Chapter 4. Release of Hsps from immune cells

4.1 Introduction

In the previous chapter it was demonstrated that it is possible for Hsps to influence the OPG/RANKL system of bone regulation in osteoblasts. In the case of bacterial Hsps it is clear how localised infection could trigger bone loss by this mechanism. However, for self Hsps to affect bone resorption via the RANKL/OPG system or an alternative system they would need to be present in the extracellular environment. Hsps and antibodies to Hsps are known to be present in human serum (Pockley et al., 1998, Pockley et al., 1999), and the source of serum Hsps can only be from two possible sources; cell damage/lysis, or active release from living cells.

Though not a widely accepted fact, active Hsp release has been reported to occur in a range of different mammalian cell types. Hsp90α has been reported to be released from vascular smooth muscle cells (Liao et al., 2000). Hsp110 and Hsp70 release has been reported from cultured rat embryo cells following heat stress (Hightower and Guidon, 1989), and Hsp70 release has been reported to occur from glial cells (Guzhova et al., 2001), human carcinoma cell lines (Broquet et al., 2003., Evdonin et al., 2004., Wang et al., 2004), and in PBMCs following heat stress and exposure to rheumatoid arthritis synovial fluid (Martin et al., 2003). One mechanism by which Hsp70 release has been demonstrated to occur is via a non-classical protein secretion pathway involving lipid rafts (Broquet et al., 2003). There are no reports of active Hsp60 release from mammalian cells, though a study has reported the release of myocardial Hsp60 into the circulation following myocardial injury (Schett et al., 1999).

It was decided that the most likely source of Hsps that could affect bone resorption in situations of disease induced bone loss would either be bone cells themselves, or immune cells. Initial work indicated that there was no significant release of Hsp60 or Hsp70 from the MG63 osteoblast-like cell line in normal cell culture conditions or following heat shock (data not shown), and it was considered much more likely that immune cell self-Hsp release would have a role in disease related bone loss. This was further supported by the elevation in Hsp gene expression that was found to occur following PHA activation in PBMCs (section 5) and the previous study (Martin et al., 2003) indicating Hsp70 release from cells of the immune system may be possible.
This chapter therefore aims to investigate the following:

Is it possible for Hsp 60 and Hsp70 be actively released from human immune cells in vitro?

Which immune cells release Hsp?

Is Hsp release affected by inhibitors of classical and non-classical protein secretion?
4.2 Methods

4.2.1 Measurement of Hsp release from immune cells in normal and heat shock conditions

4.2.1.1 Cell preparation

U937 human monocytic leukaemia cells and Jurkat human T-cell leukaemia cells were cultured as described in section 2.3.3 and section 2.3.4 respectively. PBMCs were isolated from whole blood using the method described in section 2.3.6. Prior to treatment cells were re-suspended in 10 ml supplemented RPMI (section 2.2.13) and washed by centrifugation at 500 g for 3 min at room temperature (RT). Media was removed from the pellet, and the cells were re-suspended in supplemented RPMI to give a cell density of ~6 x 10^6 cells/ml (Jurkat and U937) or ~2 x 10^7 cells/ml (PBMCs).

4.2.1.2 Heat treatment

Re-suspended cells (section 4.2.11) were transferred to 15 ml centrifuge tubes (1 ml per tube, triplicate samples) and the cells were heat shocked for 2 hours at 37°C, 42°C and 45°C, followed by a 3 hour incubation at 37°C. Samples were taken at t0 and after treatment. Cells and media were harvested and stored as described in section 4.2.3.

4.2.1.3 Hsp release time course

Re-suspended cells (section 4.2.11) were transferred to 12 well plates (1 ml/well) and the plates were incubated at 37°C, 5% CO2 for up to 24 h. At t0, 2 h, 4 h, 6 h, and 24 h time points, cells and media were collected, stored and analysed as described in section 4.2.3.

4.2.2 Treatment of PBMCs with inhibitors of protein release

Re-suspended PBMCs (section 4.2.11) were transferred to 15 ml centrifuge tubes (1 ml/tube). A t0 control sample was harvested immediately, and the remaining cells
were treated to give control and inhibitor treated samples. The inhibitors used were Monensin (8μg/ml) (section 2.2.82), Brefeldin A (25μg/ml) (section 2.2.83), Methyl-β-cyclodextrin (25μg/ml) (section 2.2.81) and Methylamine (30 mM) (section 2.2.84). The carrier control for inhibitors dissolved initially in ethanol was 0.05% (the highest concentration in any sample). Following addition of inhibitors the cells were incubated at 37°C, 5% CO₂ for 5 hours. Cells were harvested and release measured as described in section 4.2.3.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Function</th>
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<tr>
<td>Monensin</td>
<td>Inhibitor of classical protein secretion. Acts as a Na⁺ ionophore that collapses intracellular Na⁺ and H⁺ gradients within the Golgi, therefore blocking glycoprotein secretion.</td>
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<tr>
<td>Brefeldin A</td>
<td>Inhibitor of classical protein secretion. Inhibits ER/Golgi dependent protein secretion</td>
</tr>
<tr>
<td>Methyl-β-cyclodextrin</td>
<td>Inhibitor of non-classical protein secretion. Disrupts protein secretion that occurs via lipid rafts in the cell membrane.</td>
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<tr>
<td>Methylamine</td>
<td>Inhibitor of non-classical protein secretion. Disrupts endocytosis.</td>
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Table 4.1 Summary of inhibitor functions (Broquet et al., 2003. Nickel et al., 2003)

### 4.2.3 Collection, storage and analysis of cellular and media samples

Following treatments, the cells from each sample were pelleted by centrifugation (500 g, 3 min, RT). The media was carefully removed from each cell pellet and transferred to clean centrifuge tubes. The pelleted cells from each sample and any adherent cells remaining on the tube or plate were resuspended in a total quantity of either 1 ml PBS (for ELISA analysis), 1 ml western extraction buffer (for blots) or 0.4 ml mRNA extraction buffer (section 2.2.17). The samples were frozen and stored at -20°C until analysis was carried out.
The media obtained from each sample was centrifuged (500 g, 3 min, RT), before being drawn off and transferred to clean tubes once more. This was repeated once more, and the media was checked using a haemocytometer to ensure no cells remained in the media samples. The media samples were frozen and stored at −20°C until analysis was carried out.

Media and intracellular samples were analysed using one or more of the following: Hsp60 ELISA (section 2.3.17), Hsp70 ELISA (section 2.3.18), Western Blot (section 2.3.15), RT-PCR (section 2.3.12). Cell viability counts (section 2.3.7) and LDH measurements (section 2.3.19) were carried before and after treatments.

4.2.4 Isolation of T and B cells

B- and T- cells were isolated by negative selection from isolated PBMCs (section) using Dynal Biotech B- or T cell negative isolation kit (section 2.2.62 and 2.2.61) according to the manufacturers protocol. In summary, PBMCs (section 2.3.6) were resuspended in PBS containing 0.1% BSA to give a density of 1 x 10^7 cells in 200 μl, and 20 μl Antibody Mix was added per 200 μl. The cells were incubated with the antibody mix for 10 mins at 2 – 8°C, and following incubation they were washed by adding 1 ml PBS, and centrifuging for 8 min at 500 g. The supernatant was removed and the cells were resuspended in 0.9 ml PBS per 1 x 10^7 cells and washed Dynabeads (100 μl beads per 1 x 10^7 cells) were added. The cells were incubated at 2 – 8°C for 15 minutes with gentle shaking. After incubation 1 ml PBS per 1 x 10^7 cells was added, and the beads were resuspended by careful pipetting. The preparation was then placed over the magnet for 2 minutes, and the supernatant containing the isolated T- or B cells was removed by pipette and transferred to a fresh tube.

4.2.5 Hsp release from T and B cells

Prior to treatment isolated T and B cells (section 4.2.4) were pelleted by centrifugation (500 g, 3 min) and resuspended in supplemented RPMI (section 2.2.13) to give a density of 8 x 10^5 cells/ml and the cells were transferred to 15 ml centrifuge tubes (1 ml per tube, triplicate samples). A t0 control sample was harvested by centrifugation
(section 4.2.3), and the remaining samples were incubated for 24 h at 37°C, 5% CO₂. Samples were then prepared for storage and analysis (section 4.2.3. Hsp70 levels in cells and media were quantified by Hsp70 ELISA (section 2.3.1.8).
4.3 Results

4.3.1 Hsp60 release from U937, Jurkat and PBMCs

Hsp60 was released from U937, Jurkat and PBMCs under normal cell culture conditions and following heat stress. The amount released varied between the cell types; following a 5 h incubation at 37°C Jurkat cells released the most Hsp60 (7.70 ± 0.07 ng/10⁶ cells) followed by U937 cells (2.42 ± 0.03 ng/10⁶ cells) and PBMCs (0.13 ± 0.007 ng/10⁶ cells) (table 4.2) (figure 4.1, 4.3 and 4.5). When released Hsp60 is calculated as a percentage of total cell Hsp60 it can be seen that U937, Jurkat and PBMCs release a relatively similar proportion of their total Hsp60 (9.16 %, 7.71% and 5.06 % respectively) (table 4.2).

Hsp60 release was elevated following 42°C heat shock in U937 cells, but not in Jurkat or PBMCs; release decreased following 45°C heat shock in all cell types (figure 4.1, 4.3 and 4.5). Intracellular Hsp60 increased significantly following 42°C heat shock in U937 cells and PBMCs (figure 4.2, 4.6), but showed no change in Jurkat cells (figure 4.4). Following a 45°C heat shock intracellular Hsp60 showed no change from the control in U937 cells (figure 4.2), decreased in Jurkat cells (figure 4.4), and significantly increased in PBMCs (figure 4.6).

4.3.2 Hsp70 release from U937, Jurkat and PBMCs

Hsp70 was released from all cell lines tested under normal cell culture conditions, and following heat shock. As with Hsp60, the amount of Hsp70 release varied between cell types; following 5 h incubation at 37°C Jurkat cells released the largest amount of Hsp70 (0.98 ± 0.09 ng/10⁶ cells), followed by U937 cells (0.58 ± 0.11 ng/10⁶ cells) and PBMCs (0.30 ± 0.001 ng/10⁶ cells) (table 4.2) (figure 4.7, 4.9 and 4.11). When release of Hsp 70 is calculated as a percentage of total cell Hsp70 it can be seen that U937, Jurkat and PBMCs release different proportions of their total Hsp70 (1.16%, 5.01% and 11.4% respectively) (table 4.2).
Hsp70 release from PBMCs under normal cell culture conditions showed a steady increase up to 24 h (figure 4.13). Intracellular Hsp70 levels initially decreased significantly within the first 2 h, but then returned to initial concentrations (figure 4.14).

Hsp70 release was increased following 42 °C and 45°C heat shock in all cell lines when compared to the 37°C control (figure 4.7, 4.9 and 4.11). Intracellular Hsp70 in U937 cells did not show a significant change from the control following 42°C heat shock, but showed a significant decrease following a 45°C heat shock (figure 4.8). Jurkat and PBMCs showed significant increase in intracellular Hsp70 after a 42°C heat shock and a significant decrease following a 45°C heat shock when compared to the 37°C control (figure 4.10, 4.12).

4.3.3 Western blot and gene expression

Western blots of culture medium and cell extracts confirmed the identity of the released Hsp60 and Hsp70 (figure 4.15 and 4.16). Gene expression for Hsp60 in U937 and Jurkat showed little change in response to a 42°C heat shock, a decrease in expression was seen at 45°C (figure 4.17 and 4.18). Hsp70 expression increased in response to a 42°C and 45°C heat shock in U937 cells, and in response to a 42°C heat shock in Jurkat cells (figure 4.17 and 4.18).

4.3.4 LDH and cell viability counts

The intracellular enzyme LDH was used as a control for release of intracellular proteins caused by cell damage/lysis. The percentage of total cell LDH found in the media was low for U937, Jurkat and PBMCs (0.7 %, 0.9 % and 0.4 % respectively) (table 4.2) when compared to the percentage of total cell Hsp60 and Hsp70 released under the same conditions and time (table 4.2). These results indicate that significant release is occurring that is not due to cell damage under normal cell culture conditions. Percentage cell death remained low in all cell lines (<0.1%) following incubation for 5 h at 37°C (table 4.2).
4.3.5 Protein release inhibitors

In order to further investigate if the Hsp release being observed was active release rather than cell damage related a range of protein release inhibitors were added prior to cell incubation. The maximum inhibitor concentrations that could be used without significantly reducing cell viability were selected by exposing cells to a range of concentrations followed by MTS analysis (data not shown).

4.3.5.1 Inhibition of Hsp60 release

Release of Hsp60 from PBMCs under normal cell culture conditions (37°C, 5 h) was significantly inhibited by both Monensin and Brefeldin A; the ethanol control showed no significant difference to the control (figure 4.20). Intracellular Hsp60 levels were also significantly affected by Monensin and Brefeldin A1, though Monensin had the most marked effect (figure 4.21).

4.3.5.2 Inhibition of Hsp70 release

The range of inhibitors tested for effect on Hsp70 release was extended to include inhibitors of non-classical protein secretion as initial work suggested that Hsp70 release was not inhibited by classical release inhibitors (data not shown). Hsp70 release was significantly reduced by Monensin, Methyl-β-cyclodextrin, and Methylamine but not by Brefeldin A (figure 4.22). Intracellular Hsp70 levels were significantly reduced by Monensin, Brefeldin A and Methyl-β-cyclodextrin (figure 4.23). Methylamine was the only inhibitor to cause no reduction in intracellular levels (figure 4.23).

4.3.6 T and B cell Hsp release

Though the monocyte (U937) cell line and T-cell line (Jurkat) both showed release it was decided it was important to determine if release occurred in non-transformed isolated T and B cells. Hsp60 release was not accurately detectable in -T and -B cell samples; this was most likely due to low cell numbers obtained by the isolation method
and the detection limit of the assay being used. Hsp70 release was observed in both T and B cells (figure 4.19). Isolated B cells released a significantly higher level of Hsp70 (8.2 ng/10^6 cells) compared to isolated T-cells (4.4 ng/10^6 cells).
Table 4.2. Release of Hsp60 and Hsp70 from cultured U937, Jurkat, and PBMCs (all cells).

<table>
<thead>
<tr>
<th></th>
<th>U937</th>
<th>Jurkat</th>
<th>PBMC</th>
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<tr>
<td><strong>Hsp60 release into medium (ng/10^6 cells) after 5h at 37°C. n=3</strong></td>
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<td>U937</td>
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<td><strong>Hsp60 release as percentage of total cell Hsp60. n=3</strong></td>
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<td>Jurkat</td>
<td>5.01</td>
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<td>PBMC</td>
<td>11.4</td>
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<td><strong>LDH release as % of total cell LDH. n=3</strong></td>
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<tr>
<td>U937</td>
<td>0.7 ± 0.14</td>
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<tr>
<td>Jurkat</td>
<td>0.9 ± 0.22</td>
<td></td>
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<tr>
<td>PBMC</td>
<td>0.4 ± 0.1</td>
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<td><strong>% cell death following incubation for 5 h at 37°C (determined by trypan blue).</strong></td>
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<tr>
<td>U937</td>
<td>&lt; 0.1 %</td>
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<tr>
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<td>&lt; 0.1 %</td>
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<tr>
<td>PBMC</td>
<td>&lt; 0.1 %</td>
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Figure 4.1. Release of Hsp60 into cell culture media from U937 cells in normal and heat shock conditions. The results after culture at 37°C, 42°C and 45°C for 2 h followed by 3 h incubation at 37°C are shown (minus t0 control). Data presented as mean ± SEM, n = 3. * p <0.05, ** p <0.01, *** p <0.001 (significant difference from 37°C control).
Figure 4.2. Intracellular Hsp60 levels in U937 cells cultured in normal and heat shock conditions. The results at time zero (t.0) and after culture at 37°C, 42°C and 45°C for 2 h followed by 3 h incubation at 37°C are shown. Data presented as mean ± SEM, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from 37°C control).
Figure 4.3. Release of Hsp60 into cell culture media from Jurkat cells in normal and heat shock conditions. The results after culture at 37°C, 42°C and 45°C for 2 h followed by 3 h incubation at 37°C are shown (minus t0 control). Data presented as mean ± SEM, n = 3. * p <0.05, ** p <0.01, *** p <0.001 (significant difference from 37°C control).
Figure 4.4. Intracellular Hsp60 levels in Jurkat cells cultured in normal and heat shock conditions. The results at time zero (t.0) and after culture at 37°C, 42°C and 45°C for 2 h followed by 3 h incubation at 37°C are shown. Data presented as mean ± SEM, n = 3.

* p < 0.05,  ** p < 0.01,  *** p < 0.001 (significant difference from 37°C control).
Figure 4.5. Release of Hsp60 into cell culture media from PBMCs in normal and heat shock conditions. The results after culture at 37°C, 39°C, 42°C and 45°C for 2 h followed by 3 h incubation at 37°C are shown (minus t0 control). Data presented as mean ± SEM, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from 37°C control).
Figure 4.6. Intracellular Hsp60 levels in PBMCs cultured in normal and heat shock conditions. The results at time zero (t.0) and after culture at 37°C, 39°C, 42°C and 45°C for 5 h are shown. Data presented as mean ± SEM, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from 37°C control).
Figure 4.7. Release of Hsp70 into cell culture media from U937 cells in normal and heat shock conditions. The results after culture at 37°C, 42°C and 45°C for 2 h followed by 3 h incubation at 37°C are shown (minus t0 control). Data presented as mean ± SEM, n = 3. * p < 0.05, **p < 0.01, ***p < 0.001 (significant difference from 37°C control).
Figure 4.8. Intracellular Hsp70 levels in U937 cells cultured in normal and heat shock conditions. The results at time zero (t.0) and after culture at 37°C, 42°C and 45°C for 5 h are shown. Data presented as mean ± SEM, n = 3. * p <0.05, ** p <0.01, *** p <0.001 (significant difference from 37°C control).
Figure 4.9. Release of Hsp70 into cell culture media from Jurkat cells in normal and heat shock conditions. The results after culture at 37°C, 42°C and 45°C for 2 h followed by 3 h incubation at 37°C are shown (minus t0 control). Data presented as mean ± SEM, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from 37°C control).
Figure 4.10. Intracellular Hsp70 levels in Jurkat cells cultured in normal and heat shock conditions. The results at time zero (t.0) and after culture at 37°C, 42°C and 45°C for 5 h are shown. Data presented as mean ± SEM, n = 3. * p <0.05, ** p <0.01, *** p <0.001 (significant difference from 37°C control).
Figure 4.11. Release of Hsp70 into cell culture media from PBMCs in normal and heat shock conditions. The results after culture at 37°C, 39°C, 42°C and 45°C for 2 h followed by 3 h incubation at 37°C are shown (minus t0 control). Data presented as mean ± SEM, n = 3. * p <0.05, ** p <0.01, *** p <0.001 (significant difference from 37°C control).
Figure 4.12. Intracellular Hsp70 levels in PBMCs cultured in normal and heat shock conditions. The results at time zero (t.0) and after culture at 37°C, 39°C, 42°C and 45°C for 5 h are shown. Data presented as mean ± SEM, n = 3. * p <0.05, ** p <0.01, *** p <0.001 (significant difference from 37°C control).
Figure 4.13. Release of Hsp70 into cell culture media from PBMCs after culture at 37°C for 2, 4, 6 and 24 h (minus t0 control). Data presented as mean ± SEM, n = 3.

* p <0.05,  ** p <0.01,  *** p <0.001 (significant difference from initial timepoint).
Figure 4.14. Intracellular Hsp70 in PBMCs after culture at 37°C for 2, 4, 6 and 24 h. Data presented as mean ± SEM, n = 3. * p <0.05, ** p <0.01, *** p <0.001 (significant difference from initial timepoint).
Figure 4.15. Western blot results for Hsp60 and Hsp70 in media and cell lysates from heat shocked U937 cells.

Lane 1. t.0 (time zero). U937 media.
Lane 2. 37°C, 5 h. U937 media.
Lane 3. 42°C, 5 h. U937 media.
Lane 4. 45°C, 5 h. U937 media.
Lane 5. t.0. U937 cell lysate.
Lane 6. 37°C, 5 h. U937 cell lysate.
Lane 7. 42°C, 5 h. U937 cell lysate.
Lane 8. 45°C, 5 h. U937 cell lysate.
Lane 9. Positive control.
Figure 4.16. Western blot results for Hsp60 and Hsp70 in media and cell lysates from heat shocked Jurkat cells.

Lane 1. t.0 (time zero). Jurkat media.
Lane 2. 37°C, 5 h. Jurkat media.
Lane 3. 42°C, 5 h. Jurkat media.
Lane 4. 45°C, 5 h. Jurkat media.
Lane 5. t.0. Jurkat cell lysate.
Lane 6. 37°C, 5 h. Jurkat cell lysate.
Lane 7. 42°C, 5 h. Jurkat cell lysate.
Lane 8. 45°C, 5 h. Jurkat cell lysate.
Lane 9. Positive control.
Figure 4.17. Hsp60 and Hsp70 expression in U937 cells in response to a 2 h heat shock.

Lane 1. Time zero (t.0) control.
Lane 2. 37°C (2 h).
Lane 3. 42°C (2 h).
Lane 4. 45°C (2 h).
Figure 4.18. Hsp60 and Hsp70 expression in Jurkat cells in response to a 2 h heat shock.

Lane 1. Time zero (t.0) control.
Lane 2. 37°C (2 h).
Lane 3. 42°C (2 h).
Lane 4. 45°C (2 h).
Figure 4.19. Release of Hsp70 into cell culture media from T and B-cells after culture at 37°C for 5 h at 37°C (minus t0 control). Data presented as mean ± SEM, n = 3.

* p <0.05, ** p <0.01, *** p <0.001 (significant difference from T-cells).
Figure 4.20. Effect of protein release inhibitors on Hsp60 release from PBMCs during 5 h incubation at 37°C. Data presented as mean ± SEM, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 4.21. Effect of protein release inhibitors on intracellular Hsp60 in PBMCs during 5 h incubation at 37°C. Data presented as mean ± SEM, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 4.22. Effect of protein release inhibitors on Hsp70 release from PBMCs during 5 h incubation at 37°C. Data presented as mean ± SEM, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
Figure 4.23. Effect of protein release inhibitors on intracellular Hsp70 in PBMCs during 5 h incubation at 37°C. Data presented as mean ± SEM, n = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 (significant difference from control).
4.4 Discussion

The aim of the work in this chapter was to determine whether Hsp60 and Hsp70 could be released from immune cells, and if so, to investigate which cells release Hsps, and if it is possible for the release to be affected by protein release inhibitors.

The results of the work demonstrated that both Hsp60 and Hsp70 are released from immune cells in both normal conditions, and in response to heat stress. Significant release of both Hsp60 and Hsp70 was observed from U937 cells, Jurkat cells and PBMCs; the release of complete Hsp60 and Hsp70 proteins was confirmed by western blot. Isolated T and B cells were found to release significant levels of Hsp70.

After it was determined that Hsps were being released, the main aims of the work were to determine whether this release was active, or due to cellular damage. The initial control for damage related release was a t0 sample, which was subject to the same initial isolation treatment and centrifugation treatment as the samples that were harvested following incubation. The media from these samples was consistently found to contain very low levels of Hsp60 and Hsp70, and the value obtained was subtracted from total release in all experiments. Total intracellular Hsp values at t0 were found not to be significantly different from intracellular Hsp levels following 5 h incubation at 37°C in the majority of experiments, indicating the cells were not losing Hsps due to damage/lysis. Trypan blue viability counts confirmed that no significant damage to the cells was occurring during the incubation period at 37°C.

As a further control for damage related release, the percentage release of the intracellular enzyme LDH into the culture media was determined. The results showed that the percentage release of LDH was significantly lower than that of Hsp60 and Hsp70 in all cell types, therefore providing further evidence that some of the observed release was active rather than related to damage. LDH has been previously used to correct Hsp 70 release values (Liao et al., 2000) and percentage LDH values higher than those seen in this work have been reported (Broquet et al., 2003).
As the evidence in the results was pointing strongly to the possibility of active release, the next step was to determine if inhibitors of protein release could block the secretion of Hsps. Initially inhibitors of classical protein secretion were tested; Brefeldin A which blocks transport of polypeptides from the ER to the Golgi, and Monensin which is a Na\(^+\) ionophore that collapses intracellular Na\(^+\) and H\(^+\) gradients within the Golgi, therefore blocking glycoprotein secretion. Both Monensin and Brefeldin A significantly inhibited Hsp60 release, while Hsp70 release was inhibited by Monensin, but not by Brefeldin A. As the results for Hsp70 were contradictory it was decided that inhibitors of non-classical protein secretion should be tested. It had already been proposed that Hsp70 release occurs via lipid rafts, and that this release was inhibited by Methy-β-cyclodextrin (Broquet et al., 2003). Methyl-β-cyclodextrin which disrupts membrane rafts was therefore tested along with Methylamine, which is known to disturb endocytosis (Nickel, 2003). The results showed both Methyl-β-cyclodextrin and Methylamine significantly inhibited Hsp70 release from PBMCs.

The results for Hsp70 could be interpreted as being contradictory, as an inhibitor of classical secretion (Monensin) inhibited release along with two inhibitors of non-classical secretion (Methyl-β-cyclodextrin, Methylamine). As Brefeldin A did not inhibit it is possible that Hsp70 secretion involves the ER but not the Golgi. Another possibility could be a non-specific inhibition of Hsp70 release caused by monensin; monensin has the potential to cause cell cycle arrest and apoptosis at relatively low concentrations (Park et al., 2002), and whilst MTS and trypan blue staining did not indicate that cell death occurred during the 5h experiment, it is possible that more general effects of monensin caused the reduction in release, rather than specific inhibition at the point of the Golgi. Overall the results of the inhibitor work suggested a classical secretion route for Hsp60 and a non-classical secretion route for Hsp70, though the limitations created by the potential of the protein release inhibitors to affect the cells in non-specific ways needs to be considered.

The results of the previous chapter (Chapter 4) demonstrated that it is possible for Hsps to affect the RANKL/OPG system of bone regulation in a way that could lead to increased bone resorption. The results of this chapter provide a possible mechanism by which bone cells could be exposed to Hsps in vivo; either through an elevation in serum
hspS caused by immune cell release, or by a local increase in hspS caused by a high presence of Hsp releasing immune cells as would occur in the case of autoimmune disease such as rheumatoid arthritis (Martin et al., 2003), or in the case of localised infection. The next step in this thesis will therefore be to investigate whether Hsp release can be affected by other factors, such as immune cell activation, suppression of activation, or by exposure to other HspS. The effect of these treatments on the expression of other cytokines that may influence bone resorption will also be examined.