. T-2 attenuates caspase 1 activity and IL-1β expression

Caspase1 is activated by recruitment to a molecular platform called inflammasome, resulting in IL-1β maturation. Luminometric assessment of caspase 1 activity showed a decrease at 0.2μM relative to the control (Figure 3A). IL-1β mRNA levels were significantly decreased at 0.2μM relative to the control (Figure 3B).

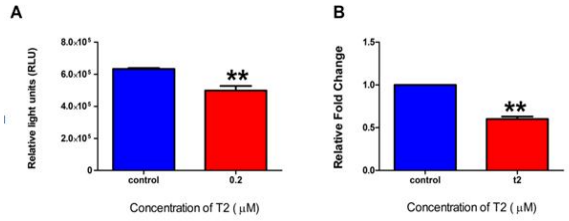


Fig 3: Caspase 1 activity (A) and IL-1β mRNA (B) decreased in HepG2 cells treated with T-2 toxin at 6h relative to the control (P< 0.005).**

2.4. Expression and activity of NFκB is down regulated in inflammatory primed HepG2 cells exposed to T-2.

NF-κB regulates the expression of pro-inflammatory mediators. When analyzed by western blot, the phosphorylated and transcriptionally active p65 subunit was found to be significantly decreased at the IC50 concentration (Figure 4B). Concomitantly T-2 significantly decreased total p65 transcript levels (Figure 4A). These results strongly suggest that T-2 affects post-translational activation of the NF-κB p65 subunit.

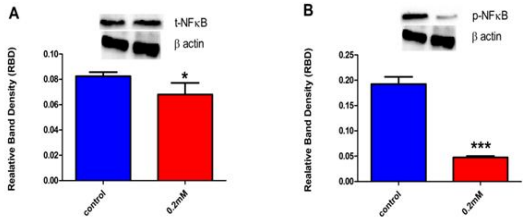


Fig 4: Assessment of NF-κB p65 subunit showed decreased expression (A) and activation (B) in cells treated with T-2 despite LPS priming (*P < 0.05,*P< 0.001).**

2.5. T-2 enhances mitochondrial maintenance

Mitochondrial dysfunction is linked to oxidative stress as these organelles are both generators and targets of ROS. The NRF2 transcription factor translocates to the nucleus under oxidative stress conditions and upregulates cytoprotective genes. Phosphorylation of NRF2 at serine 40 promotes its dissociation from Kelch-Like-ECH Associated Protein 1 (KEAP1). Further investigations revealed a similar trend in total NRF2 protein expression in T-2 stimulated cells (Figure 5A). SOD2 expression is regulated by NRF2. SOD2 mRNA expression level was significantly elevated at 0.2μM relative to the control (Figure 5D).

Situin 3 is the primary mitochondrial deacetylase that functions tomprevent the accumulation of reactive oxygen species by activating SOD2. T-2 Significantl enhanced SIRT3 protein expression (Fig 5B). Accumulation of PINK1 recruits Parkin which results in enhanced clearance of damaged mi-
tochondria. T-2 significantly increased PINK1 expression when compared to the control (Figure 5C).

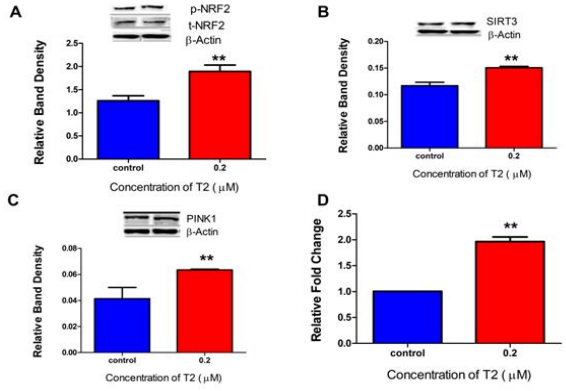


Figure 5: T-2 enhanced the expression of mitochondrial defence mechanisms against oxidative stress.

III. DISCUSSION

The liver is adept at prompting an immune response owing to first pass metabolism, and as a result comes in contact with pro-inflammatory stimuli [9]. T-2 is a known modulator of the NFκB response [2, 3] and is associated with mitochondrial dysfunction.

The MTT assay measures cell viability relative to the number of metabolically active cells by measuring mitochondrial reducing equivalents. T-2 toxin drastically decreased cell viability and ATP levels. It

has been established that T-2 inhibits mitochondrial protein synthesis [10] as well as the function of mitochondrial complexes in the electron transport chain [11]. Mitochondrial depolarization is a good indicator of mitochondrial health. T-2 toxin significantly increased the percentage of depolarised mitochondria, a clear indication of mitochondrial dysfunction. Our data is in agreement with that of [11]. Thus, suggesting that T-2 can directly or indirectly inhibit mitochondrial enzymes as well as reducing equivalent metabolism, thus reducing ATP synthesis.

The synthesis and maturation of IL-1β is tightly controlled by a two-step mechanism. The transcription of IL-1β is regulated by NF-κB. In the inactive state, NF-κB is sequestered in the cytoplasm by its inhibitor, IκB. The activation and nuclear translocation of NF-κB requires the phosphorylation and degradation of IκB by IKKβ. T-2 down-regulated the expression of the transcriptionally active p65 subunit. Phosphorylation is required for p65 nuclear translocation resulting in NF-kB activation [12].

The maturation of IL-1β is regulated by the NLRP3 inflammasome, a multiprotein platform that activates caspase 1. This inflammatory caspase cleaves the IL-1β zymogen into its biologically active form [7]. NLRP3 inflammasomes require a priming step in which the components of this inflammasome are up-regulated. NF-κB is responsible for inducing the expression of NLRP3 and caspase 1. T-2 inhibited the activation of caspase 1. Caspase 1 activation is an essential mediator of inflammasome function and its activity is considered a marker of inflammasome activation. This further lends support to caspase 1 dysfunction and aberrant IL-1β processing by T-2.

The most common mitochondrial ROS is superoxide (O2). A key component of the mitochondrial antioxidant response is the enzyme SOD2, which catalyzes the conversion of O2 to hydrogen peroxide (H2O2). Excess mitochondrial ROS is a potent inducer of oxidative damage within the organelle itself. T-2 increased the expression of SOD2. SOD2 expression is regulated by the activation of NF-κB [13]. These results are in inconsistent with our findings as we showed elevated SOD2 expression despite a decrease in NF-κB. We therefore explored the possibility of NRF2 being an alternate transcriptional regulator of SOD2 in response to T-2. Many studies have established that the Keap1/NRF2/ARE redox-sensitive signaling system increases SOD2 expression [14].The binding of NRF2 to the ARE, leads to transcriptional activation of antioxidant enzymes making it an important transcriptional regulator of oxidative stress responses. NRF2 further acts as a transcription factor for SIRT3, the activator of SOD2. T-2 toxin upregulates defence mechanisms to counteract elevated mitochondrial oxidative stress. Recent studies have proposed enhanced mitophagy as a quality control process to negatively regulate inflammation. Postulating that the anti-inflammatory effect of T-2 is mediated through NRF2 we investigated NRF2 gene products involved in autophagy. Sequence analysis have indicated the existence of ARE in the promoter region of PINK1 [15].

The serine/threonine kinase PINK acts as a molecular sensor of mitochondrial dysfunction. Under basal environments PINK1 is degraded in the mitochondria. However, upon detection of mitochondrial dysfunction PINK1 selectively accumulates on damaged mitochondria where it signals for the recruitment and activation of Parkin. Parkin functions as an E3- ubiquitin ligase to recruit additional proteins necessary for removal of damaged mitochondria [16]. T-2 significantly increased the expression of PINK1 (Fig 5B) suggesting that PINK1/Parkin mediated clearance of damaged mitochondria is initiated and this prevents the accumulation of toxic mitochondrial products that would normally induce inflammation.

CONCLUSION

This work contributes significantly to the clarification of dietary toxins modulating the cross talk between NRF2 and inflammation. These results provide evidence that T-2 decreased NF-κB transcriptional activity despite a pro-inflammtory state being induced by LPS.

REFERENCES

[1] Bin-Umer, M.A., et al., Elimination of damaged mitochondria through mitophagy reduces mitochondrial oxidative stress and increases tolerance to trichothecenes. Proceedings of the national academy of sciences, 2014. 111(32): p. 11798-11803.

[2] Fu, Y., et al., The effect of T-2 toxin on IL-1β and IL-6 secretion in human fetal chondrocytes. International orthopaedics, 2001. 25(3): p. 199-201.

[3] Tian, J., et al., T-2 toxin enhances catabolic activity of hypertrophic chondrocytes through ROS-NF-κB-HIF-2α pathway. Toxicology in vitro, 2012. 26(7): p. 1106-1113.

[4] García-Ruiz, C., et al., Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcription factor nuclear factor-kappa B: studies with isolated mitochondria and rat hepatocytes. Molecular pharmacology, 1995. 48(5): p. 825-834.

[5] Li, N. and M. Karin, Is NF-κB the sensor of oxidative stress? The FASEB Journal, 1999. 13(10): p. 1137-1143.

[6] Xiao, C. and S. Ghosh, NF-κB, an evolutionarily conserved mediator of immune and inflammatory responses, in Mechanisms of lymphocyte activation and immune regulation X. 2005, Springer. p. 41-45.

[7] Mankan, A.K., et al., The NLRP3/ASC/Caspase□1 axis regulates IL□1β processing in neutrophils. European journal of immunology, 2012. 42(3): p. 710-715.

[8] Salminen, A., et al., Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging. Ageing research reviews, 2008. 7(2): p. 83-105.

[9] Gao, B., W.I. Jeong, and Z. Tian, Liver: an organ with predominant innate immunity. Hepatology, 2008. 47(2): p. 729-736.
[10] Pace, J., M. Watts, and W. Canterbury, T-2 mycotoxin inhibits mitochondrial protein synthesis. Toxicon, 1988. 26(1): p. 77-85.
[11] Liu, J., et al., The role of mitochondria in T-2 toxin-induced human chondrocytes apoptosis. PloS one, 2014. 9(9): p. e108394.
[12] Doyle, S.L., C.A. Jefferies, and L.A. O'Neill, Bruton's tyrosine kinase is involved in p65-mediated transactivation and phosphorylation of p65 on serine 536 during NF-κB activation by LPS. Journal of Biological Chemistry, 2005.
[13] Xu, Y., et al., An intronic NF-kappaB element is essential for induction of the human manganese superoxide dismutase gene by tumor necrosis factor-alpha and interleukin-1beta. DNA and cell biology, 1999. 18(9): p. 709-722.
[14] Gwarzo, M.Y., Nrf2 transcription factor gene regulates basal transcription of mitochondrial superoxide dismutase enzyme in mouse brain. African Journal of Biotechnology, 2009. 8(20).
[15] Murata, H., et al., NRF2 regulates PINK1 expression under oxidative stress conditions. PloS one, 2015. 10(11): p. e0142438.
[16] Nguyen, T.N., B.S. Padman, and M. Lazarou, Deciphering the molecular signals of PINK1/Parkin mitophagy. Trends in cell biology, 2016. 26(10): p. 733-744.
[17] Funding
The authors would like to acknowledge the college of Health sciences (UKZN) and the NRF (South Africa) for funding this study

★★★