

## Does Hsp60 provide a link between mitochondrial stress and inflammation in Diabetes Mellitus?

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## **Abstract**

The focus of this review is to summarise the known relationships between the expression of Heat Shock Protein 60 (Hsp60) and its association with the pathogenesis of Type 1 and Type 2 Diabetes Mellitus. Hsp60 is a mitochondrial stress protein that is induced by mitochondrial impairment. It is known to be secreted from a number of cell types and circulating levels have been documented in both Type 1 and 2 Diabetes mellitus patients. The biological significance of extracellular Hsp60 however, remains to be established. We will examine the links between Hsp60 and cellular anti and pro- inflammatory processes and specifically address how Hsp60 appears to affect immune inflammation by at least two different mechanisms: as a ligand for innate immune receptors and as an antigen recognized by adaptive immune receptors. We will also look at the role of Hsp60 during immune cell activation in atherosclerosis, a significant risk factor during the pathogenesis of Diabetes Mellitus.

## **Introduction**

Diabetes mellitus is a spectrum of metabolic disorders characterised by chronic hyperglycaemia and abnormalities within the metabolism of proteins, fats and carbohydrates (Polonsky 2012). The two most common form of diabetes are classified as Type 1 and 2. Type 1 also known as insulin-dependent diabetes (IDDM) is characterised by the autoimmune destruction of the  $\beta$ -cells of the pancreatic islets which ultimately results in loss of insulin production leading to hyperglycaemia (Roep & Tree, 2014). On the other hand, Type 2 diabetes is linked to disorders of both insulin secretion and insulin action (Olokoba, Obateru, & Olokoba, 2012). It is becoming increasingly clear that Type 2 diabetes is also associated with a progressive destruction of  $\beta$ -islet cells by autoimmune processes linked to inflammation (Itariu & Stulnig, 2014). However, the key triggers and molecular mechanisms responsible for the loss of  $\beta$ -cells have not yet been elucidated. Since mitochondria play a key role in the secretion of insulin from  $\beta$ -islet cells (Maechler, 2013), in this review we will examine the role of the mitochondrial molecular stress protein Hsp60 in the pathogenesis of both Type 1 and 2 diabetes and the potential links between Hsp60 expression and inflammation.

## **Role of mitochondrial Hsp60 in Type 1 & 2 Diabetes Mellitus**

Heat shock protein 60 (Hsp60) is a molecular stress protein predominantly localised to the mitochondrion where it is known to play a role in the folding of proteins in the mitochondrial matrix. Hsp60 is upregulated in response to mitochondrial impairment (Pellegrino, Nargund, & Haynes, 2013) and is considered to be an indicator of mitochondrial stress. Interestingly, Hsp60 has also been shown to be secreted from a variety of mammalian cell types (Gupta & Knowlton; Merendino et al., 2010; Swaroop, Sengupta, Suryawanshi, Adlakha, & Basu, 2016) and is known to be found at elevated levels in both Type 1 and 2 Diabetes Mellitus (Jing Yuan, Peter Dunn, & Ryan Dennis Martinus, 2011). The physiological/pathological consequence of having elevated levels of Hsp60 in systemic circulation and the cell types responsible for secretion of Hsp60 into circulation in Diabetes Mellitus is not yet known.

For many years Hsp60 has been observed in non-obese (NOD) mouse model of diabetes and has been linked to play a role in the destruction of pancreatic  $\beta$ -islet cells caused by the spontaneous development of autoimmune T-lymphocytes (Birk, Douek, et al., 1996). Several epitopes has been identified to have significant anti-Hsp60 T cells responses, but one in particular p277 peptide has shown the greatest anti-Hsp60 T cells responses (Abulafia-Lapid et al., 1999). The p277 peptide of Hsp60 has been widely studied over the years, it is 24

amino acid peptide within amino acid residue 427-460 derived from monocytes human Hsp60 (Elias et al., 1991). Hyperglycaemia and insulinitis developed when standard strains of mice, not prone to spontaneous diabetes were immunized with p277 covalently conjugated to a foreign immunogenic carrier molecule (Elias, Marcus, Reshef, Ablamunits, & Cohen, 1995).

Interestingly, Hsp60 and p277 peptides can also lead to protection of  $\beta$ -cell functions (Raz et al., 2001; Sarikonda et al., 2015). This protection is thought to be due to modulation of the autoimmune process responsible for  $\beta$ -cell destruction. Therapeutic vaccination with p277 has been documented to slow and inhibit the destruction of  $\beta$ -cells both in NOD mice and in humans. Administration of p277 has been shown to down regulate T-cell reactivity to  $\beta$ -cell antigens. This process is thought to be associated with a shift in cytokine profile from the pro-inflammatory T-helper 1 (Th1) phenotype to the anti-inflammatory T-helper-2 (Th2) phenotype. Thus, p277 has been shown to increase IL-4 and IL-10 secretion and a decrease in  $\gamma$ -IF secretion and is thought to be mediated by p277 binding to TLR2 receptors (Kim, Lee, Lee, Kim, & Lee, 2012; Nussbaum, Zanin-Zhorov, Quintana, Lider, & Cohen, 2006).

Therefore, it is evident that Hsp60 is able to influence T-cell responses in two ways: as a ligand of toll-like receptor 2 signalling and as an antigen. But how can T-lymphocytes target Hsp60 (which is ubiquitous) and p277 epitope be involved with the destruction and protection of pancreatic  $\beta$ -cells? One explanation for this is molecular mimicry. Hsp60 and p277 could possibly mimic a tissue specific antigen of the  $\beta$ -islet cells of the pancreas that has a p277 like epitope (Jones, Coulson, & Duff, 1993). A study by (Birk, Elias, et al., 1996) reported that mouse Hsp60 molecules in  $\beta$ -cells of NOD mouse is the target of anti-H-p277. However this finding poses the question of how a ubiquitous molecule such as Hsp60 can be the target of a tissue-specific autoimmune disease? There is no evidence to suggest that tissue-specific Hsp60 exist, given that there is a fibroblast homologue which contains identical sequence to the pancreatic  $\beta$ -islet Hsp60 (Birk, Elias, et al., 1996). However, several suggestions have been proposed in order to address this question; Hsp60 are present in a unique way in secretory vesicles of the  $\beta$ -cells, and in the event of insulin secretion, these vesicles fuse with the  $\beta$ -cell membrane causing Hsp60 to be presented on the  $\beta$ -cell membrane and/ or even secreted in the absence of mitochondrial stress, causing differences in secretion characteristic from other cells (Birk, Elias, et al., 1996; Brudzynski, Martinez, & Gupta, 1992). When C9 cells (a diabetogenic NOD T-cell clone that responds to p277) are injected into NOD mice, it migrates to the pancreas and kidney (Birk, Elias, et al., 1996). Another experiment showed that there are 8-12 genes for Hsp60 in the vertebrate genome, however when sequenced, all but one was a pseudogene (Venner, Singh, & Gupta, 1990). All of these experiments suggest that although  $\beta$ -cell Hsp60 is not tissue

specific, there may be tissue specific post-translational modifications of Hsp60 (Birk, Elias, et al., 1996). Experiments have also shown how T-cells are still able to proliferate in the presence of  $\beta$ -cell and absence of other antigen-presenting cells suggesting that Hsp60 in  $\beta$ -cell may be processed specifically to that tissue, enabling the  $\beta$ -cell to present its own Hsp60 to the T-cells, and ultimately present specific immunogenic peptide like p277 (Birk, Elias, et al., 1996). Even though T-cells targets non-tissue specific molecules, it may have a greater preference over vulnerable, damaged  $\beta$ -cells, ultimately causing distress to  $\beta$ -cells, and allowing the release of tissue specific antigens (Birk, Elias, et al., 1996). Expression of Hsp60 in  $\beta$ -cell may also be augmented by environmental tissue-specific trigger such as viral infection via  $\gamma$ -interferon, thus causing  $\beta$ -cell to be targets for anti-Hsp60 T-cells (Birk, Elias, et al., 1996).

Thus what is the purpose and what can the immune system gain from recognising Hsp60? Hsp60 is a highly conserved protein, therefore both bacterial (foreign) and endogenous Hsp60 (self) can act as an antigen for B cells (Kaufmann, 1989). At first, production of antibodies against Hsp60 was thought to be a mechanism to fight bacterial infection or vaccination (Kaufmann, 1989), however it was subsequently found that autoantibodies against self-Hsp60 was also found to be associated for various autoimmune diseases such as Type 1 Diabetes (F. J. Quintana & Cohen, 2011).

Various studies with human patients have shown that treatment of Type 1 diabetes with p277 epitope can preserve part of the endogenous insulin production by arresting the destruction of the  $\beta$ -cell mass in the pancreas (Huurman et al., 2008; Stuart et al., 2012), The paper by Stuart et al., 2012 states that a number of Hsp60 peptide epitopes that can bind multiple allelic variants of the human major histocompatibility complex molecule HLA-DR called pan-DR epitopes which can induce low peptide-specific proliferative responses and peptide specific production of intracellular cytokines such as interleukin-10 (IL-10) an anti-inflammatory cytokine, and interferon-  $\gamma$  (IFN- $\gamma$ ) in type 1 diabetes (Stuart et al., 2012). This suggests that Hsp60 and peptides derived from the full length molecule can induce both pro-inflammatory and anti-inflammatory cytokines. This confirms Hsp60 as an important modulator of inflammation in Type 1 Diabetes Mellitus. It should be noted that Hsp60 is also thought to down-regulate inflammation via activated effector T cells up-regulating Hsp60 and presenting their own Hsp60 epitopes to anti-ergotypic regulatory T cells (Francisco J. Quintana, Mimran, Carmi, Mor, & Cohen, 2008)

Evidence is also accumulating which seem to suggest that Hsp60 may also be involved in the pathogenesis of Type 2 Diabetes Mellitus. A number of studies have shown elevated levels of Hsp60 protein in systemic circulation in Type 2 diabetes patients. Yuan et al. (2011)

reported the elevated presence of Hsp60 in both serum and saliva of Type 2 diabetics compared to a non-diabetic control subjects. Salivary Hsp60 were found to be fourfold higher in type 2 diabetics compared to non-diabetics, and serum Hsp60 was found to be 16 fold higher than the salivary Hsp60 in Type 2 diabetics. The presence of Hsp60 as a molecular marker that represents mitochondrial stress opens up the opportunity for a non-invasive diagnostic route to further investigate the relationship of Hsp60 and diabetes (J. Yuan, P. Dunn, & R. D. Martinus, 2011). Evidence has been documented which show that when human HeLa cells are grown in the presence of mitochondrial inhibitors (such as sodium azide, hydrogen peroxide and high glucose) there is a significant up-regulation of Hsp60 at the protein level (Hall & Martinus, 2013). This suggests that the increased level of serum Hsp60 detected in Type 2 Diabetes Mellitus patients might also be due to mitochondrial stress.

### **Hsp60 & Inflammation**

A number of studies suggest that extracellular Hsp60 play a role as a cellular “danger” signal for cellular and humoral immune reactions (Calderwood, Mambula, Gray Jr, & Theriault, 2007; de Haan, Smeets, Pasterkamp, & Arslan, 2013). In 1997, a hypothesis was proposed suggesting that elevated acute-phase/ stress reactants (such as Hsp60) and their major cytokine are associated with Type 2 Diabetes (Pickup, Mattock, Chusney, & Burt, 1997). Since then, many studies have been conducted looking at circulating markers of inflammation, and their association with Type 2 Diabetes Mellitus (Pickup, 2004). Inflammation has been found to be an important causative factor in the pathogenesis of Type 2 Diabetes and insulin resistance (Wellen & Hotamisligil, 2005). There is also an observable association between the pathogenesis of insulin resistance, diabetes and atherosclerosis, and the activation of innate immune system by toll-like receptors (TLRs) (Curtiss & Tobias, 2009; Shi et al., 2006; Wong & Wen, 2008). Interestingly, a link between TLR2 and TLR4 polymorphisms and Type 2 diabetes has been documented, suggesting that TLRs may play a causative role in diabetes (Bagarolli, Saad, & Saad, 2010; Gülden & Wen, 2014). Other studies have shown the increase expression of TLR2 and TLR4 in conventional insulin resistance target tissues like skeletal muscle and adipose tissue of Type 2 diabetics (Creely et al., 2007; Reyna et al., 2008).

TLRs are a family of protein that senses the invasion of microorganism. This in turn stimulates the TLRs and initiates a range of host defence mechanisms (Takeda, Kaisho, & Akira, 2003). Each member of the TLR family are set to recognise a specific pathogen component, and when activated it will create a signalling cascade that ultimately leads to the

production of cytokine (such as; IL-1 $\beta$ , IL-6, IL-8, (monocyte chemoattractant protein-1 (MCP-1), and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )) and adaptive immune response (Takeda et al., 2003). Ligands for TLR2 and TLR4 includes; Hsp60, Hsp70, high mobility group B1 protein, endotoxin, hyaluronan, advance glycation end products and extracellular matrix components (Tsan & Gao, 2004). In 2009, it was postulated that the effects of Hsp60 on the innate immune system may be due to the presence of bacterial contaminants (LPS, lipopolysaccharide, a major cell wall component of gram negative bacteria) in preparations of the recombinant mammalian Hsp60 preparations (Tsan & Gao, 2009), however it is now clear that activation of innate immune receptors can be caused by Hsp60 on its own and not by associated contaminants (Henderson et al., 2010). A study done in 2010 by Dasu et al. confirmed that expression of TLR2 and TLR4 in Type 2 diabetics is greatly increased when compared to non-diabetics. Furthermore, due to the increase of TLR2 and TLR4 expression, there was a subsequent increase of inflammation, which was mediated by nuclear factor Kappa Beta (NF- $\kappa$ B) p65 (Dasu, Devaraj, Park, & Jialal, 2010). The concentrations of pro-inflammatory mediators; IL-1 $\beta$ , IL-6, IL-8, MCP-1, and TNF- $\alpha$  in the serum was also found to be significantly increased in Type 2 diabetics, compared to non-diabetics. This novel finding suggests that Hsp60 could be playing modulatory responses in inflammation, a metabolic characteristic of Type 2 diabetes, through the activation of TLRs.

Interestingly, anti-human Hsp60 small-hairpin RNAs (shRNAs) has been documented to down-regulate the expression of endogenous Hsp60 mRNA 48 hours post-transfection in human cells (Corydon, Hansen, Bross, & Jensen, 2005). The study proves that Hsp60 can be regulated using RNAi and opens the possibility to develop RNAi based therapeutic strategies to treat Type 2 diabetes clinically.

Many studies has also shown that people suffering from Type 1 and Type 2 diabetes have accelerated atherosclerosis and are in greater risk of developing atherosclerosis (Matheus et al., 2013). Atherosclerosis is a disease where plaque builds up inside the arteries, and is the cause of a majority of cardiovascular diseases (Tabas, García-Cardena, & Owens, 2015). Early atherosclerosis is characterised by the penetration of agranulocyte or mononuclear cells, in particular monocytes, macrophages and T-lymphocytes (Xu, Oberhuber, Gruschwitz, & Wick, 1990). In the late atherosclerosis lesions, T-lymphocytes were seen to be activated, and a substantial proportion of the cells are thought to be reacting against Hsp60 (Benagiano et al., 2005; Curry, Portig, Goodall, Kirkpatrick, & Gaston, 2000). A study done using rabbits immunised with mycobacterial Hsp60 have shown that atherosclerotic lesions can be prevented when the rabbit's T-lymphocytes are depleted (Metzler et al., 1999; Xu et al., 1992). On the other hand, when LDL-receptor deficient mice are introduced to the

Hsp60 reactive T-lymphocytes, the mice were able to induce a pronounced atherosclerotic vessel wall changes (George, Afek, Gilburd, Shoenfeld, & Harats, 2001).

A study done in 2007, found a correlation between atherosclerosis and T-cell reactivity to Hsp60 in young males but not in men aged 50 and above. This suggests that the T-cell reactivity to Hsp60 is more prominent in young and very early stages of arteriosclerosis (Knoflach et al., 2007). It is thought that T-cell reactivity to Hsp60 is less prominent in men age 50 and over because the majority of the T-cells has already formed from blood to the site of inflammation in atherosclerotic plaques and lymphocytes from peripheral blood may no longer present the specific antigen repertoire of cells in vessel walls (Knoflach et al., 2007). This T-cell reactivity to Hsp60 is capable of triggering both innate and adaptive immune responses that initiate the earliest inflammatory stage of atherosclerosis, and mitochondrial Hsp60 is increasing being recognised as a key autoantigen at the sites of endothelial inflammation (Grundtman, Kreutmayer, Almanzar, Wick, & Wick, 2011; Wick, 2016). However, the mechanisms leading to expression of Hsp60 during the initiation of arteriosclerosis due to T-cell reactivity to Hsp60 is still not well understood.

## **Conclusion**

There is a clear association between Hsp60 and Type 1 and Type 2 diabetes. In Type 1 diabetes, Hsp60 protein are able to induce the production of anti-Hsp60 antibodies as a defence mechanism against pathogens, anti-Hsp60 antibodies also targets endogenous Hsp60 (p277 epitope), and results in the destruction of  $\beta$ -islet cells. However, both Hsp60 and p277 peptide can also prevent  $\beta$ -cell destruction by up-regulation of the anti-inflammatory Th2 cytokine pathway. Since the loss of  $\beta$ -islet cells is primarily thought to be driven by a pro-inflammatory Th1 cytokine response, the shift of Th1 to Th2 by Hsp60 and p277 may be involved in attenuation of Type 1 Diabetes Mellitus (Figure 1). The high levels of Hsp60 found in the serum in Type 2 diabetic may also lead to the initiation of pro-inflammatory cytokines in target cells (such as vascular endothelial cells) by interacting with TLR2 and TLR4 receptors (Figure 2). Thus, Hsp60 acting as a pro-inflammatory signalling molecule may play a role in the non-resolved vascular inflammation, which is increasingly being recognised as a feature of Type 2 diabetes. Suggesting that, Hsp60 does indeed play a key regulatory role in modulating inflammatory processes in Diabetes Mellitus and could also provide a key link between mitochondrial stress and inflammation in Diabetes Mellitus.



“The authors declare that they have no competing interests”

#### Author contributions

“JJ was responsible for researching the areas covered under the review under the supervision of RDM” The review paper was written by JJ and edited by RDM”

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Figure 1

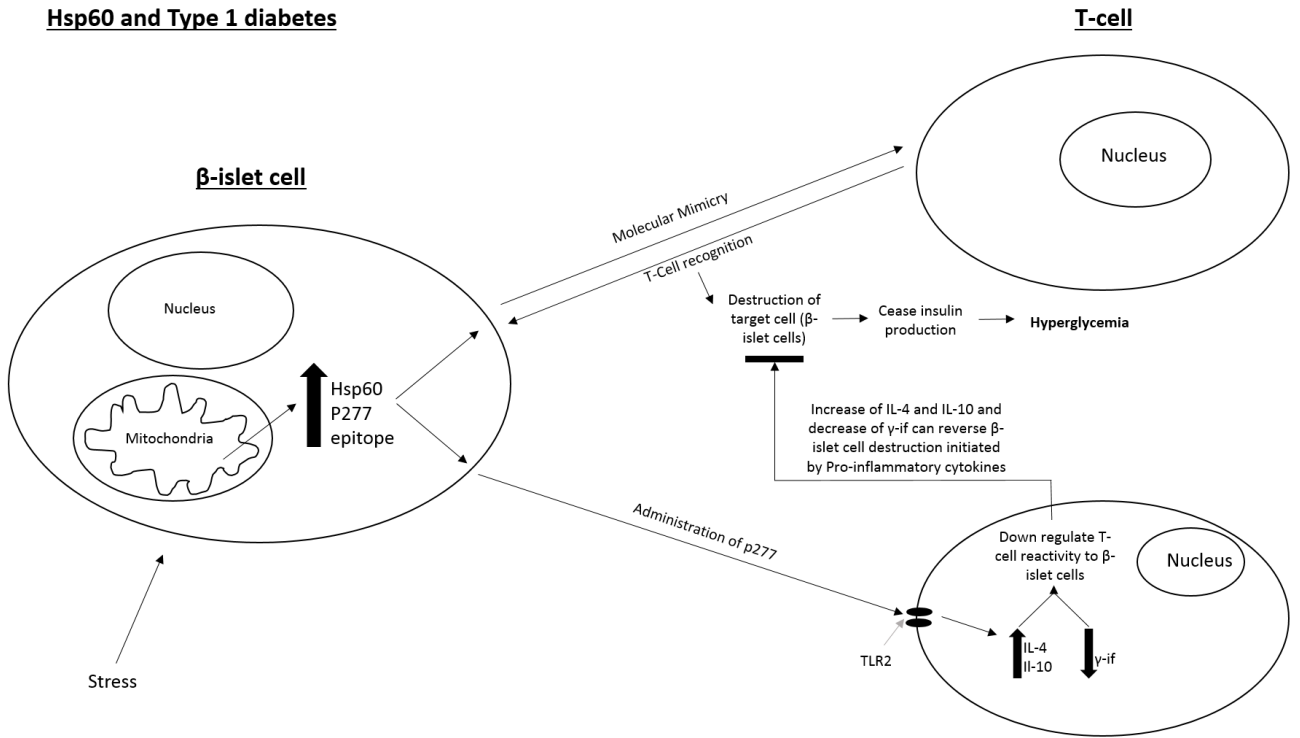


Figure 2

**Hsp60 and Type 2 diabetes**

