Chapter 4

Exercise and regulation of Hsp72 localisation in humans

4.1 Introduction

Immune cell activation and inflammation have a key role in regulating the expression of HSPs. Inflammation is activated with intensive exercise together with the release of stress hormone norepinephrine: these are signs that HSP protein expression and release would be activated. In the previous chapter the induction and production of Hsp72 in different tissues was examined and it was possible to suggest possible sources of serum Hsp72. certain mechanisms by which cells could increase Hsp72 secretion were suggested. In this chapter the attention was focused on investigating the possible mechanisms of serum Hsp72 increase by using an in vitro cell culture model and a novel in vivo exercise protocol.

Hsp72 transcription, protein synthesis and its appearance outside cells increases in response to exercise together with activation of an inflammatory response (Steensberg et al. 2006; Suzuki et al. 2006). In particular exercise increases IL-6 production and release from muscle (Febbraio et al. 2002). Many cells seem to express Hsp72 in response to exercise, in particular muscle cells (Febbraio et al. 2002) and leukocytes (Fehrenbach et al. 2000). Hsp72 gene expression, for example increases in response to strenuous exercise such as running, and remains high for the following 24 hours after the exercise (Fehrenbach et al. 2000). The expression of intracellular and plasma Hsp72 has been extensively documented when strenuous exercise has been applied (Walsh et al. 2001; Fehrenbach et al. 2005; Suzuki et al. 2006); the intensity of the exercise and the duration seemed to affect Hsp72 release with a higher increase of soluble Hsp72 for longer and more intensive exercise (Fehrenbach et al. 2005). Eccentric exercise seemed to induce an inflammatory response with plasma IL-6 increase (Toft et al. 2002) and Hsp72 increase although to a lesser degree if compared with endurance exercises where the muscle damage is more systemic if compared with eccentric exercise (Toft et al. 2002; Hirose et al. 2004). No data has been collected before regarding the Hsp72 expression and plasma levels with intensive anaerobic training, the Wingate test (Hussain et al. 1996), where IL-
12 cytokine, a NK cell stimulatory factor has been shown to be released immediately after exercise with a restoration to the initial levels with time of rest (Akimoto et al. 2000). The Wingate exercise determine also an increase in lactate level, as a marker for muscle damage, which remains high for long time after the exercise and potassium increase only straight after the exercise (Hussain et al. 1996).

Many studies suggest an association between the release of IL-6 and Hsp72, in particular in muscle where IL-6 is a marker of muscle damage (Pedersen et al. 2004; Peake et al. 2005; Fischer 2006; Suzuki et al. 2006), there is a concomitant increase of plasma Hsp72 (Febbraio et al. 2002): this increase is modulated by the amount of glycogen available in the muscle (Febbraio et al. 2004). Plasma IL-6 increases after a strenuous exercise and varies between athletes, together with LPS diffusion from intestine, cortisol release, high body temperature, oxidative stress and muscle damage, potentially all inducers of an HSP response. In order to induce an IL-6 release from muscle the exercise needs to be intensive and long enough, with many muscle contractions together with an impaired glucose availability (Fischer 2006). Glucose deprivation increased Hsp72 in cultured cells, whereas reduced glycogen availability elevates Hsp72 in contracting human skeletal muscle (Febbraio et al. 2004). Following an exercise of up to 1 hour duration, the exercise intensity appeared to have a greater effect on the anti-inflammatory cytokine production than exercise-induced muscle damage (Peake et al. 2005). After a short anaerobic exercise such the Wingate test, markers of muscles damage such as plasma lactate concentration increases (Hussain et al. 1996) together with IL-12 cytokine, a natural killer cell stimulator factor that has been shown to be released immediately after the exercise with a restoration of the initial levels with time of rest (Akimoto et al. 2000). However no evidence has been collected before regarding Hsp72 expression and release with intensive anaerobic training, the Wingate test. IL-6 release in a Wingate trial has been shown to be recorded in a study where arachidonic acid supplementation, a fatty acid main component of peanut oil, was tested in order to see if it helped anaerobic exercise in decreasing the inflammatory response (Roberts et al. 2007); however no data has been collected looking the effect of IL-6 release in plasma and Hsp72 in plasma after a Wingate exercise procedure. Anti-inflammatory drugs such as ibuprofen and aspirin have been shown to
change the expression profile of the pro- and anti-inflammatory cytokines. Ibuprofen at maximal doses of 1200 mg has no effect in inflammatory response after eccentric exercise (Peterson et al. 2003), and has no effect on muscle hypertrophy and strength and does not affect rating of muscle soreness (Krentz et al. 2008). A single dose of 1000 mg aspirin did not affect physical performance during sub-maximal and maximal exercise on a cycle ergometer (Roi et al. 1994).

Interestingly, intensive exercise together with high temperature induce the release of the stress hormone norepinephrine in the circulation (Powers et al. 1982; Schwarz and Kindermann 1990). *In vivo* models hypothesised the induction of extracellular Hsp72 as a consequence of norepinephrine release under psychological stress in rats by stimulation of α1 adrenoreceptors (Johnson and Fleshner 2006). Therefore it is reasonable to hypothesise that intense exercise together with a physiological temperature increase might induce norepinephrine release which in turn would stimulate Hsp72 release as part of the immune response to exercise induced cellular stress. The studies done so far have concentrated on *in vivo* animal models, but not on a tissue culture model that could investigate more in detail the mechanism by which α-1 adreno-receptors are activated.

Thus the aim of the study was:

- To test the ability of inflammatory cytokines to modulate the Hsp72 movement *in vitro* in activated macrophages.
- To explore the role of adreno-hormones *in-vitro* in term of the Hsp72 up-regulation and movement.
- To determine whether an intensive anaerobic exercise could induce a stress response by Hsp72 expression.
- To test whether the inflammatory response was involved in the induction of Hsp72 by analysing several cytokines release in plasma (IL-6, TNF-α, IL-10).
- To explore the effects of aspirin and ibuprofen supplementation and the release of pro- and anti-inflammatory cytokines on Hsp72 after an intensive anaerobic exercise, 30 second maximal intensity Wingate test.
4.2 Methods and experimental design

4.2.1 *in-vitro* experiments

U937 monocytic cell line was used and transformed into macrophages by treatment with phorbol-γ-myristate-acetate (PMA) as fully described in Chapter 2. A visual example of the activated macrophages is shown in Figure 4.1 below.

![Visualization of activated macrophages morphology in several pictures.](image)

Inflammatory cytokine treatments were applied to the cells at a physiological concentration compatible to the ones usually found in the blood stream after an inflammation stimulus: interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) were used at a concentration range between 5 to 150 ng/ml for 2 and 4 hours and when necessary 24 hours.

Adreno-hormones used were epinephrine and norepinephrine at concentrations compatible to the ones found physiologically in the body after a stress stimulus (~10 μM). Cells were incubated for 2 hours with the hormone
and then analysed straight after. Adreno-hormone receptor inhibitor prazosin was used in order to block the activation through a specific pathway at concentrations 1μM; a 1 hour pre-incubation with prazosin was applied to the cells before being treated with the hormones. Heat shock positive control (2h hours at 42°C) was included in each experiment in order to test the goodness of the experiment (data not shown).

Statistical analysis used was one way ANOVA with Tukey's post hoc test and two way ANOVA with Bonferroni post hoc test with a double analysis: comparing first, the difference between the control and the other time points in each concentration and another test analysing the non treated control and the different concentrations within the same time point.

4.2.2 Exercise protocol method

Chester University research ethics committee approval was obtained for the study, an informed consent form were completed by each volunteer participating to the trial.

A group of trained males (N=12) were subjected to the Wingate test which is considered an anaerobic test that does not involve any aerobic workout. The protocol, was organised according with the standard protocol used previously (Hussain et al. 1996): voluntary people were subjected to 30 sec maximum intensity cycling exercise with a pre-set friction load of 7.5 % of the subject's body weight applied to each bike; each subject was allowed to warm up for 10 min at 80 watts power; the exercise although short is very intensive, and to achieve the maximal work, the subjects were encouraged to cycle at their maximal attainable pedalling frequency throughout the test. 10 minutes of recovery after the exercise at 80 watts was allowed to each subject. Participants were divided into 3 groups and administered Ibuprofen (IBU, 1200mg) or Aspirin (ASP, 2700mg) and compared to a control group (CON) with no anti-inflammatory treatment. Drugs were administered into 3 doses of 400 mg and 900 mg of ibuprofen and aspirin respectively. The first 2 doses were dispensed the day before 24 hours and 12 hours before the test; the last one was administered in the morning 2 hours before the exercise with in conjunction with a light breakfast in between.

Blood samples were taken before the exercise, 10 minutes after, and 1 hour after the second blood taking when the recovery from the exercise should be
completed. Hsp72 mRNA was purified first with m-RNA extraction kit, c-DNA synthesis was performed and real time RT-PCR was carried out, using Hsp72 gene expression assay with Taqman® technology and protocols were followed according the methods section in Chapter 2. Intracellular protein expression was measured by flow cytometry techniques using a monoclonal anti-Hsp72-FITC antibody (Assay design) and it was carried out a comprehensive study of all the different leukocytes sub populations; therefore CD markers were used in conjunction with Hsp72: CD19-PE, CD4-PE/Cy7, CD8-APC/Cy7, CD56-APC all from BDBiosciences were used to detect B-lymphocytes, T-helper lymphocytes, T-cytotoxic lymphocytes, NK-lymphocytes respectively, while monocytes and granulocytes populations were distinguished from their size from the FSC/SSC dot plot. 10000 events were acquired, populations were gated using the CD markers and FSC/SSC and percentage of cells expressing Hsp72 was recorded in each population. An example of the analysis method is represented in Figure 4.1. Total Plasma concentrations of IL-6, TNF-α, and IL-10 was measured using quantitative sandwich ELISA (e-Bioscience). Data are expressed as mean SD and statistical analysis was performed using graph-pad; analysis of variance was done using one sample t-test and two way ANOVA with Bonferroni post hoc test.
Figure 4.2: Gating strategy for cell analysis
The Table below show the population gating that has been chosen for the study: (A) leukocytes are distinguished by their CD45 marker fluorescence intensity; (B) Hsp72 positive leukocytes are analysed looking the percentage of the cells positive to Hsp72; (C) T-Helper lymphocytes positive to Hsp72 have been analysed by using the CD4 marker; (D) T-Cytotoxic lymphocytes are analysed by using the CD8 marker; (E) NK cells lymphocytes have been analysed by using the CD56 marker; (F) B-lymphocytes have been analysed by using the CD19 marker. The lymphocyte subtypes are all gated on the red lymphocyte gate from graph A.
4.3 Results

4.3.1 *in-vitro* experiments

4.3.1.1 IL-6 stimulation of macrophages

Activated macrophages were treated with IL-6 as above described. The treatment was not cytotoxic as expected, with no change in cell viability, necrosis (PI) and apoptosis (Figure 4.3A-B-C). However, Hsp72 levels and movement towards the external environment vary with IL-6 treatment and is up-regulated: intracellular Hsp72 levels increased with the highest IL-6 concentration, \( P<0.05 \) at 150 ng/ml IL-6 (Figure 4.4A). Surface Hsp72 increased slightly \( P<0.05 \) at 5 and 150 ng/ml (Figure 4.4B) and extracellular Hsp72 release is induced when macrophages are treated with the highest IL-6 concentration \( P<0.05 \) for 150 ng/ml (Figure 4.4C).
Figure 4.3: Cell activity, necrosis and apoptosis test in IL-6 treated U937 activated macrophages. Activated U937 macrophages were treated with increasing concentrations of IL-6 (5-150 ng/ml) for 4 hours (A) cell activity was tested by MTS assay. (B) propidium iodide fluorescence signal was tested by fluorimetric assay. (C) caspase-3 activity was tested by fluorimetric assay.
Figure 4.4: Intracellular, surface and extracellular Hsp72 levels following IL-6 treatment in activated U937 macrophages. Activated U937 macrophages were treated with increasing concentrations of IL-6 (5-150 ng/ml) for 4 hours (A) intracellular Hsp72 was examined by flow cytometry. (B) surface Hsp72 was examined by flow cytometry (C) extracellular Hsp72 was tested by ELISA in cell culture supernatant. *=P<0.05
4.3.1.2 Treatment with TNF-α

Treatment with TNF-α decrease cell activity, with all the concentrations used (P<0.01 with both concentrations) (Figure 4.5A). Concomitantly there is an increase of cell death by necrosis (P<0.05 with 5 ng/ml and P<0.01 with 150 ng/ml TNF-α) (Figure 4.5B) but not by apoptosis: in fact caspase-3 activity remains unchanged (Figure 4.5C).

Hsp72 localization seemed to be influenced by TNF-α treatment: despite intracellular Hsp72 remaining stable there is a decrease of surface Hsp72 presentation (P<0.05 with 150 ng/ml TNF-α) (Figure 4.6B) with a concomitant increase of Hsp72 release (P<0.05 with 150 ng/ml TNF-α) (Figure 4.6C); this is probably due to necrosis cell death so it cannot be caused by any active transport.
Figure 4.5: Cell activity, necrosis and apoptosis detection in U937 activated macrophages treated with TNF-α. Activated U937 macrophages were treated with increasing concentrations of TNF-α (5-150 ng/ml) for different 4 hours (A) cell activity was tested with MTS assay. (B) necrosis was tested with propidium iodide (PI) fluorescence. (C) apoptosis was tested detecting activated Caspase-3 with a fluorimetric assay. *=P<0.05; **=P<0.01.
Figure 4.6: Intracellular, surface and extracellular Hsp72 detection after TNF-α treatment in U937 activated macrophages. Activated U937 macrophages were treated with increasing concentrations of TNF-α (5-150 ng/ml) for 4 hours (A) intracellular Hsp72 was tested by flow cytometry. (B) surface Hsp72 was tested by flow cytometry. (C) extracellular Hsp72 was tested in cell culture supernatant by ELISA. *=P<0.05
4.3.1.3 Treatment with IFN-γ

IFN-γ treatment on activated U937 cells slightly decreased cell activity at 5 ng/ml (P<0.05) but did not seem to be cytotoxic for the cells since necrosis is not present (Figure 4.7).

Hsp72 localization seemed to be influenced by IFN-γ treatment: despite intracellular Hsp72 remain stable there is a decrease of surface Hsp72 presentation (P<0.05 with 5 and P<0.001 with 150 ng/ml IFN-γ) (Figure 4.8B) with a concomitant increase of Hsp72 release (P<0.05 with 150 ng/ml IFN-γ) (Figure 4.8C); hence as confirmed by the literature, IFN-γ can induce Hsp72 release from cells, in this case macrophages.
Figure 4.7: Cell activity, necrosis, apoptosis detection after IFN-\(\gamma\) treatment in U937 activated macrophages. Activated U937 macrophages were treated with increasing concentrations of IFN-\(\gamma\) (5-150 ng/ml) for 4 hours (A) cell activity was tested by MTS assay. (B) necrosis was tested with propidium iodide (PI) fluorescence. (C) apoptosis was tested through active caspase-3 by fluorimetric assay. *\(=P<0.05\)
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Figure 4.8: Intracellular, surface, extracellular Hsp72 detection after IFN-γ treatment in U937 activated macrophages. Activated U937 macrophages were treated with increasing concentrations of IFN-γ (5-150 ng/ml) for 4 hours (A) intracellular Hsp72 was detected by flow cytometry. (B) surface Hsp72 was detected by flow cytometry. (C) extracellular Hsp72 was detected in tissue culture supernatant by ELISA. *=P<0.05; **=P<0.01; ***=P<0.001.
4.3.1.4 Epinephrine and prazosin treatment

U937 activated macrophages were treated with 10 μM of epinephrine in the presence or absence of α1-adrenoreceptor antagonist prazosin at 1 μM. Treatments were not cytostatic or cytotoxic since viability and necrosis levels remain stable (Figure 4.9). However Hsp72 levels are affected by the treatments: epinephrine only increase the intracellular Hsp72 level (P<0.05) (Figure 4.10A) and decreased surface levels (P<0.01) (Figure 4.10B) with no significant change in the release level (Figure 4.10C), suggesting that Hsp72 remains inside the cells. Co-treatment with the α1-adrenoreceptor antagonist prazosin determined a reduction of the intracellular Hsp72 up to non treated level (Figure 4.10A), a surface Hsp72 induction, although the levels are less than the control sample (Figure 4.10B) and increase release (P<0.05).
Figure 4.9: Cell activity, necrosis, apoptosis test in Epinephrine (E) and Prazosin treated U937 activated macrophages. Activated U937 macrophages were treated with 10 μM epinephrine with and without 1μM prazosin (A) cell activity was tested by MTS assay. (B) necrosis was tested by propidium iodide fluorescence staining assay. (C) apoptosis was tested by active caspase-3 fluorescence detection assay.
Figure 4.10: Intracellular, surface and extracellular Hsp72 levels on U937 activated macrophages after epinephrine and prazosin treatment. Activated U937 macrophages were treated with 10 μM epinephrine with and without 1μM prazosin (A) intracellular Hsp72 was tested by flow cytometry. (B) surface Hsp72 was tested by flow cytometry. (C) extracellular Hsp72 was tested in cell culture supernatant by ELISA. * = P<0.05; ** = P<0.01.
**4.3.1.5 Norepinephrine treatment and Prazosin treatment**

U937 activated macrophages were treated with 10 μM of norepinephrine in the presence or absence of α1-adrenoreceptor antagonist prazosin at 1 μM. The two compounds are not cytotoxic since cell viability and necrosis showed no variation when activated macrophages were treated with the two compounds (Figure 4.11A, B). Hormones modify the Hsp72 levels: norepinephrine induces intracellular Hsp72 (P<0.05) (Figure 4.12B) and in turn decreases surface (P<0.05) and extracellular Hsp72 (P<0.01) (Figure 4.12B, C) while co-treatment with prazosin blocks the intracellular induction restoring a similar level to the non treated control (Figure 4.12A), decreasing more the surface expression (P<0.001) (Figure 4.12B) and in extracellular Hsp72 (Figure 4.12C).
Figure 4.11: Cell activity, necrosis and apoptosis on U937 activated macrophages after norepinephrine and prazosin treatment. Activated U937 macrophages were treated with 10 μM norepinephrine with and without 1μM prazosin (A) cell activity was tested by MTS assay. (B) necrosis was tested by propidium iodide fluorescence assay. (C) apoptosis was tested by active caspase-3 fluorescence assay. *=P<0.05.
Figure 4.12: Intracellular, surface and extracellular Hsp72 level on U937 activated macrophages after norepinephrine and prazosin treatment. Activated U937 macrophages were treated with 10 μM norepinephrine with and without 1μM prazosin (A) Hsp72 level was tested by flow cytometry. (B) surface Hsp72 was tested by flow cytometry. (C) extracellular Hsp72 level was tested in cell culture supernatant by ELISA. *=P<0.05; **=P<0.01; ***=P<0.001.
4.3.2 Exercise experiments

4.3.2.1 Hsp72 gene expression after anti inflammatory drug administration.

The first parameter that was considered in the study was the Hsp72 gene expression in purified white blood cells with the Wingate exercise itself, therefore the Control (CON) group was analysed separately for Hsp72 activation. No previous studies had analysed the effect of an anaerobic exercise on the activation of the heat shock response. Hsp72 gene expression showed a significant activation of the Hsp72 transcription straight after the exercise (P<0.05) and a restoration of the normal levels of gene expression after the hour of recovery, which return to initial levels of gene expression (Figure 4.13). However, anti-inflammatory drug administration in subjects showed a significant decrease of Hsp72 gene expression (P<0.005) independently from the exercise (Figure 4.14). This decrease is shown in both ASP group and IBU group, before, after and 1 hour after the exercise suggesting an inhibitory role of both drugs in the Hsp72 gene expression rather than a significant effect of the exercise itself. The analysis of the single parameter showed that there were only differences after the exercise between the three groups. Firstly the expression was lower in controls and that treatments eliminated the effect of exercise (P<0.05 in CON-ASP, P<0.01 in CON-IBU).
**Figure 4.13: Hsp72 gene expression in Wingate exercise.**
RT-PCR in CON group was obtained from blood cells purified with the whole blood method; statistic performed was one sample t-test with Tukey’s post-hoc test in order to compare the first blood test with the others after the exercise; *=P<0.05; **=P<0.01.

**Figure 4.14: Hsp72 gene expression in Wingate anaerobic exercise after administration of anti-inflammatory drugs**
RT-PCR was performed in blood cells purified with the whole blood technique from CON, ASP and IBU volunteers. Two way ANOVA with Bonferroni post-hoc test was performed in order to test the effect of the anti inflammatory drugs compared with the CON group on the Hsp72 gene expression; ***=P<0.001.
4.3.2.2 Effects of the Wingate test and anti-inflammatory drug administration: Hsp72 protein in different blood populations

Analysis was extended to the different leukocyte populations in order to check the Hsp72 intracellular protein level in all of the population subtypes. Most of the populations did not seem to possess intracellular Hsp72 or experience a change in its expression (Table 4.1): in fact most of the leukocyte subtypes did not seem to be affected by the exercise procedure itself. However a significant intracellular Hsp72 level could be found in monocytes of CON group which showed a significant decrease straight after the exercise (P<0.01) followed by an increase back to baseline levels after 1 hour (Figure 4.15). The ASP group showed the same trend found in CON with a decrease of intracellular Hsp72 levels straight after the exercise and a restoration of the initial level during the recovery time. However there was no difference between CON and ASP and IBU in term of Hsp72 levels: the two way ANOVA which has been performed to test the interaction between the different treatments showed no difference between the three groups, suggesting that the anti-inflammatory drugs did not affect the Hsp72 intracellular levels in monocytes. Ibuprofen administration however did not seem to affect the Hsp72 levels as shown in the IBU group where no significant differences could be found (Figure 4.15).
Table 4.1: Lymphocyte sub-populations and Hsp72 protein during the exercise in different treated groups. Cells were gated using CD markers and percentage of cells positive to Hsp72 ± Standard Deviation between the 4 people forming the groups is represented.

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<td>CON</td>
<td>ASP</td>
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<tr>
<td><strong>Before Ex</strong></td>
<td>1.35 ±1</td>
<td>1.8 ±1.6</td>
<td>0.5 ±0.1</td>
<td>10.0 ±11.3</td>
</tr>
<tr>
<td><strong>After Ex</strong></td>
<td>1.0 ±1.1</td>
<td>0.6 ±0.5</td>
<td>0.4 ±0.3</td>
<td>7.1 ±7.8</td>
</tr>
<tr>
<td><strong>1h After Ex</strong></td>
<td>1 ±0.6</td>
<td>0.6 ±0.4</td>
<td>2.5 ±3.4</td>
<td>4.6 ±4.4</td>
</tr>
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Figure 4.15: Percentage of monocytes positive to Hsp72
Blood cells was gated by CD marking and FSC/SSC parameters; monocytes percentage of intracellular Hsp72 positive cells were analysed by flow cytometry in CON, ASP and IBU group. Statistical analysis performed were one way ANOVA with Tukey’s post hoc test performed separately in each data set. An additional two way ANOVA was performed in order to check the interaction between the treatments; *=P<0.05; **=P<0.01.
4.3.2.3 Effects of anti-inflammatory drug administration in subjects undertaking the Wingate exercise: pro- and anti-inflammatory cytokine release in plasma

The study was carried out to test whether the anaerobic exercise would change pro-inflammatory and anti-inflammatory cytokine release when the subjects were supplemented with anti-inflammatory drugs. The release of pro-inflammatory cytokines IL-6, also a marker of muscle damage and TNF-α, and the anti-inflammatory cytokine IL-10 have been analysed.

IL-6 plasma levels showed that in the ASP group the level of IL-6 was significantly elevated (P<0.05) (Fig 4.16) before the exercise and after the exercise but not after the recovery time when the level was comparable to the CON group. However no differences across time, between the different blood samples have been shown either in the CON nor in the ASP and IBU groups. In other words the exercise itself does not induce a change in term of IL-6 release in plasma as it is possible to highlight when looking at the CON group. The IBU group did not show any change of IL-6 levels when the exercise was performed and was maintained during the Wingate exercise (Figure 4.16).

Plasma TNF-α did not change level with exercise and with drug administration either, it was not possible to show statistical differences between the groups (Figure 4.17)

Plasma IL-10 showed no statistical differences between the drug groups: moreover in each group the exercise did not change the level of the anti-inflammatory cytokine, although it was possible to see a trend of an increase in the IBU group in response to exercise (Figure 4.18).
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Figure 4.16: Plasma IL-6 levels (pg/ml) in subjects treated with anti-inflammatory drugs.
Plasma from Wingate exercise subjects was analysed for IL-6 content. Statistical analysis performed was a two way ANOVA with Bonferroni post-hoc test comparing the amount of IL-6 released in CON with the two treated group ASP and IBU in every blood sample collected; **=P<0.01.

Figure 4.17: Plasma TNF-α levels (pg/ml) in subjects treated with anti-inflammatory drugs.
Plasma from subjects undergone the Wingate exercise was analysed for TNF-α content. Statistical analysis performed was a two way ANOVA with Bonferroni post-hoc test comparing the amount of TNF-α released in CON with the two treated group ASP and IBU in every blood sample collected.
Figure 4.18: Plasma IL-10 levels (pg/ml) in subjects treated with anti-inflammatory drugs.
Plasma from subjects undergone the Wingate exercise was analysed for IL-10 content. Statistical analysis performed was a two way ANOVA with Bonferroni post-hoc test comparing the amount of IL-10 released in CON with the two treated group ASP and IBU in every blood sample collected.
4.3.2.4 Serum Hsp72 level after anaerobic exercise

Serum Hsp72 was measured with the in-house Hsp72 ELISA and showed that drugs and exercise together can modify blood Hsp72 levels: CON groups showed a slight increase of serum Hsp72 straight after the exercise although the difference is not significant, but when looking at the ASP group, the exercise and in particular the rest time after the exercise reduced the Hsp72 levels (P<0.01) when compared to the non exercised samples; on the other hand the serum Hsp72 increased with exercise and ibuprofen as shown in the IBU group, especially after 1 hour rest (P<0.05)(Figure 4.19).

![Figure 4.19: Serum Hsp72 levels (ng/ml) in exercised subjects treated with anti-inflammatory drugs.](image)

Serum from subjects undergone the Wingate exercise was analysed for Hsp72 content. Statistical analysis performed was a two way ANOVA with Bonferroni post-hoc test comparing the amount of serum Hsp72 in CON with the two treated group ASP and IBU in every blood sample collected; *=P<0.05; **=P<0.01.
4.3.2.5 Hsp72 release in plasma after aerobic and anaerobic exercise

Hsp72 plasma content in the CON group was preliminarily analysed and showed that when plasma samples were analysed straight away no statistical difference between the samples could be seen with the exercise, while, when whole blood was left at 37°C for 1 hour and plasma was collected, an increase was seen in each sample type but also within the exercise timing, with an increase in particular after the recovery time (P<0.001), but also if each sample was compared with the non released samples (P<0.001) (Figure 4.20).

![Figure 4.20: Effect of exercise on Hsp72 in plasma straight from blood cells.](image)

Plasma samples of CON groups were collected and divided into 2 aliquots: one was kept in the -80°C until the analysis and the other was incubated for 1 hour at 37°C and then frozen at -80°C until the analysis. Statistical analysis performed were 2 way ANOVA with Bonferroni post hoc test, in order to test the difference between the plasma incubated at 37°C and the one not, in each data set, and to test the difference with the exercise; ***=P<0.001.
4.4 Discussion

In vitro cytokines and stress-hormones stimulation

The aim of the present study was to evaluate the in vitro effects of cytokine stimulations and adreno-receptor treatment in cell culture in order to investigate the role of these in terms of Hsp72 localization and aimed to build an effective model to study the mechanism. Activated macrophages were chosen for their primary characteristic of possessing the ability to produce cytokines and adreno-hormone receptors and for their primary role in heat shock protein release and uptake from the extracellular environment (Sondermann et al. 2000; Gao and Tsan 2004; Vega et al. 2008) and also because they play a key role in the first steps of the immune system activation. As previously shown, cytokine actively participate in the activation and release of HSP (Stephanou et al. 1998; Bajramovic et al. 2000; Ripley et al. 2001; Febbraio et al. 2002; Barreto et al. 2003). The present study concentrated on Hsp72 movement and release and, as shown from the results, cytokines induced a change in Hsp72 protein levels. IL-6 treatment up-regulated intracellular Hsp72 levels, as well as surface and contribute to an extracellular release at high concentrations.

IFN-γ treatment induced Hsp72 release from cells as previously demonstrated (Barreto et al. 2003; Bausero et al. 2005) and a concomitant decrease of surface Hsp72, whilst the intracellular levels remained stable, suggesting a possible de-novo synthesis that keeps the intracellular level stable. However TNF-α affected cell viability of activated macrophages and increased necrosis: hence the resultant increase of extracellular Hsp72 can only be explained by cell destruction by necrosis.

Hence pro-inflammatory cytokines, in particular IL-6 and IFN-γ interfere with Hsp72 release and movement of a cell compartments and association with membranes.

Adreno-hormones, such as epinephrine and norepinephrine have been implicated in Hsp72 release by in vivo studies where mice were psychologically stressed, and suggested a model where the induction of extracellular Hsp72 was a consequence of norepinephrine release via α1-adrenoreceptors activation (Johnson and Fleshner 2006). Intensive exercise together with high temperature also induces the release of the stress hormone norepinephrine in the blood circulation (Powers et al. 1982; Schwarz and
Kindermann 1990). Hence exercise stress can induce Hsp72 release not only by activation of the immune response, but also as a consequence of the norepinephrine release in blood. The studies done so far were concentrated on *in vivo* animal models, not in a tissue culture model that could investigate more in detail the mechanism by which α-1 adreno-receptors are activated. The activated macrophages cell model results suggest that epinephrine and norepinephrine induce accumulation of Hsp72 inside the cells actually inhibit surface Hsp72 presentation and release. However co-treatment with the α1 adreno-receptor blocker prazosin, which was the postulated receptor involved in Hsp72 activation (Johnson and Fleshner 2006) gave surprising results: despite blocking the α1 adreno-receptor, prazosin treatment caused a surface Hsp72 induction and a release at least similar to the non-treated samples, while it reduced the intracellular Hsp72 content. Therefore prazosin it is certainly a membrane interacting agent, since it modifies the level of Hsp72 present on the cells. In this cell model is it possible that prazosin interferes with other membrane associated structures for example ABC transporters, since it was found to be a substrate of one of them, ABCG2 (Nishimura et al. 1986; Litman et al. 2000). It has been suggested that Hsp72 can interact with several ABC transporters (Mambula and Calderwood 2006), hence it could be hypothesised that the modification of the ABCG2 transporter by prazosin could improve the Hsp72 surface presentation although it can’t be ruled out a possible effect of prazosin on its own, that will be explored in future work. Thus, Hsp72 release in this cellular model is not α1-adrenoreceptor mediated.

**In-vivo exercise model of anti-inflammatory modulation**

This study was planned to explore the effects of anaerobic, intensive exercise on Hsp72 expression and activation of the immune system, and to check whether it was possible to manipulate the expression of Hsp72 by altering the body inflammatory status by using anti-inflammatory drugs such aspirin and ibuprofen. In fact, Hsp72 was synthesised following exercise together with activation of an inflammatory response (Steensberg et al. 2006; Suzuki et al. 2006). *Hsp72* gene expression and activation of the immune response, increases in response to strenuous exercise (Fehrenbach et al. 2000) and eccentric exercise (Toft et al. 2002) although to a lesser degree if compared with endurance exercises where the muscle damage is more systemic (Toft et
al. 2002; Hirose et al. 2004). Hsp72 protein expression and release outside the cells has been extensively documented when strenuous exercise has been applied (Walsh et al. 2001; Fehrenbach et al. 2005; Suzuki et al. 2006). However no evidence has been collected before regarding the Hsp72 expression and release with intensive anaerobic training. Our results showed that the Wingate anaerobic exercise did significantly induce the \textit{Hsp72} gene expression straight after the exercise and the low level of gene expression was restored after the recovery time. Both the anti-inflammatory drugs aspirin and ibuprofen significantly decreased \textit{Hsp72} gene expression, independently of the exercise test and remain low in all the samples taken, suggesting, in this context, a possible inhibition mechanism of \textit{Hsp72} gene expression.

Activation of immune cells has been shown to occur when the Wingate test is performed; in particular NK cells are likely to be activated, as IL-12, a natural killer cell stimulator factor has been shown to be released immediately after exercise with a restoration of the initial levels with time of rest (Akimoto et al. 2000); therefore it was hypothesised that NK could express markers of stress such as Hsp72. However leukocyte populations, including the lymphocyte subsets of T-helper lymphocytes, T-cytotoxic lymphocytes, B-lymphocytes, NK-cells did not significantly change their intracellular Hsp72 content in CON, IBU and ASP treated subjects as shown in Table 4.2. The results shown here suggest that this kind of exercise protocol, which utilises an anaerobic workout is not capable of inducing an accumulation of Hsp72 in leukocytes. However when looking at the monocyte population, the Hsp72 intracellular levels decreased in response to exercise in CON group, but again as seen in the gene expression data, not in ASP or IBU groups. The decrease of Hsp72 in the monocyte population after exercise in CON group, followed by a level similar to the initial one after the recovery time, is suggestive that this cell type is affected by the physiological changes that can occur with anaerobic exercise.

Despite the increase of \textit{Hsp72} gene expression after exercise the monocyte population possesses decreased protein levels: this is probably due to a delayed response when the gene expression is activated. It could be also hypothesised that the protein is synthesised but is then released straight away into the extracellular compartment to be able to circulate and act as a danger signal: the release data confirms this hypothesis showing that in the CON
group there is a trend of increase after the exercise, although the difference is not significant and is in line with previous findings which showed that serum Hsp72 increased after intensive exercise (Walsh et al. 2001); these results also suggest that part of the serum Hsp72 could come from cells from of monocyte lineage.

Pro-inflammatory cytokines are produced when intensive exercise is applied. IL-6 release has been considered a marker of muscle damage (Pedersen et al. 2004; Peake et al. 2005; Fischer 2006; Suzuki et al. 2006), and it has been shown in association with Hsp72 expression (Febbraio et al. 2002). IL-6 synthesis and release requires an intensive and long exercise, together with an impaired glucose availability (Fischer 2006). Exercise intensity is shown to produce a higher level of anti-inflammatory cytokines if compared with exercise-induced muscle damage (Peake et al. 2005). After a short anaerobic exercise such as the Wingate test, markers of muscle damage such as plasma lactate concentration increases (Hussain et al. 1996) together with IL-12 that has been shown to be released immediately after exercise (Akimoto et al. 2000). IL-6 release in a Wingate trial has been shown to be recorded in a study where arachidonic acid supplementation, a fatty acid main component of peanut oil was tested in order to see if it was beneficial when anaerobically exercised resulting in a decrease of an inflammatory response (Roberts et al. 2007); however no data has been collected looking the effect of IL-6 release in plasma and Hsp72 in plasma after a Wingate exercise procedure.

The results here showed that there was no change in IL-6, TNF-α, IL-10 levels after the Wingate exercise providing evidence that the Wingate anaerobic exercise did not induce any significant inflammatory response in the subjects. Anti-inflammatory drugs such as ibuprofen and aspirin have been shown to change the expression profile of the pro- and anti-inflammatory cytokines. Aspirin attenuated the stress-induced increase in plasma IL-6 levels in psychosocial stress risk of athero thrombotic events (von Kanel et al. 2008), but, regarding exercise an early work showed that a single dose of 1000 mg aspirin did not affect physical performance during sub-maximal and maximal exercise on a cycle ergometer (Roi et al. 1994). Ibuprofen at maximal doses of 1200 mg had no effect in inflammatory cell concentrations after eccentric exercise (Peterson et al. 2003), and had no
effect on muscle hypertrophy and strength and does not affects rating of muscle soreness (Krentz et al. 2008).

The results obtained show an increase of IL-6 release with aspirin but not with ibuprofen. No significant differences in IL-6, TNF-α, IFN-γ plasma levels were found with exercise. Previous literature showed a modulation of IL-6 depending on the glycogen availability (Febbraio et al. 2004), the unchanged levels of IL-6 with the exercise could be explained by considering that the glycogen levels do not change significantly in a very short exercise such as Wingate. The anti-inflammatory drugs together with intensive and anaerobic exercise, despite them not significantly activating the immune response, they modify the level of Hsp72 in the serum of subject: aspirin cause a decrease of serum Hsp72, specially after the exercise despite previous *in vitro* work showing that it induces Hsp72 release from mast cells (Mortaz et al. 2006), while ibuprofen caused an increase of serum Hsp72 after the anaerobic exercise. This could be explained by an activation of *Hsp72* gene expression by aspirin that might block the release from the cells.

Further work needs to be done increasing the number of subjects involved, and testing different exercise protocols either predominantly aerobic or anaerobic.
4.5 Summary

- Pro-inflammatory cytokines such as IL-6 and IFN-γ at high concentrations stimulate the Hsp72 movement from the intracellular space towards the surface and secretion in the extracellular environment.

- Adreno-hormones induce accumulation of Hsp72 inside the cells but stop the surface Hsp72 presentation and release. Co-treatment with α1-adrenoreceptor blocker prazosin, induce the surface Hsp72 accumulation and secretion suggesting a possible involvement of ABC transporters in the Hsp72 release pathway.

- Despite a lack of inflammatory activation following intensive anaerobic exercise, blood monocytes express Hsp72 together with a slight increase of Hsp72 serum level and a temporary decrease of intracellular level in monocytes: this suggests a mild activation of the heat shock response with release of Hsp72 already present in the cells and protein synthesis from the gene level. Monocytes could be a partial source of serum Hsp72.

- Anti-inflammatory drug supplementation with aspirin and ibuprofen significantly decreased Hsp72 gene expression, independently from the exercise, suggesting a possible inhibition mechanism. However the two drugs cause a differential effect in the serum Hsp72 content, especially after the exercise, which increase with ibuprofen supplementation and decrease with aspirin, suggesting indirectly that Hsp72 can be regulated by agents that interact with the immune response.