General discussion

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Part of this chapter is based on:


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Chapter 10

This thesis explores the modulation of the immune system during natural torpor and pharmacologically induced torpor, with the ultimate aim to exploit such mechanisms to limit organ injury in the clinical setting. Therefore, we first explored one of the obvious clinical conditions that likely benefits from insights obtained from hibernators, i.e. cardiac surgery employing cardiopulmonary bypass (CPB). In a retrospective study of patients that underwent CPB, we identified several factors related to the occurrence of acute kidney injury and mortality during follow-up (chapter 2). In this study, we identified higher age, preexisting renal dysfunction, lowered pre-operative hemoglobin concentration, longer perfusion duration, low blood bicarbonate content and increased arterial oxygen pressure during perfusion to be associated with the occurrence of post-operative renal dysfunction. Although mild hypothermia (>30°C) has beneficial effects on post-operative kidney function and survival, moderate and deep hypothermia (<30°C) is associated with an increased incidence of acute kidney injury and mortality. To examine the molecular aspects of CPB induced damage to the kidney, we determined changes in the gene expression profile in the kidney following normothermic CPB in the rat (chapter 3). In this model, we demonstrated that CPB induces a local inflammatory response in kidney. This response is thought to result from systemic activation of complement, leukocytes, platelets, coagulative and oxidative pathways (Landis, 2007; Heyn et al., 2011), due to surgical trauma, ischemia, reperfusion and contact between leukocytes and the foreign-body surface of the machine (Biglioli et al., 2003). In addition, the use of hypothermia during CPB leads to a more extensive systemic inflammatory response after surgery (Ohata et al., 1995). Thus, hypothermia and inflammation seem to be an important players in the etiology of renal injury following CPB. Therefore, we studied which mechanisms underlie the protection of organ injury during mammalian hibernation. One such mechanism might be the reversible clearance of circulating leukocytes during torpor, which we demonstrated in chapter 4. Neutropenia during torpor is probably due to transient margination of neutrophils induced by low body temperature (chapter 5). Lymphopenia on the other hand, is secondary to reduced egress of lymphocytes from secondary lymphoid organs due to a decreased plasma level of Sphingosine-1-phosphate (S1p) during torpor (chapter 6). Likely, the reduced plasma level S1p results from a decreased transport of S1p from erythrocytes at low body temperature. Although the number of circulating lymphocytes restores during rewarming upon arousal towards counts observed in summer euthermic animals, the function of the humoral immune system remains partly suppressed, because of a reduced capacity to induce a T-cell independent immune response during the hibernation season (chapter 7). Pharmacological induction of a torpor-like state with increased resistance to hypothermia and a reduced immune function may be of therapeutic use to improve outcome following CPB. Currently tested methods to pharmacologically induce a torpor-like state are reviewed in chapter 8. To assess whether pharmacologically induced torpor induces changes in lymphocyte dynamics similar to natural hibernation, we tested one such a strategy using 5'-AMP in mice. In chapter 9 we demonstrate that 5'-AMP administration in mice leads to a reversible reduction in the body temperature and lymphopenia, due to the retention of lymphocytes in lymph nodes caused by activation of adenosine 2b (A2b) receptors.
Cardiopulmonary bypass: a case for hibernation

As briefly described in the introduction of this thesis, hibernators do not show gross signs of organ injury, despite the repetitive cooling and rewarming (Zancanaro et al., 1999; Arendt et al., 2003; Sandovici et al., 2004; Fleck and Carey, 2005; Talaei et al., 2011). Experimental cold ischemia/reperfusion of livers derived from torpid, aroused and summer thirteen-lined ground squirrels and rats revealed that the hibernation phenotype is associated with an increased ex vivo resistance to cold ischemia/reperfusion-injury, as demonstrated by a better preservation of mitochondrial respiration, bile production, and sinusoidal lining cell viability, and a decrease in vascular resistance and Kupffer cell activation (Lindell et al., 2005). The increased resistance to ischemia/reperfusion induced injury (I/R-injury) was later confirmed in vivo, by showing decreased mucosal damage in hibernators following intestinal warm ischemia/reperfusion (Kurtz et al., 2006). Not only the winter phenotype, but possibly the general phenotype of hibernating species is associated with increased tolerance to ischemic stress. Cerebral ischemia induced by cardiac arrest in summer normothermic Arctic ground squirrels leads to less brain injury as compared to rats (Dave et al., 2006). Balancing energy production and consumption as well as the upregulation of specific protective pathways appear to play key roles in limiting cell death in these models and hence, tissue injury due to cooling, low oxygen supply, reperfusion, and oxidative stress. Specific adaptations of hibernators that allow them to survive periods of low body temperature and decreased oxygen supply without signs of gross organ injury seem rooted in alterations in cellular respiration and anti-oxidative pathways (Lindell et al., 2005; Kurtz et al., 2006), production of anti-oxidants such as ascorbate and genes under control of Nrf2 (Drew et al., 1999; Morin, Jr. et al., 2008), production of several chaperones such as the heat shock proteins to prevent accumulation of misfolded proteins (van Breukelen and Martin, 2002; Carey et al., 2003a; Storey, 2010) and also downregulation of apoptotic pathways (Fleck and Carey, 2005). In addition, once cellular stress does occur, alterations in the immune system of hibernating animals may prevent exaggeration of tissue injury (Bouma et al., 2010a). Reviewing these features of hibernation, medical doctors will readily think of a number of conditions in which hibernation could limit organ damage. Of these conditions, cardiopulmonary bypass (CPB) stands out as an excellent candidate, because the associated organ damage is thought due to both ischemia/reperfusion injury and systemic inflammation. The importance of inflammation in the etiology of acute organ injury following CPB has also been demonstrated experimentally, as the extent of post-operative myocardial and pulmonary injury can be reduced by the use of leukocyte depleting filters (Gu et al., 1996; Fabbri et al., 2001; Zhang et al., 2010). Organ damage following CPB is readily observed in the kidney. Acute kidney injury may occur in up to 30 % of patients following cardiac surgery with cardiopulmonary bypass (CPB) (Loef et al., 2009). The occurrence of post-operative kidney injury is an important predictor of short-term and long-term mortality (Loef et al., 2005; Karkouti et al., 2009; Loef et al., 2009) (chapter 1).
Factors associated with acute kidney injury following CPB

To analyze which factors are involved in the etiology of post-operative renal dysfunction, we performed a retrospective database study among patients that underwent CPB in our center during the last 15 years (1997-2012) (chapter 2). To obtain more insight into the etiology of renal injury following CPB, a multivariate analysis was performed to identify pre- and perioperative factors determining the transient renal function loss, expressed as minimum estimated creatinine clearance (eCCr) during the first post-operative week. In this analysis, we found that lower pre-operative eCCr and Hb, increased age and pre-operative leukocyte count are associated with an impaired post-operative renal function. Possibly, the higher pre-operative number of circulating leukocytes induces a more extensive inflammatory response during extracorporeal circulation, which is thought to play a role in the pathogenesis of renal injury (Asimakopoulos, 2001; Caputo et al., 2002; Holmes et al., 2002; Kourliouros et al., 2010). The analysis further shows that perioperative factors have far less bearing on the post-operative renal function than pre-operative factors. However, perioperative factors may be of higher importance, as they are potentially modifiable. We identified several perioperative factors to be associated with the occurrence of acute kidney injury, including cross-clamp time, perfusion time and hypothermia. Although mild hypothermia (>30°C) has beneficial effects on the kidney function following CABG, these protective effects were not observed following valve surgery or combined surgery. Although low body temperature may have beneficial effects because of the induction of leukopenia as we demonstrated in this thesis, these seem to be outruled by hazardous effects of hypothermia such as poor tissue perfusion, leading to ischemia and subsequent reperfusion injury upon rewarming (Mand'ak et al., 2004; Gordan et al., 2010). Furthermore, moderate to deep hypothermia (<30°C) has detrimental effects on the kidney function after valve surgery. Thus, oxidative stress due to ischemia/reperfusion and hypothermia seem to play an important role in the etiology of post-operative renal injury following CPB.

Inflammation as etiological factor in acute kidney injury following CPB

Although we demonstrated that CPB leads to perioperative renal function loss in up to one third of the patients, the local response in the kidney to extracorporeal perfusion itself had not been explored in depth. Therefore, we performed a genome-wide microarray analysis on kidney samples derived from rats that underwent either (normothermic) CPB or Sham surgery (chapter 3). We show that CPB induces an acute inflammatory response in the kidney. Our results indicate the major involvement of gp130-cytokine-receptor mediated signaling by interleukin-6 (IL-6) provoking the renal inflammatory response. This local inflammatory response in the kidney is likely to be initiated by the activation of resident tissue macrophages, leading to the production of chemotactic cytokines (chemokines) and subsequent influx of other types of leukocytes, such as neutrophils, lymphocytes and monocytes. The plasma level of IL-6 correlates with kidney dysfunction (Gueret et al., 2009b) and a reduced lung function (Halter et al., 2005) in patients after CPB. However, the predictive value of inflammatory markers such as cytokine plasma levels on mortality after CPB is less clear and remains a matter of controversy (Larmann and Theilmeier, 2004; Ganem et al., 2011). Our data suggest that a local organ-based (i.e. kidneys/lungs) inflammatory response sets the stage for further systemic immune activation. In addition to the local inflammatory response, other factors might be involved in the induction of the systemic inflammation following CPB including the contact between leukocytes and plastic surfaces of the machine, mechanical trauma, endotoxin released from inflamed intestine and
low body temperature (Asimakopoulos, 2001; Caputo et al., 2002; Holmes et al., 2002; Larmann and Theilmeier, 2004; Kourliouros et al., 2010). In addition to ischemia/reperfusion and hypothermia, also inflammation seems to be involved in the pathogenesis of kidney injury following CPB.

**Acute kidney injury and long-term mortality following CPB**

The extent of perioperative renal function decline, defined as the proportional perioperative rise in serum creatinine, is associated with the mortality risk of patients that underwent CPB (chapter 2). This finding confirms that the occurrence of post-operative renal injury represents an important risk factor for mortality following CPB assisted cardiac surgery (Loef et al., 2005; Loef et al., 2009). Although acute kidney injury after CPB might be a transient event, recovery of renal function at hospital discharge towards pre-operative values does not offset the risk of mortality (Loef et al., 2009). Unfortunately, the cause of death could not be retrieved from the data available in our study (chapter 2). Therefore, the relationship of a transient decline in kidney function on mortality after CPB remains speculative. Our data does confirm that the major determinant of that post-operative renal function loss is the pre-operative renal function. Kidney function decline is an important predictor for mortality in the overall population of 65 years and older (Manjunath et al., 2003). Potentially, the occurrence of kidney injury might reflect a frail health status and/or be due to a more complicated surgical course of these patients. Future studies that take the cause of death into account might provide more information about the effect of transient renal injury on long-term mortality.

**Towards prevention of acute kidney injury following CPB**

Although corticosteroids are able to suppress the inflammatory response following CPB (Morariu et al., 2005), administration of corticosteroids does not affect CPB induced myocardial, pulmonary or renal injury or influence mortality (Loef et al., 2004; Morariu et al., 2005; Dieleman et al., 2011). Likely, adverse effects on glucose metabolism overrule the beneficial effects of corticosteroids as suppressors of inflammation. Hyperglycemia is associated with organ injury, e.g. in critically ill patients as demonstrated by mitochondrial dysfunction and production of reactive oxygen species in hepatocytes (Vanhorebeek et al., 2005). Hence, current therapeutic strategies are not able to sufficiently reduce inflammation induced by CPB to protect organs from acute dysfunction. Receptors of the signal transduction pathways activated by CPB as identified in our microarray data (chapter 3) may be promising pharmacological targets in the prevention of acute renal injury following CPB. In addition, previously identified factors (i.e. ischemia/reperfusion, hypothermia and oxidative stress) might well represent upstream inducers of a local inflammatory response, which amplifies the extent of renal injury (chapter 3). Therefore, a potential successful strategy to limit renal injury following CPB should limit both the ischemia/reperfusion injury and the inflammation in response to cellular injury, contact between leukocytes and the machine and endotoxin leakage from the gut. Hibernation is associated with increased resistance against ischemia/reperfusion injury, reduced metabolism and depressed immune function. Therefore, induction of torpor represents an attractive strategy to limit CPB induced organ injury. A torpor-like state with reduction of metabolism can be induced pharmacologically by different compounds, including 5’-AMP (chapter 8). Besides activating adenosine receptors, 5’-AMP activates adenosine-monophosphate kinase (AMPK), which is a molecular sensor of the cellular energy status. Upon activation, AMPK induces an energy-
saving state that activates several important molecular pathways that are involved in the protection against cellular injury induced by ischemic preconditioning and thereby potentially increases resistance against ischemia/reperfusion injury (Bouma et al., 2010b). Thus, the mechanism of action of 5’-AMP to lower metabolic rate, increase resistance to ischemia/reperfusion and hypothermia and induce leukopenia are generally known (chapters 9 and 10). Therefore, after investigating its interaction with general anesthesia, it would be highly interesting to test efficacy of the compound in the rat CPB model.

**The hibernating immune system**

Table 10.1: The effect of body temperature on leukocyte dynamics during hibernation

<table>
<thead>
<tr>
<th>Body temperature</th>
<th>State (chapter)</th>
<th>Species</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>± 8°C</td>
<td>deep torpor (4)</td>
<td>European ground squirrel</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>± 8°C</td>
<td>deep torpor (5,6,10)</td>
<td>Syrian hamster</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓</td>
</tr>
<tr>
<td>± 9°C</td>
<td>hypothermia (5,6,10)</td>
<td>Syrian hamster</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓</td>
</tr>
<tr>
<td>± 19°C</td>
<td>hypothermia (5,6)</td>
<td>rat</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓</td>
</tr>
<tr>
<td>± 23°C</td>
<td>pharmacological torpor (9)</td>
<td>C57/Bl6 mice</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>± 24°C</td>
<td>hypothermia (9)</td>
<td>C57/Bl6 mice</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>± 25°C</td>
<td>daily torpor (5,6,10)</td>
<td>Djungarian hamster</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>± 25°C</td>
<td>daily torpor (10)</td>
<td>CD1 mice</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
</tr>
<tr>
<td>± 36°C</td>
<td>pharmacological torpor (9)</td>
<td>C57/Bl6 mice</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
</tr>
</tbody>
</table>

The numbers of circulating neutrophils, lymphocytes and monocytes are reduced during deep torpor in the European Ground Squirrel and the Syrian hamster, but also during forced hypothermia to ± 19°C and ± 9°C in the rat and Syrian hamster, respectively. In contrast, the number of circulating monocytes is not reduced during daily torpor in the Djungarian hamster. Daily torpor in mice, either workload or pharmaco logically induced by 5’-AMP reduces the number of circulating lymphocytes only. Cooling of mice to a similar body temperature as observed during daily torpor in mice reduces the number of circulating lymphocytes as well, without affecting the number of circulating neutrophils or monocytes. ↓/↓↓ represents a significant decrease up to 90% / > 90% as compared to euthermic values, respectively.

In chapter 4, we demonstrate that torpor is associated with a significant decline in the number of thrombocytes and leukocytes, without affecting the number of circulating erythrocytes. The unaltered number of circulating erythrocytes implicates that the observed thrombo- and leukopenia cannot be attributed to a fluid shift into the intravascular compartment. Although the occurrence of leukopenia during torpor is demonstrated to occur in all species of hibernating animals studied thus far (Spurrier and Dawe, 1973; Suomalainen and Rosokivi, 1973; Reznik et al., 1975; Frerichs et al., 1994; Toien et al., 2001; Bouma et al., 2010a; Bouma et al., 2011), our studies provide additional information about its dynamics as we compared blood cell counts at multiple time-points during torpor and arousal. Notably, the rapid decrease and restoration of normal numbers of circulating leukocytes upon arousal, suggests a temporary retention of cells at certain locations, rather than apoptosis-and-reproduction as underlying mechanism (chapter 4). Therefore, we studied the underlying mechanisms of the reduced number of major subtypes of circulating leukocytes,
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Neutrophil and lymphocytes during torpor in chapters 5 and 6, respectively. In this thesis, we demonstrate that deep torpor reduces the number of circulating neutrophils (chapters 4 and 5), lymphocytes (chapter 6) and monocytes (box 1). Natural daily torpor on the other hand, leads to a reduced number of circulating neutrophils (chapter 5) and lymphocytes (chapter 6), while no decrease in the number of monocytes was observed (box 1). In order to investigate the potential to mimic the immunological aspects of natural hibernation as described in this thesis, we investigated leukocyte dynamics in energy constrained mice (Schubert et al., 2010). Energy constraint induces daily torpor in mice, as characterized by a transient drop in body temperature (box 2). Compared to Ad libitum fed euthermic animals, energy constrained mice had a lower number of circulating lymphocytes, which correlated significantly with their body temperature. Such a torpor-like state can also be induced by injection of (synthetically produced) 5’-AMP in mice (Zhang et al., 2006; Lee, 2008) (chapter 8). In chapter 9 we reveal that 5’-AMP induced torpor in mice is associated with lymphopenia as well. Forced hypothermia of anesthetized animals (chapter 5, 6, 9 and 10) to reach a body temperature of ± 24°C (mice) decreases the number of circulating lymphocytes, while cooling to ± 19°C (rats) reduces the number of circulating neutrophils as well and cooling to ± 9°C (Syrian hamsters) leads to a reduction in the number of circulating lymphocytes, neutrophils and monocytes. Hence, the decrease in numbers of circulating neutrophils and monocytes seem to depend principally on the lowering of body temperature. In contrast, although low body temperature might reduce the number of circulating lymphocytes, additional temperature-independent mechanisms seem to be involved as well (chapter 9). The different effects of torpor types and hypothermia on leukocyte dynamics are summarized in table 10.1.

Neutrophil dynamics

To obtain more information about the mechanism that governs clearance of circulating neutrophils during torpor, blood samples were drawn during entrance into torpor and rewarming from torpor, of which the results are shown in chapter 5. Lowering of body temperature induced a gradual decline in the number of circulating neutrophils. Neutropenia is not restricted to deep torpor in the Syrian hamster, but also occurs to a lesser extent during daily torpor in the Djungarian hamster. Moreover, forced hypothermia of anesthetized summer active Syrian hamsters and of rats leads to the induction of a reversible state of neutropenia as well. Injection of dexamethasone prior to forced cooling prevented the reduction in the number of circulating neutrophils. This finding suggests that neutropenia is due to margination of cells (adherence to the endothelium of the vasculature), rather than apoptosis of neutrophils. In line with this, no increase in the number of circulating immature granulocytes was found upon arousal from torpor or rewarming following forced hypothermia. The precise mechanisms that promote neutrophil margination upon lowering of the body temperature remains still elusive. We speculate that the hypothermia related depression of cardiac output and the subsequent decreased in blood flow velocity may lead to margination of cells to the endothelial wall of the vasculature (Beekhuizen and van, 1993; von and Ley, 2008). Despite the influence of cooling, also temperature-independent mechanisms of torpor might be involved in the clearance of circulating leukocytes. This speculation is based on the fact that incubation of rat endothelial cells at 37°C in vitro with plasma from hibernating, but not from euthermic thirteen-lined ground squirrels, leads to upregulation of ICAM-1 expression and increased monocyte adhesion to these cells (Yasuma et al., 1997).
Figure 10.1. The number of circulating monocytes is reduced by low body temperature in deep torpor, but are unaffected by daily torpor. The mRNA-expression was measured using primersets for CD14 (monocytes): (F)ACCAATCTGGCTTGGATCT, (R)GTGCTTGCTTGCTGTTT and CD68 (macrophages): (F)AAGCAGCACAGTGGACATTC, (R)ATGATGAGAGGCAGCAAGAG. EU = winter euthermic; DT = daily torpor; TO = deep torpor; AR = arousal. Bars represent means ± SEM of n = 4-8 animals per group. Groups were compared using an independent samples Two-Tailed Student’s t-test, a one-way ANOVA and post-hoc least significant difference or Kruskal-Wallis and Mann-Whitney U. * means $p < 0.05$.

In line with the dynamics of other subtype of leukocytes, the number of circulating monocytes decreases from 0.08 ± 0.04 in winter euthermic Syrian hamsters to 0.01 ± 0.00 (x 10^6/ml) ($p < 0.05$) during torpor, followed by restoration to 0.16 ± 0.05 (x 10^6/ml) ($p < 0.01$; Figure 10.1A-C) upon arousal. The number of circulating monocytes during entrance into torpor correlates strongly with the body temperature (Pearson Rho^2 = 0.474, $p < 0.01$), thus suggesting a potential role of low body temperature in the reduction of blood monocytes. Indeed, clearance of circulating monocytes could be induced by forced hypothermia in Syrian hamsters (Figure 10.1B) and its level was gradually restored upon arousal from torpor or rewarming from forced hypothermia in Syrian hamsters (Figure 10.1C). Interestingly, the number of circulating monocytes was not affected by daily torpor in the Djungan hamster (Figure 10.1D). To assess the role of the spleen as potential retention site for monocytes in torpor, we first measured the expression of CD14 and CD68 (markers for monocytes and macrophages, respectively) in spleens from torpid and aroused Syrian hamsters. Although we did not find a difference in the expression of CD68 (Figure 10.1E), the expression of CD14 was significantly higher during torpor than during arousal ($p < 0.05$, Figure 10.1E). Increased expression of CD14 during torpor might either reflect influx of CD14+ cells into the spleen or upregulated CD14-expression. To further substantiate the role of spleen, monocyte counts from splenectomized animals were analyzed. Resection of the spleen during summer did not affect clearance of circulating monocytes during torpor (Figure 10.1F). Further, resection of the spleen in torpid animals did not preclude restoration of normal monocyte counts upon arousal (Figure 10.1G). Thus, although some monocytes might be retained in the spleen as suggested by the expression of CD14, the spleen does not serve a key role in the retention of monocytes during torpor.
The reduced number of circulating neutrophils and monocytes may explain in part the reduced innate immune function during torpor, which is illustrated by the fact that a febrile response is not induced by intraperitoneal injection of LPS during torpor. Injection of LPS during arousal on the other hand, does lead to the induction of a febrile response (Prendergast et al., 2002). However, it should be noted that the intraperitoneal administration route might lead to an inferior absorption during torpor, since cardiac output is low and blood is shunted away from the periphery. Nevertheless, systemic margination of leukocytes leads to a depletion of the circulating pool and precludes their migration to specific sites of inflammation during infection or cellular injury. The spleen plays an important role in the induction of an immune response against blood-borne infections. We found that in spleen, the expression of CD16b (a marker for neutrophils) (chapter 5) and CD68 (a marker for macrophages) (box 1) does not differ between torpor and arousal, while the expression of CD14 (a marker for monocytes) (box 1) is reduced during arousal as compared to torpor. Further, splenectomy of animals before hibernation and during torpor, revealed that the spleen does not play a key role in torpor-associated reduction in the number of circulating neutrophils (chapter 5), monocytes (box 1) or lymphocytes (chapter 6). Our data suggest that, despite the substantial reduction in the number of circulating neutrophils and monocytes, no extreme changes in the number of these leukocytes occur in the spleen during hibernation. Thus, it is unlikely that the reduced immune function during torpor is caused by clearance of neutrophils and monocytes by the spleen. In addition to mechanisms that affect leukocyte migration, alterations in the responsiveness of leukocytes might affect the immune function as well. Such changes are for example the reduced production of TNF-α by macrophages derived from torpid ground squirrels following in vitro stimulation with LPS at 37°C as compared to macrophages from euthermic animals (Novoselova et al., 2000). Taken together, the function of the innate immune system is reduced during torpor, which may (at least in part) be due to the systemic margination of neutrophils and monocytes.

**Lymphocyte dynamics**

Lymphopenia was observed during deep torpor in the Syrian hamster and daily torpor in the Djungarian hamster and could also be induced by forced hypothermia in a hibernator (the Syrian hamster) and in a non-hibernating animal (the rat) (chapter 6). Interestingly, also daily torpor induced by energy constraints in mice leads to a reduced number of circulating lymphocytes as compared to *ad libitum* fed euthermic animals (box 2). By splenectomizing hamsters and injecting labeled lymphocytes, we revealed that lymphocytes are retained in secondary lymphoid organs during torpor (chapter 6). The plasma level of Sphingosine-1-phosphate (S1p) is significantly reduced during torpor, followed by an increase upon arousal. S1p is a bioactive lipid that is involved in regulation of lymphocyte egress from secondary lymphoid organs (Mandala et al., 2002; Matloubian et al., 2004; Pappu et al., 2007). To assess whether S1p is involved in lymphocyte recirculation during hibernation, we injected a specific S1p₁-receptor antagonist prior to arousal. This experiment prevented restoration of normal numbers of circulating lymphocytes upon arousal. Erythrocytes are considered to be the main source of plasma S1p (Pappu et al., 2007). This is in line with the strong negative correlation found between the S1p level in erythrocytes and the plasma level S1p during torpor and arousal. *Ex vivo* rewarming of erythrocytes induced release of S1p into the medium, which did not occur at 4°C and could largely be blocked by inhibitors of ATP binding cassette (ABC) transporters. Likely, the reduced plasma level S1p during torpor is
due to a reduced release from erythrocytes secondary to lowered body temperature and leads to the retention of lymphocytes in peripheral lymphoid organs. Although the mechanisms that underlie clearance of neutrophils and lymphocytes are different, lowering of the body temperature thus leads to a profound reduction in the number of circulating cells of all major subtypes of leukocytes.

**Box 2: Leukocyte dynamics during daily torpor in mice**

Figure 10.2. Energy constraints leads to the induction of daily torpor and lymphopenia in mice. Bars represent mean ± standard error of the mean (SEM); AR = arousal, TO = torpor; */** means $p < 0.05 / 0.01$ as analysed by a One-Way ANOVA and post-hoc LSD; ($n = 7-8$ animals per group).

Although fasting-induced daily torpor has been described previously in CD1 mice housed in constant darkness, it is not known whether this type of torpor behavior can be induced by metabolic stress under normal light:dark (L:D) conditions and whether this is associated with leukopenia, as is the case during torpor in the hamster. Therefore, a group of mice was forced to work for food, while being housed under normal L:D-cycle (12:12). Running wheels that were installed in the cages were connected via a digital counter to the food pellet dispenser (Hut et al., 2011). After an initial learning phase, an energy constraint was induced by increasing the workload/reward-ratio. During the experiment, body weight was measured every week, while body temperature was monitored continuously using telemetric sensors implanted peritoneally. Mice fed ad libitum served as control animals. The body mass of energy constrained animals was significantly lower as compared to ad libitum fed control animals ($p < 0.01$), due to a combination of weight gain in the control group and loss in the experimental groups (Figure 10.2A). All animals in the experimental groups showed daily torpor behavior, leading to significant drops in body temperature (Figure 10.2B). Blood analysis (similar method applied as used in chapters 4, 5 and 6) showed no significant changes in the number of circulating erythrocytes (Figure 10.2C), neutrophils (Figure 10.2D) or monocytes (Figure 10.2F), but revealed a significant drop in the number of circulating lymphocytes in the euthermic ($p < 0.05$; Figure 10.2E) and torpid ($p < 0.01$; Figure 10.2E) animals from the energy constrained group as compared to ad libitum fed control animals. Although the body temperature correlated with the number of circulating lymphocytes (Pearson Rho$= 0.53$, $p < 0.05$), no difference in the number of circulating lymphocytes was observed between torpid and aroused energy constrained animals. Taken together, working for food can induce a state of daily torpor and lead to a reduced number of circulating lymphocytes. As is the case during deep and daily natural torpor in Syrian and Djugarian hamsters, lymphopenia during workload induced daily torpor in mice seems to be due to a lowered body temperature as well.
Not only natural torpor, but also pharmacological torpor leads to a reduced number of circulating lymphocytes. We show in chapter 9 that injection of 5'-AMP leads to the induction of a torpor-like state characterized by a substantial and reversible drop in body temperature. During torpor, the number of circulating lymphocytes is reduced, due to retention of cells in lymph nodes, as blocking of homing receptors prevented the induction of lymphopenia. Our data suggest that lymphopenia is driven by the temperature-independent activation of the purinergic A2b receptor inhibiting lymphocyte motility and precluding their egress from lymph nodes. In addition, the plasma level S1p is reduced during 5'-AMP induced torpor and restores upon arousal. Since restoration of normal lymphocyte counts following arousal is blocked by injecting a specific S1p_1 receptor antagonist prior to arousal, the plasma level S1p likely stimulates egress of circulating lymphocytes. Both during natural and pharmacological torpor, the number of lymphocytes is reduced due to retention of cells in lymph nodes. Although the lowered body temperature seems to induce retention of lymphocytes during natural torpor through a decreased plasma level S1p (chapter 6), this is likely of minor relevance during 5'-AMP induced pharmacological torpor as A2b agonism induced retention of cells in lymph nodes, independent of body temperature. Further, in contrast to natural torpor, no reduction in the number of circulating neutrophils or monocytes was observed during pharmacological torpor. Taken together, although the underlying mechanisms that lead to a reduced number of circulating lymphocytes, lymphopenia is not restricted to natural torpor or forced hypothermia, but also occurs during pharmacologically induced torpor in mice.

**Adaptive immune function**

Previous studies demonstrated a substantial reduced function of the adaptive immune system during hibernation. This finding is illustrated by the observation that rejection of transplanted skin allografts is delayed (Shivatcheva, 1988) and antigen-induced antibody production is depressed (Sidky et al., 1972; Burton and Reichman, 1999) as compared to summer-active animals. The retention of lymphocytes in lymphoid organs likely limits their capacity to interact with other immune cells, which is crucial for a proper immune function. Since the number of circulating lymphocytes increases rapidly upon arousal, we hypothesized that retention of lymphocytes during torpor does not necessarily lead to a reduced function of the adaptive immune system throughout hibernation and that transient lymphopenic states during torpor might not be the sole explanation for the reduced adaptive immune function. Therefore, in chapter 7 we injected ground squirrels either during the summer or the hibernation season with a T-cell dependent (TD) antigen or a T-cell independent type 2 (TI-2) antigen and measured antigen-induced antibody production following primary and secondary immunization. We found that in contrast to TI-2 antigens, TD antigens can induce a humoral immune response during hibernation. We speculate that the reduced plasma concentration of complement during torpor (Maniero, 2002), might represent one of the factors that is involved in the reduced capacity to induce a TI-2 humoral immune response. Typical TD antigens are proteins, while TI-2 antigens are carbohydrate. Although pathogens bear both TD and TI-2 antigens, humoral TI-2 responses seem to be specifically important in the defense against fast replicating pathogens in the circulation (Ochsnebein et al., 2000). We speculate that the inability to mount a humoral immune response to a TI-2 antigen (carbohydrates such as endotoxins), might be of relevance for humans undergoing CPB. Endotoxin leakage from the gut is suggested to be involved in the
pathogenesis of acute kidney injury following CPB (Larmann and Theilmeier, 2004). To date, attempts to reduce the inflammatory response following CPB have been taken by capturing circulating endotoxins and pre-operative selective gut decontamination. Although capturing of endotoxins results in lower levels of circulating complement and cytokines (i.e. IL-1b, IL-6 IL-8) (Ohki et al., 2008; Blomquist et al., 2009), pre-operative selective gut decontamination shows controversial results on the inflammatory response following CPB (Bouter et al., 2002; Yu et al., 2007). The induction of a torpor-like state during CPB may alleviate organ injury due to endotoxin leakage from the gut.

**Exploiting identified targets for the prevention of acute organ injury: a case for 5’-AMP**

The ability to pharmacologically induce a torpor-like state that is associated with an increased resistance to ischemia/reperfusion, hypothermia and a reduced capacity to induce an inflammatory response, might improve the outcome of various medical conditions, including major (cardiac) surgery, organ transplantation and trauma. Interestingly, specific adjustments that allow animals to safely undergo torpor do not seem to be specifically constrained to natural hibernators, but may be operational in humans as well. First, torpor is observed in many mammals including primates (Dausmann et al., 2004). Second, we demonstrated in this thesis that major adaptations in the immune system in hibernators are secondary to low body temperature and were observed in non-hibernators as well. Third, a torpor-like state can be induced by pharmacological agents (e.g. 5’-AMP, H₂S) in non-hibernators and is protective against stress such as ischemia/reperfusion.

**Box 3: Effects of 5’-AMP on IL-6 production by HUVEC**

![Figure 10.5. 5’-AMP limits TNF-α induced IL-6 production by HUVEC. Bars represent means ± SEM of n = 6 wells per group. Groups were compared using a one-way ANOVA and post-hoc least significant difference test. ** means p < 0.01 as compared to the positive control treated with TNF-α in the absence of 5’-AMP.](image)

IL-6 is a pleiotropic cytokine that is involved in acute phase response, such as can be seen following cardiopulmonary bypass (CPB) as demonstrated in chapter 3. To assess whether addition of 5’-AMP to cells influences immune activation of endothelial cells, HUVEC were treated with 1 ng/ml TNF-α in the absence or presence of varying concentrations of 5’-AMP for 24 hours followed by measurement of interleukin-6 (IL-6). While co-incubation with 0.3 mM 5’-AMP did not affect the production of IL-6, dosages from 1 – 10 mM significantly reduced the production of IL6 by HUVEC (p < 0.01; Figure 10.5) (ELISA DuoSet IL-6 human, R&D Systems). Although it should be noted that much more cell types and cytokines are involved in inflammatory responses as demonstrated here, these results warrant further investigation into the effects of 5’-AMP on inflammation.
Box 4: In vitro effects of 5′-AMP on hypothermia-induced cellular injury

Figure 10.4. 5′-AMP reduces hypothermia induced cell death in HUVEC preserved for 24 hours at 4°C. Bars represent means ± SEM of n = 6 wells per group. Groups were compared using a student’s t-test or a one-way ANOVA and post-hoc least significant difference test. */**/*** means p < 0.05/0.01/0.001 as compared to baseline (t = 0 hr.) or negative control.

To assess whether 5′-AMP protects from hypothermia-induced injury, primary human umbilical vein endothelial cells (HUVEC) were isolated by the Endothelial Cell Facility (University Medical Center Groningen) from two umbilical cords as previously described (Schraa et al., 2002). In this experiment, HUVEC with and without 5′-AMP were preserved under hypothermic conditions for 24 hours and subsequently rewarmed during 4 hours. The intracellular ATP concentration and MTS turnover were measured according to the manufacturers’ instructions, while the extracellular concentration of LDH was determined by measuring turnover of beta-nicotinamide adenine dinucleotide (NADH) turnover during incubation of 100 µl supernatant with two equal volumes of 3.24 µM sodium pyruvate and 0.68 µM NADH in 0.08 M Tris and 0.2 M NaCl (pH = 7.8), which was read at an OD of 340 nm during 30 minutes at 37°C and was compared to a standard curve with a known LDH concentration. In this experiment, we found that cold preservation of untreated HUVEC leads to a transient rise in the intracellular ATP level at 2 hours (p < 0.05) followed by a reduction at 24 hours (p < 0.001) (ATP Bioluminescence assay kit CLS II, Roche diagnostics) and the extracellular amount of LDH is increased from 4 hours hypothermic storage (p < 0.01) (Figure 10.4A). Next, cells were preserved for 24 hours in the presence of 1 mM 5′-AMP added 30 minutes prior to hypothermic storage. Due to interference between ATP and 5′-AMP in the assay, the amount of ATP was not directly determined here, but MTS turnover was used as a surrogate marker (CellTiter 96 AQueous One Solution Cell Viability, Promega Benelux). Addition of 1 mM 5′-AMP 30 minutes prior to cold preservation to the cell culture medium limits the release of LDH (p < 0.05; Figure 10.4B) and increases the turnover of MTS (p < 0.001; Figure 10.4C) after 24 hours of cold preservation, thus suggesting an increased viability of the cells. These data suggest that 5′-AMP might improve hypothermic preservation of cells.

As described, 5′-AMP not only stimulates the A2b receptor, but may also activate other adenosine receptors and AMPK. Depletion of ATP or, alternatively, increased levels of AMP can activate AMPK and induce an energy-saving state that prevents lactate accumulation and cell injury (Peralta et al., 2001), inhibits de novo protein production (an energy-consuming process) (Hardie, 2003), downregulates important anabolic pathways via inhibition of lipid biosynthesis and activates important catabolic pathways (Hardie et al., 2003; Hardie, 2004; Aymerich et al., 2006). Among the downstream effectors of AMPK are eNOS, NO and NfκB (Morrow et al., 2003; Cacicedo et al., 2004; Carling, 2005; Hattori et al., 2006; Gaskin et al., 2007). eNOS-mediated effects of AMPK are suggested to be of main importance in the protective effects of ischemic precondition (Gaskin et al., 2007) and limit endothelial cell injury following cold preservation (Schutte et al., 2001; Sola et al., 2001). In a pilot-experiment, we tested the potential benefits of AMPK activation by 5′-AMP in hypothermia-induced cellular injury and inflammation. 5′-AMP reduces TNF-α induced interleukin-6 (IL-6) production in HUVEC (box 3). Potential anti-inflammatory effects of 5′-AMP are in line
with other studies, which demonstrated that AMPK-activation reduces TNF-α-induced activation of NFκB and expression of monocyte chemoattractant protein-1 (MCP-1), E-selectin, VCAM-1 and ICAM-1 in HUVEC (Cacicedo et al., 2004; Hattori et al., 2006). Further, pretreatment of human umbilical vein endothelial cells (HUVEC) with 5′-AMP lowered extracellular lactate dehydrogenase (LDH) levels following rewarming and an increased cellular viability as measured by MTS-turnover (box 4). Since these preliminary data suggest that AMPK increases resistance to hypothermia, ischemia/reperfusion and has anti-inflammatory properties, AMPK might be expected to prevent organ injury during natural torpor. However, AMPK activity is only increased in white adipose tissue, but not in liver, skeletal muscle, brain of brown adipose tissue during torpor, which suggests that AMPK does not play a major role (Horman et al., 2005). Nevertheless, its functional downstream effects make AMPK a potential pharmacological target to optimize outcome following CPB.

Conclusions and future directions

Hibernating animals are able to withstand physiological extreme situations, which would cause organ injury in non-hibernating species. This is illustrated in this thesis, as hypothermia and inflammation play an important role in the pathogenesis of renal injury following CPB. We also demonstrated that low body temperature leads to a reduced number of all major subtypes of leukocytes and is not restricted to natural hibernating species, since leukopenia also occurs in hypothermic non-hibernating animals. Despite the effects of hypothermia on the migration of circulating leukocytes, low body temperature is associated with the induction of inflammation and organ injury following CPB (Ohata et al., 1995; Kourliouros et al., 2010). We speculate that the induction of potential beneficial effects induced by low body temperature in patients may be overwhelmed by other devastating effects of hypothermia such as low tissue perfusion and ischemia/reperfusion injury, which do not lead to organ injury in hibernators (Drew et al., 1999; van Breukelen and Martin, 2002; Carey et al., 2003b; Lindell et al., 2005; Fleck and Carey, 2005; Kurtz et al., 2006; Morin, Jr. et al., 2008; Storey, 2010). Therefore, metabolic suppression as occurs during natural hibernation, might improve outcome following hypothermic CPB. Although (forced) hypothermia as currently used during CPB leads to a reduced metabolism, additional pharmacological reduction of metabolism may represent a superior strategy to reduce the incidence of organ injury following CPB. In order to discover novel pharmacological targets that might lead to the induction of a torpor-like state, it is important to study natural mammalian hibernation. To date, the molecular mechanisms that trigger natural torpor are not known. As discussed in the introduction, in the late ‘60s of the previous century, Dawe and Spurrier found evidence that suggested the existence of a blood-borne factor that was able to induce torpor in summer active ground squirrels by injection these animals with either whole blood, erythrocytes or serum from hibernating squirrels (Dawe and Spurrier, 1969; Dawe et al., 1970). Unfortunately, other groups were not able to reproduce these results (Abbotts et al., 1979). Hence, the existence of a circulating factor (e.g. a hormone) that induces natural torpor remains still questionable. In mice however, fasting under conditions of constant darkness leads to increased plasma levels of 5′-AMP and the induction of a torpor-like state. Interestingly, injection of 5′-AMP in mice induces a torpor-like state as well (Zhang et al., 2006; Lee, 2008). Recently, it has been suggested that fasting leads to increased intracellular levels of AMP and oxidized nicotinamide adenine dinucleotide (NAD+). These molecules can switch the cellular fuel source from glucose to lipids, reduce oxidative stress,
affect circadian clock function and induce prolonged inactivity through signaling pathways in which AMPK and Sirtuins (SIRT1) are involved (Heldmaier et al., 2004; Melvin and Andrews, 2009). Although (5’-)AMP might well play an important role during torpor by increasing resistance to ischemia and hypothermia, differences in the dynamics of metabolism and body temperature suggest that 5’-AMP does not induce natural torpor (Swoap et al., 2007; Strijkstra et al., 2012). Future studies using pharmacological interventions during natural hibernation or genetic modification of hibernators might reveal important clues about molecular mechanisms of natural torpor. Despite the fact that the molecular mechanisms that induce natural torpor remain to be discovered, 5’-AMP closely mimics natural torpor with respect to the reduction in body temperature and clearance of circulating lymphocytes through the A2b receptor (this thesis). Furthermore, preliminary results also suggest that 5’-AMP limits hypothermia induced cellular injury and immune activation of endothelial cells. These effects might be due to activation of AMPK, a pharmacological intracellular receptor of 5’-AMP, which acts as a cellular energy sensor and can induce cellular protection against hypothermia, ischemia/reperfusion but also has anti-inflammatory properties. These features make 5’-AMP induced signal transduction, including A2b receptors and AMPK, promising pharmacological targets to limit organ injury in conditions when ischemia/reperfusion, hypothermia or inflammation are involved in the pathogenesis, as is the case during CPB.

Taken together, mammalian hibernation represents a unique model of organ preservation. Discovery of the signal transduction pathways that are involved in the induction of natural torpor will have major clinical potential and might lead to the development of novel pharmacological strategies to improve outcome following CPB. Furthermore, molecular mechanisms of natural hibernation represent pharmacological targets that are of potential interest for conditions featuring metabolic or immunologic disorganization. Molecular approaches (i.e. genetic modification and pharmacological intervention) might reveal important information about the mechanisms involved in the induction of natural torpor. Since (1) leukopenia during torpor is induced by low body temperature and is not restricted to hibernating species, (2) torpor is widely conserved throughout the entire animal kingdom, including primates and (3) pharmacological interventions can closely mimic torpor, we speculate that either the induction of a transient torpor-like state or targeting of specific signal transduction routes in patients undergoing CPB might reduce post-operative morbidity and mortality. A human hibernation-like state might not remain science-fiction.