Pharmacokinetics of Leptin in Female Mice

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Short Title

Leptin distribution in mice

Summary

Pharmacokinetics of leptin in mammals has received limited attention and only one study has examined more than two time points and this was in *ob/ob* mice. This study is the first to observe the distribution of leptin over a time course in female mice. A physiologic dose (12ng) of radiolabelled leptin was injected in adult female mice via the lateral tail vein and tissues were dissected out and measured for radioactivity over a time course up to two hours. Major targets for administered leptin included the liver, kidneys, gastrointestinal tract and the skin while the lungs had high concentrations of administered leptin per gram of tissue. Leptin was also found to enter the lumen of the digestive tract intact from the plasma. Very little of the dose (< 1 %) was recovered from the brain at any time. Consequently we confirm that the brain is not a major target for leptin from the periphery, although it may be very sensitive to leptin that does get to the hypothalamus. Several of the major targets (GI tract, skin and lungs) for leptin form the interface for the body with the environment, and given the ability of leptin to modulate immune function, this may represent a priming effect for tissues to respond to damage and infection.

Key Words

Leptin, distribution, pharmacokinetics, elimination, periphery

Introduction

Leptin is a 16 kDa cytokine originally identified as a hormone secreted from adipose tissue (Zhang *et al.* 1994) but is also known to be synthesised in other tissues including the placenta (Masuzaki *et al.* 1997), skeletal muscle (Wang *et al.* 1998) and the stomach (Bado *et al.* 1998). Leptin circulates at concentrations that correlate with fat mass (Considine *et al.* 1996) and signals via its alternately spliced receptors (Lee *et al.* 1996), notably LepRb, which has the greatest signalling capacity (Bjørbæk *et al.* 1997). Animals that lack functional leptin, *ob/ob*, or LepRb, *db/db*, express a phenotype of voracious appetite and obesity and correction of the deficit corrects this (Campfield *et al.* 1995, de Luca *et al.* 2005, Halaas *et al.* 1995, Kowalski *et al.* 2001, Pelleymounter *et al.* 1995, Weigle *et al.* 1995). Furthermore, LepRb has been found to be expressed in the hypothalamus at high density in nuclei thought to regulate energy balance (Chen *et al.* 1996, Lee *et al.* 1996). Consequently, leptin was hypothesised to signal information from the periphery to the brain about energy stored as fat (Friedman and Halaas 1998).

In addition to leptins central effects it is increasingly being recognised as having roles in the periphery, which include regulating aspects of reproduction, immune function, energy substrate preference and regulation of nutrient absorption. During reproduction, leptin attenuates testosterone secretion (Tena-Sempere *et al.* 1999) and prepares the endometrium for embryo implantation (Malik *et al.* 2001), it is involved in regulating immune responses (Loffreda *et al.* 1998) and may be involved in the development of visceral obesity (Duffield *et al.* 2009). In muscle leptin has been reported to induce a preference for fatty acids as a fuel substrate (Muoio *et al.* 1997), while in the digestive tract leptin increases the activity of glucose transporters (GLUT) 2 and 5 (Pearson *et al.* 2001, Sakar *et al.* 2009), reduces the activity of sodium-glucose cotransporter (SGLT) 1 (Ducroc *et al.* 2005, Iñigo *et al.* 2007) and has been postulated to regulate the gut microbiome by altering the secretion of anti-microbial proteins in the colon (Rajala *et al.* 2014).

Despite the wealth of literature about leptin, most papers describing leptin distribution have focussed on local movement, e.g. transport across the blood brain barrier (Banks *et al.* 1996) or kidneys

(Cumin *et al.* 1996), rather than a broad examination of leptin distribution. Indeed, to date the authors are aware of only five pharmacokinetic studies, with four of these having examined leptin distribution from the circulation (Ceccarini *et al.* 2009, Hill *et al.* 1998, Li *et al.* 2013, McMurty *et al.* 2004, Van Heek *et al.* 1996). In chickens a time course study has been conducted (McMurty *et al.* 2004), whereas in mammals only one study has reported tissue distribution at more than two time points for comparison in a number of tissues, however this examined distribution in *ob/ob* mice at a supraphysiologic dose via intraperitoneal administration (Van Heek *et al.* 1996). The locations of leptin binding in the periphery of 'normal' animals after a physiologic dose over a time course are largely unknown. Therefore, a time course experiment may provide detailed information about major targets for peripheral leptin and its profile in the target tissues. Here we describe the distribution of radiolabelled leptin at a physiologic dose over a two hour time period in female mice following intravenous administration.

Materials and Methods

Animals

Female Swiss mice, aged 8-16 weeks weighing 37.92 ± 2.33 g, were obtained from a colony maintained at the University of New England Animal House. Mice were kept in same sex litter groups with *ad libitum* access to commercial chow and water. A 12 h light dark cycle was maintained with lights on at 07:00 h AEST in a room kept at 22 ±0.5 °C. All work was approved by the University of New England Animal Ethics Committee and conformed to the NHMRC Code of Practice for the Care and Use of Animals for Scientific Purposes.

Experimental Protocol

Recombinant bovine leptin (Kauter et al 2000) was labelled with ^{125}Iodine (ANSTO, Lucas Heights, NSW, Australia) using the Iodogen method (Thermo Fisher Scientific, Rockford, IL). Mice were injected via the lateral tail vein with 12 ng of radiolabelled leptin (37.02 kBq) in a total volume of 100 µl made up with phosphate buffered solution. Animals were then placed in an individual cage with access to food and water until the specified time when the animal was euthanised by CO_2 asphyxiation at 5, 15, 30, 60 (n = 2 each) and 120 min (n = 1) after injection to observe the radiolabelled leptin distribution over time.

Tissues were dissected and weighed, with duplicate samples placed in polypropylene tubes and measured for total γ-radioactivity (1470 Wizard, Perkin Elmer, Turku, Finland). Background radiation was subtracted from all samples. Measurements from replicates were averaged and multiplied across the total mass of the relevant tissue to calculate total tissue accumulation.

Cardiac puncture was performed immediately after euthanasia to collect blood, which was transferred into a heparinised tube before organs were dissected out and weighed. The digestive tract tissues were measured with their respective contents. Two samples of small intestine contents were precipitated with 30 % trichloroacetic acid to determine intactness of the radiolabelled leptin. Skin was removed with the exception of that around the snout and ‘cuffs’ around the paws and tail of the animals. Four

segments (fore limb, hind limb, interscapular region and dorsal cervical region) were collected, with radioactivity measured and averaged for these samples. This average was then multiplied for the mass of the whole skin to estimate the total recovered from the tissue. Similarly for the blood, duplicate samples of blood were measured and this was averaged and multiplied to estimate the total in circulation based on a total blood volume estimated at 96.3 ml/kg of body weight as previously reported (Riches *et al.* 1973).

Data Analysis

Plasma and whole body clearance of leptin was calculated by using the area under the curve method, fitting a second order exponential decay curve to the respective data using Origin 4.10 (Microcal Software Inc. 1996) using the formula: $y = A\,e^{-x/t_1} + B\,e^{-x/t_2}$ where y represents the radioactivity per ml of blood or total radioactivity recovered from the body at time x (min), A and B are the radioactivity present in each pool and t_1 is $1/\alpha$ and t_2 is $1/\beta$ where α and β are the decay constants for the respective pools.

All data are expressed as mean ± standard error, unless raw data are presented.

Results

After intravenous administration of leptin to female mice at a physiologic dose, major targets were the liver, kidneys, digestive tract and skin. Radiolabelled leptin was recovered from other tissues and these were pooled as "other tissues". These tissues included the brain, submandibular salivary glands, spleen, heart, lungs, ovaries, uterus and perirenal fat (figure 1).

Radiolabelled leptin from the blood, liver, kidneys and the pooled 'other tissues' was rapidly cleared and was cleared from the skin slightly slower. Five minutes post-injection the blood contained 42.9 ± 0.8 % of the administered dose, the liver 19.5 ± 2.5 %, the kidneys 11.1 ± 1.1 %, skin 11.4 ± 0.3 % and 'other tissues' 3.9 ± 0.1 % of the administered dose. The lungs contributed 19 – 33 % of the radiolabelled leptin recovered in the 'other tissues' over the duration of the experiment. In contrast, between 5 and 60 minutes following administration the total radiolabelled leptin recovered from the digestive tract (pooled with luminal contents) increased from 6.4 ± 1.3 % of the dose to 12.8 ± 0.7 % of the dose before a slight decrease to 10.0 % of the dose 120 minutes after injection. The contents of the small intestine (n = 2) were found to be 45.2 ± 1.6 % intact. The highest amount of radiolabelled leptin recovered from the brain was 0.267 % of the dose 5 minutes after administration (data not shown).

The total amount of radiolabelled leptin recovered from all examined tissues followed an exponential decay pattern (figure 2). A total of 95.3 ± 5.9 % of the administered dose was recovered 5 min post-injection, declining to 54.3 ± 4.1 % 15 min post-injection and decreasing to 28.1 % of the dose 120 min after administration. Using these data whole body leptin kinetics were examined, revealing administered leptin had a clearance rate of 0.23 ml/min/kg and a half-life of 47.3 min (table 1) in the whole animal with an α phase half-life of 3.6 min and a β phase half-life of 150.0 min.

Radiolabelled leptin recovered from the blood rapidly dropped from 14.01 ± 0.38 % of the administered dose per ml (dose/ml) 5 min post-injection to 5.28 ± 0.94 % of dose/ml 15 min after administration. Following this a slower decline was seen to 3.09 % of dose/ml 120 min post-injection (figure 3). Using these data the plasma clearance rate was calculated to be 1.58 ml/kg/min and the

half-life of administered leptin in the plasma to be 32 minutes (table 1) with an α phase half-life of 2.9 min and a β phase half-life of 230.1 min.

In most of the tissues examined the radiolabelled leptin per gram of tissue (dose/g) decreased over the course of the experiment. Five minutes after injection concentrations in the kidneys and liver contained 22.71 ± 3.70 % of dose/g and 10.19 ± 1.34 % of dose/g, respectively, while the skin had 2.54 ± 0.10 % of dose/g. In the lungs a rapid drop was seen 5-15 min after administration from 7.69 ± 0.49 % of dose/g to 2.45 ± 0.45 % of dose/g; this then remained relatively stable until 60 min post-injection, followed by a drop 120 min after injection to 1.46 % of dose/g. In the brain there was also a decrease in exogenous leptin per gram 5-30 min post-injection from 0.55 ± 0.05 % of dose/g to 0.15 ± 0.02 % of dose/g, followed by a slight increase 60 min post-injection to 0.19 ± 0.03 % of dose/g and then a slight decrease 120 min after administration to 0.11 % of dose/g. Radiolabelled leptin recovered per gram of perirenal fat 5 min after administration was 3.55 ± 0.10 % of dose/g, dropping to approximately 0.74 % of dose/g, which was maintained to 60 min post-injection before decreasing to 0.51 % of dose/g 120 min post-injection. Muscle from the left quadriceps and biceps femoris were examined 15, 30 and 60 min post-injection (n = 1 animal each) and were found to have 0.67 % of dose/g, 0.62 % of dose/g and 0.43 % of dose/g, respectively.

Generally, when radiolabelled leptin per gram of tissue was examined the pattern was similar to that reported in total dose recovery above, with highest concentrations recovered from the blood (figure 3), kidneys, liver, skin and perirenal fat (figure 4) and a decrease in radioactivity detected. One notable difference was that the lungs displayed a high amount of radiolabelled leptin per gram.

Discussion

Leptin distribution was examined in female mice after intravenous injection of a physiologic dose of radiolabelled leptin. The tissue distribution of radiolabelled leptin reported is similar in pattern to those reported previously for intravenous administration in rats (Hill *et al.* 1998) and chickens (McMurty *et al.* 2004) and following intraperitoneal administration in mice (Van Heek *et al.* 1996). The tissues where a high amount of radiolabelled leptin was recovered included the, kidneys, liver, skin and gastrointestinal tract (and contents). Similar to the previous studies, it was found that the brain was not a major target for leptin in these mice. In most of the tissues examined there appeared to be an initial rapid clearance of leptin from the tissue before a slower phase of clearance. The radiolabelled leptin was detected in all examined tissues up to the conclusion of the experiment 120 min after the injection.

Consistent with previous reports in several vertebrates (Hill *et al.* 1998, McMurty *et al.* 2004, Van Heek *et al.* 1996), very little of the administered leptin (< 1 % of the administered dose here) was detected in the brain of the mice over the duration of the experiment. While on face value this may seem to contradict the notion of leptin from the periphery playing a major role in the central nervous system in the regulation of energy turnover and body mass (Friedman and Halaas 1998), it must be noted that LepRb is found in the hypothalamus at relatively high density compared with most other regions of the brain (Ghilardi *et al.* 1996, Lee *et al.* 1996), particularly in the arcuate, ventromedial, dorsomedial, lateral and paraventricular nuclei, which are thought to be involved in appetite regulation (Fei *et al.* 1997, Mercer *et al.* 1996). Furthermore, intravenously administered radiolabelled leptin, albeit at a supraphysiologic dose, can be recovered from the arcuate nucleus of the hypothalamus and the choroid plexus (Banks *et al.* 1996). This may indicate that these hypothalamic nuclei are extremely sensitive to the signalling of leptin from the periphery, which may be supported by the intravenous and intraperitoneal administration of leptin to mice inducing STAT3 activation in the hypothalamus at a physiologic dose (Vaisse *et al.* 1996). However, as > 99% of the administered dose of leptin was not recovered from the central nervous system it would appear that the major roles for leptin lie in the periphery.

The total administered leptin recovered from the mice declined over the course of the experiment. This is consistent with leptin being eliminated from the system via the kidneys (Cumin *et al.* 1996), but also suggests that leptin may also enter tissues that were not sampled here. As 4.68 – 71.92 % of the administered dose was unaccounted for over the duration of the experiment some representative samples of muscle were examined. In female mice skeletal muscle has been reported to account for 20 % of body mass (Griffin and Goldspink 1979) , if this were assumed and that 0.6 % of the dose was recovered per gram of muscle (an approximation of the findings) there may be 4.55 % of the dose in skeletal muscle over the entire animal. Both muscle (De Matteis *et al.* 1998, Hoggard *et al.* 1997, Löllmann *et al.* 1997) and bone (osteoblasts and chondrocytes) express leptin receptors (Steppan *et al.* 2000). In the muscle leptin attenuates insulin induced lipogenesis (Muoio *et al.* 1997) and stimulates fuel oxidation (Dulloo *et al.* 2002, Muoio *et al.* 1997). In bone leptin has been shown to stimulate growth in *ob/ob* mice (Steppan *et al.* 2000), although centrally it appears to inhibit bone growth (Ducy *et al.* 2000). Therefore, it seems reasonable to speculate that a portion of the leptin not recovered over the course of the experiments was sequestered into the musculoskeletal system.

The plasma half-life for the administered leptin in the plasma was found to be 32 min. This is comparable with reported endogenous human leptin half-life, at 24.9 min (Klein *et al.* 1996). The α phase half-life, 2.9 min, is similar to reports in rats of approximately 5.1 min (Zeng *et al.* 1997) and 3.4 min (Hill *et al.* 1998), less than reported for human leptin in monkeys at 10.4 min (Ahrén *et al.* 2000) and intermediate to the early phase half-lives (1.2 – 7.2 min) reported in rats in a third order model (Cumin *et al.* 1996). In contrast, the terminal phase plasma half-life was 230.1 min and is much higher than the values reported in mice (Ahrén *et al.* 2000) and rats (Zeng *et al.* 1997) of approximately 49 min, or other reported values in rats ranging from 71 min (Hill *et al.* 1998) to 90 min (Cumin *et al.* 1996) and is also higher than that reported for human leptin in rhesus monkeys of 96 min (Ahrén *et al.* 2000). However, a number of these studies were performed at pharmacologic doses ranging from 0.25 mg/kg (Cumin *et al.* 1996) to 10 mg/kg (Ahrén *et al.* 2000). The clearance from the blood reported is consistent with leptin removal by the kidneys, as has been identified as the primary mechanism of elimination (Cumin *et al.* 1996, 1997). The reported data may have been

improved if the experiment was run over a longer period with more early time points, which may have enabled the fitting of a third order exponential decay curve, as reported previously (Cumin *et al.* 1996). Due to the sexual dimorphism of circulating leptin concentrations (Saad *et al.* 1997) it may be interesting to determine whether the parameters examined here are similar in male mice.

Interestingly, the whole body half-life for administered leptin was approximately 1.5 times longer than that for plasma borne leptin at 47.3 min, the reason for this is not clear. However, as hypothesised previously (Hill *et al.* 1998) there seems to be a large pool of more slowly cleared leptin that is thought to include leptin bound to receptors in tissues of the periphery . As indicated earlier, the musculoskeletal system appears to be a target for leptin. Additionally, adipose tissue may retain some peripheral leptin, although this may be transient, as indicated by the rapid drop in concentration seen in the perirenal fat. Another large sink for circulating leptin was identified in the digestive tract, with 12.8 % of the administered dose recovered 60 min after administration. This, as well as the slow clearance from the blood 15-120 min after administration may indicate that leptin enters these tissues for a period of time before re-entering the circulation. This possibility is supported by the finding that LepRa-d facilitate the endocytosis and subsequent exocytosis of intact leptin from cells *in vitro* (Tu *et al.* 2007). Further investigation would be needed to confirm this possibility.

Generally the pattern in total leptin recovery and leptin per gram in tissues examined followed a similar pattern, with a notable exception in the lungs. The highest total recovery detected from the lungs was 1.3 ± 0.01 % of the administered dose 5 min post-injection (data not shown), in comparison > 10 % of the radiolabelled leptin administered was recovered from the blood, liver, kidneys, skin and digestive tract, respectively, at various times observed. However, when concentration was examined 5 min post-injection 7.69 ± 0.49 % of dose/g was recovered from the lungs and only the blood, liver and kidneys exhibited higher amounts per ml or gram. It is possible that a portion of the radiolabelled leptin recovered from the lungs is actually in the blood, however cardiac puncture would be expected to have removed most of this and the data presented are consistent with distribution reported in rats (Hill *et al.* 1998). This would seem to suggest that leptin plays a particularly important role in the lungs. Leptin receptor mRNA is found at a relatively high abundance in the lungs (Ghilardi *et al.*

1996, Löllmann *et al.* 1997) and both long and short isoforms are expressed as proteins in the tissue, with immunohistochemistry showing club cells, muscle and veins stain strongly for LepRb (De Matteis *et al.* 1998). Furthermore, leptin has been shown to have physiologic effects in the lungs including regulation of tissue maturation and possibly increasing surfactant secretion (Kirwin *et al.* 2006). Radiolabelled leptin concentrations were apparently maintained at approximately 2.36 % dose/g for 45 min during the experiment, possibly indicating that leptin has a normal maintenance role in the lungs and as LepRb is expressed in club cells (De Matteis *et al.* 1998), which can modulate inflammation (Snyder *et al.* 2010), this may be related to immune function.

The total recovery of exogenous leptin from the skin was examined for the first time and showed that the skin is a major target for circulating leptin. Five min post-injection 11.4 ± 0.3% of the radiolabelled leptin was detected in the skin. It should be noted that skin was removed intact from animals with some underlying tissue and may therefore also be a proxy for subcutaneous fat, as leptin is known to accumulate in fat (Ceccarini *et al.* 2009, Li *et al.* 2013). However, as the skin is the largest organ of the body, it seems reasonable to suggest that with such a high recovery of the administered dose from the tissue that this could be the skin itself. In support of this LepRb is expressed in human fibroblasts (Glasow *et al.* 2001). Leptin is capable of stimulating skin growth and enhances wound healing, with LepRb expression reported at the margins of wound sites (Frank *et al.* 2000). Furthermore, leptin has been found in the skin and is reduced in response to injury (Stallmeyer *et al.* 2001), coupled with the high proportion of leptin recovered from the skin here, leptin appears to play a role in the maintenance of skin homeostasis. As a high concentration of leptin was also found in the lungs and large amounts were in the digestive tract, all tissues that constitute the interface of the body with the environment, it may be that leptin primes these tissues ready to respond to insult, but more work would be required to confirm this.

In summary, plasma leptin half-life in female mice was found to be shorter than whole body half-life and is presumed to be due to accumulation of leptin in peripheral tissues. A total of 12.8 % of the dose was found to be in the digestive tract tissues and contents 60 min after administration, while 11.4 % was recovered from the skin 5 min after administration, representing major targets for leptin in the

circulation and possibly indicating far more prominent roles for leptin in these tissues. A larger sample size and longer time course may allow the use of more complex modelling, such as the use of a third order exponential decay curve, to accurately describe leptin pharmacokinetics in the female mouse.

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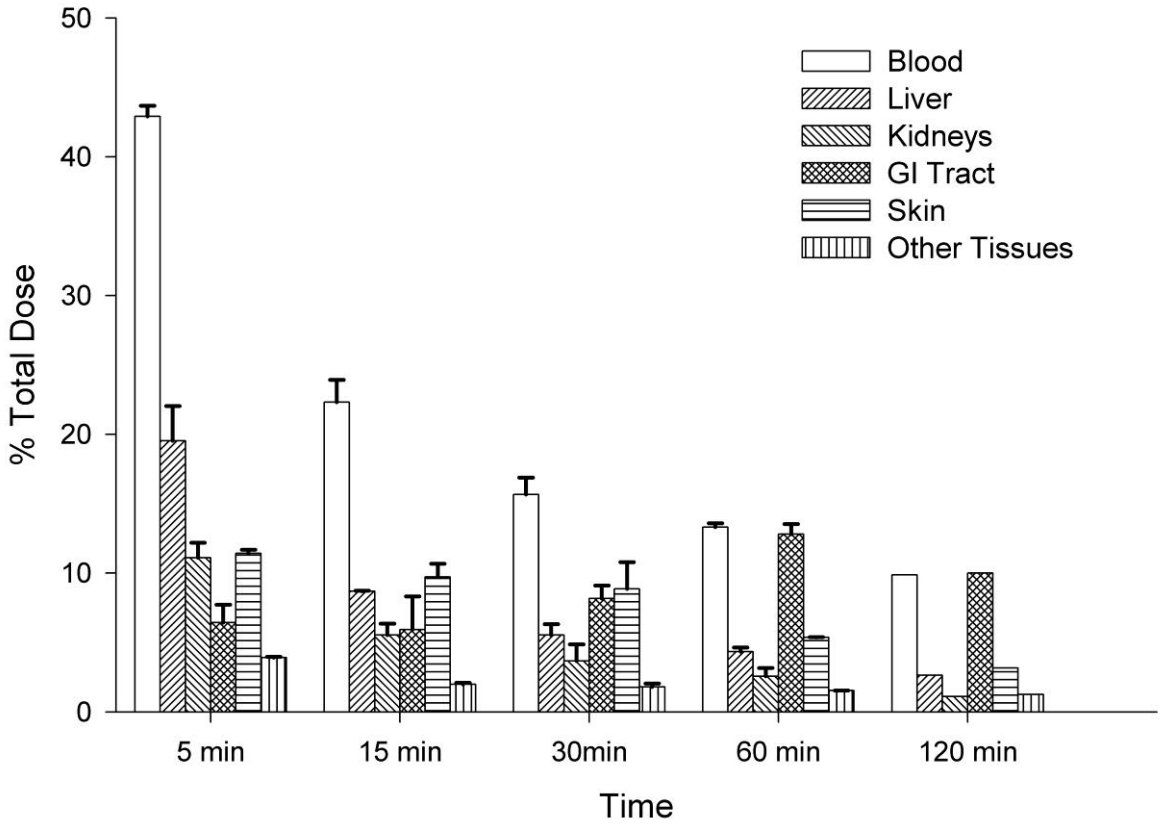
433 **List of Figure Captions**



434

435 **Figure 1** – Major targets for leptin in female mice after intravenous administration as the percentage
436 of initial dose recovered from each tissue (GI Tract – gastrointestinal tract and contents; Other Tissues
437 – Pooled data for brain, submandibular salivary glands, spleen, heart, lungs, ovaries, uterus and
438 perirenal fat)

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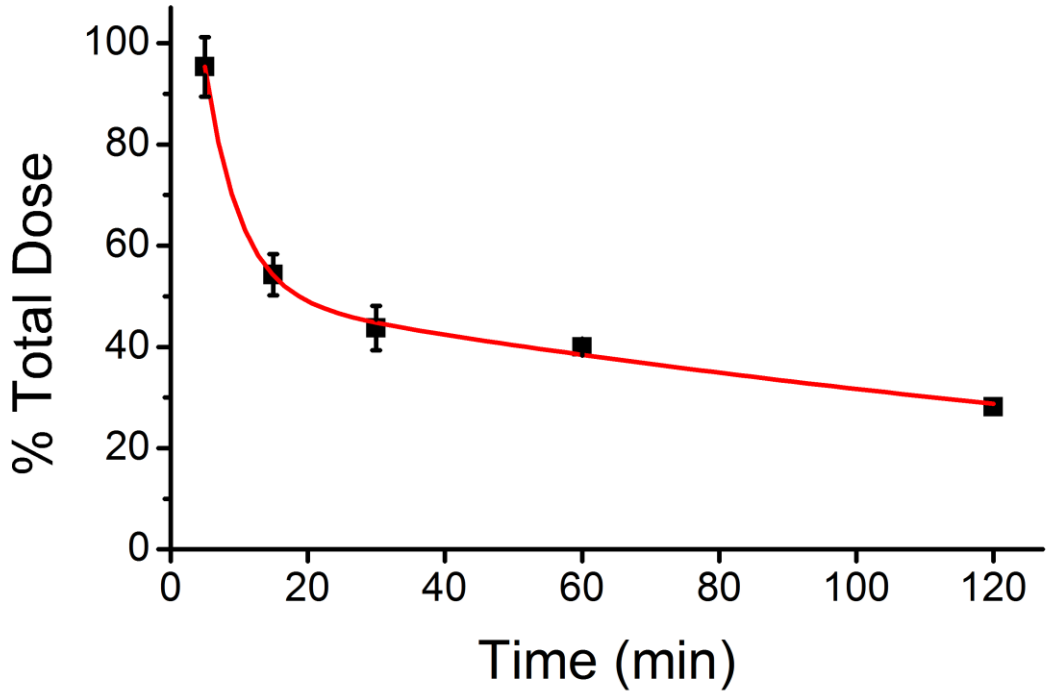

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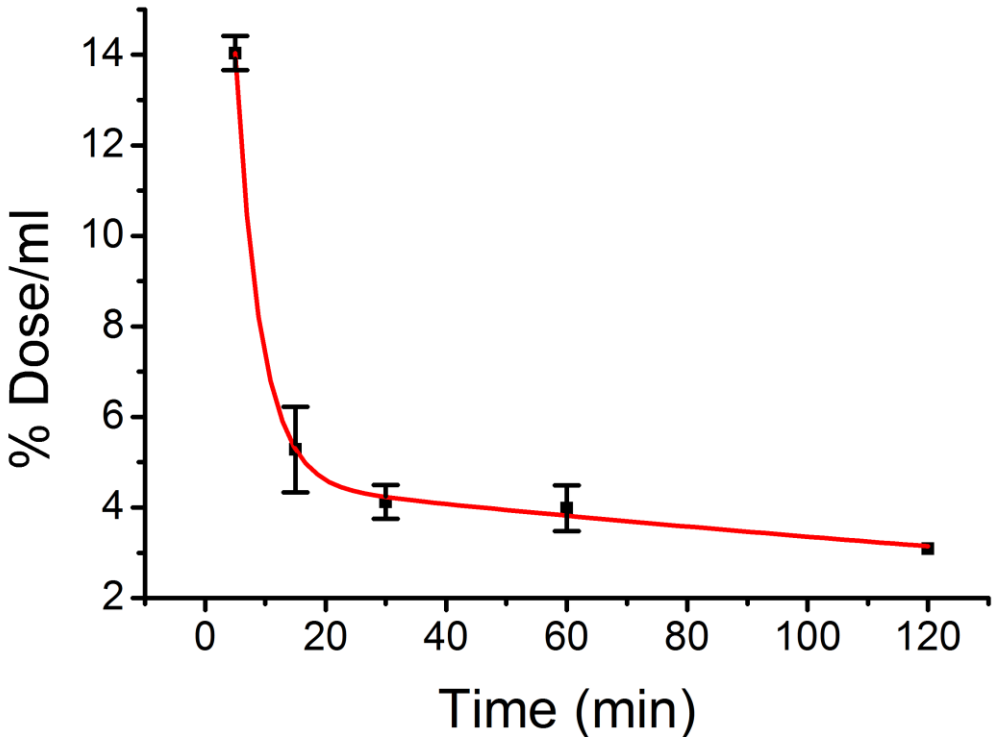
441 **Figure 2:** Radiolabelled leptin clearance from all examined tissues of female mice following
442 intravenous administration presented as percentage of total administered dose with a second order
443 exponential decay curve fit

444



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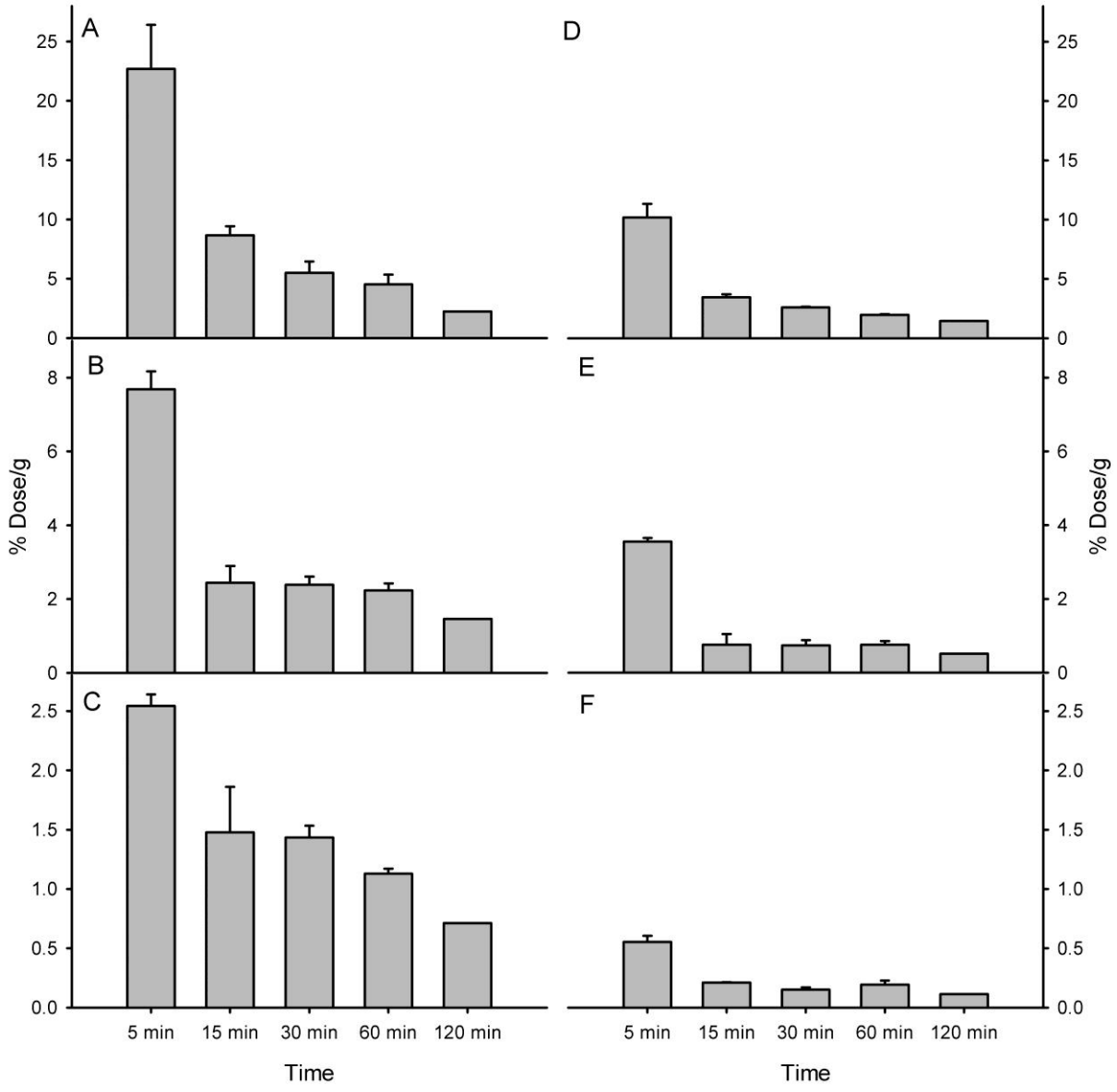
446 **Figure 3** – Radiolabelled leptin in the blood of female mice after intravenous administration

447 measured as percentage of dose per ml with a second order exponential decay curve fit

448



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450 **Figure 4** – Radiolabelled leptin after intravenous administration to female mice presented as

451 percentage of administered dose per gram of tissue (A – Kidneys; B – Lungs; C – Skin; D – Liver; E –

452 Perirenal Fat; F – Brain)

453

List of Tables

Table 1 – Radiolabelled leptin pharmacokinetic parameters in female mice

	Plasma	Whole Body
Clearance Rate (ml/kg/min)	1.59	0.23
Half-Life (min)	32.0	47.3