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Effects of wintertime fasting and seasonal adaptation on AMPK and ACC in hypothalamus, adipose tissue and liver of the raccoon dog (*Nyctereutes procyonoides*)

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ABSTRACT

The raccoon dog (*Nyctereutes procyonoides*) is a canid with autumnal fattening and passive wintering strategy. We examined the effects of wintertime fasting and seasonality on AMP-activated protein kinase (AMPK), a regulator of metabolism, and its target, acetyl-CoA carboxylase (ACC) on the species. Twelve farmed raccoon dogs (eleven females/one male) were divided into two groups: half were fasted for ten weeks in December–March (winter fasted) and the others were fed *ad libitum* (winter fed). A third group (autumn fed, eight females) was fed *ad libitum* and sampled in December. Total AMPK, ACC and their phosphorylated forms (pAMPK, pACC) were measured from hypothalamus, liver, intra-abdominal (iWAT) and subcutaneous white adipose tissues (sWAT). The fasted animals lost 32% and the fed 20% of their body mass. Hypothalamic AMPK expression was lower and pACC levels higher in the winter groups compared to the autumn fed group. Liver pAMPK was lower in the winter fasted group, with consistently decreased ACC and pACC. AMPK and pAMPK were down-regulated in sWAT and iWAT of both winter groups, with a parallel decline in pACC in sWAT. The responses of AMPK and ACC to fasting were dissimilar to the effects observed previously in non-seasonal mammals and hibernators. Differences between the winter fed and autumn fed groups indicate that the functions of AMPK and ACC could be regulated in a season-dependent manner. Furthermore, the distinctive effects of prolonged fasting and seasonal adaptation on AMPK–ACC pathway could contribute to the wintering strategy of the raccoon dog.

1. Introduction

Mammals living at northern latitudes have evolved diverse physiological adaptations to endure wintertime food scarcity and cold temperatures. While some species continue active foraging or rely on their food caches, other species cease eating after autumnal fattening and hibernate to survive the winter. Hibernation is a state of a reduced body temperature (T_b) and metabolic rate (IUPS Thermal Commission, 2001). A shallow form of wintertime passivity is exhibited by a few larger species, such as bears (*Ursus* spp.) and badgers (*Meles meles* and *Taxidea taxus*; Harlow, 1981; Hissa, 1997). Robust seasonal fluctuations in food intake and body mass (BM) are characteristic of hibernating or passively wintering species.

The raccoon dog (*Nyctereutes procyonoides*) is an omnivorous canid species originating from eastern Asia. Animals of the subspecies *N. p. ussuriensis*, native to Amur and Ussuri regions of Siberia and eastern China (Novikov, 1962), were widely introduced to northwestern U.S.S.R. between the 1930s and 1950s and in a few decades the raccoon dogs rapidly spread to parts of northern and eastern Europe (Kauhala, 1996). In Finland, the northern limit of distribution lies at the Arctic Circle (Kauhala and Saeki, 2004). Raccoon dogs have been bred for commercial fur farming in Finland since the 1970s, the farmed population originating from captured wild animals (Mäkelä, 1973).

In the native range of *N. p. ussuriensis* the winters are cold and snowy, which makes it well pre-adapted to the cold climate of northern Europe. To survive the harsh conditions, the raccoon dog has evolved a unique wintering strategy among canids. It displays autumnal fattening and long fasting periods during winter. In Finland, the BM of the raccoon

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Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; BM, body mass; T_b, body temperature; iWAT, intra-abdominal white adipose tissue; sWAT, subcutaneous white adipose tissue.

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dog varies 30–40% throughout the year, being the highest in the late autumn (Oct–Dec) and the lowest in the summer (Siivonen, 1972; Kauhala, 1992; Asikainen et al., 2004). The BM ranges between 3.5 and 10.5 kg in the wild (Kauhala, 1992) and between 5.5 and 14.5 kg in the farm animals (Asikainen et al., 2002). Due to favorable feeding conditions and lack of exercise, farmed raccoon dogs are usually larger and have more abundant adipose tissue, observed as higher body mass indexes (BMI). The highest BMI in autumn is approximately 35–40 kg/m^3 in farmed and 25–30 kg/m^3 in wild animals (Asikainen et al., 2004; Mustonen et al., 2004, 2007a; Nieminen et al., 2005). In contrast to hibernating species, raccoon dogs show only a moderate, 1–2 °C reduction in T_b during the winter rest (Nieminen et al., 2005; Mustonen et al., 2007b, 2012). Furthermore, depending on the snow cover and food availability, they can forage actively during winter (Siivonen, 1972; Asikainen et al., 2004; Mustonen et al., 2012). Raccoon dogs can remain normoglycemic and defend their bone mass as well as muscle protein concentration and function during prolonged wintertime fasting (Mustonen et al., 2004; Nieminen et al., 2011; Kinnunen et al., 2015). Raccoon dogs raised in Finnish fur farms do not experience distinct winter lethargy mostly due to the regular feeding and lack of nest boxes (Korhonen, 1987; Asikainen et al., 2002), but show seasonal fluctuations in food intake with autumnal fattening and voluntary decrease in food intake in winter (Nieminen et al., 2002; Mustonen et al., 2004). The circulating levels of hormones that regulate energy homeostasis, such as leptin, ghrelin and insulin, participate in the seasonal regulation of BM (Nieminen et al., 2002; Mustonen et al., 2004). Previous studies show that the observed seasonal physiological responses are quite similar in wild and farmed raccoon dogs (Nieminen et al., 2002, 2005; Asikainen et al., 2004; Mustonen et al., 2004, 2007a, 2007b, 2007c, 2012).

AMP-activated protein kinase (AMPK) is an intracellular energy sensor that also plays a major role in the regulation of whole body energy balance (Hardie and Carling, 1997; Andersson et al., 2004; Minokoshi et al., 2004). In response to high AMP/ATP ratio caused by metabolic stress factors, such as fasting or exercise, AMPK is activated by phosphorylation on threonine 172 of the catalytic α subunit. AMPK activation promotes energy producing pathways and inhibits anabolic processes that consume ATP (Hardie et al., 2003; Kahn et al., 2005). AMPK is the main regulator of the activity of acetyl-CoA carboxylase (ACC), a key enzyme in the fatty acid metabolism in the cell. AMPK inhibits ACC by phosphorylation, resulting in suppressed malonyl-CoA content, which consequently increases mitochondrial β-oxidation and decreases fatty acid synthesis (Kahn et al., 2005; Wolfgang and Lane, 2006). The AMPK–ACC pathway mediates the effects of several hormones and nutrients in the hypothalamus and peripheral tissues. Anorectic signals, such as leptin and insulin, decrease AMPK phosphorylation in the hypothalamus (Andersson et al., 2004; Minokoshi et al., 2004). In contrast, orexigenic signals, such as ghrelin and adiponectin, increase the hypothalamic AMPK phosphorylation (Kola et al., 2005; Kubota et al., 2007; Minokoshi and Kadowaki, 2007). In laboratory rodents, fasting increases the hypothalamic AMPK activity, thereby also decreasing the ACC activity and subsequently leading to increased food intake. On the other hand, low AMPK activity and decreased ACC phosphorylation under fed conditions suppress feeding (Minokoshi et al., 2004). In peripheral tissues, the regulation of AMPK differs from the hypothalamus by being tissue-specific. Leptin and adiponectin activate AMPK in the liver and white adipose tissue (WAT), initiating pathways that decrease gluconeogenesis and fatty acid synthesis and increase glucose utilization and fatty acid oxidation. In contrast, ghrelin inhibits AMPK in the liver and WAT, enhancing lipogenic pathways (Xue and Kahn, 2006). Nutrient deprivation and prolonged starvation have been shown to activate hepatic AMPK (Assifi et al., 2005; Dentin et al., 2005). In WAT, AMPK phosphorylation is also increased following food deprivation (Daval et al., 2005).

The roles of AMPK and ACC in the regulation of energy balance have been extensively studied in non-seasonal animals, such as laboratory rodents (Kahn et al., 2005; Xue and Kahn, 2006). The few existing studies with hibernating species indicate that AMPK function differs from non-seasonal animals and that AMPK might have a role in the seasonal regulation of food intake and metabolic adaptations. For example, in winter-acclimated ground squirrels (*Callospermophilus lateralis* and *Ictidomys tridecemlineatus*), the phosphorylation of hypothalamic AMPK does not occur in response to fasting and a decrease in the liver AMPK phosphorylation is observed during hibernation (Horman et al., 2005; Healy et al., 2011b). On the other hand, similar to non-seasonal animals, fasting leads to AMPK activation in WAT (Horman et al., 2005; Florant et al., 2010; Healy et al., 2011b). Previously, we observed that the skeletal muscles of the farm-bred raccoon dog are resistant to wintertime fasting as observed in the maintenance of the functions of AMPK and proteins involved in insulin signaling (Kinnunen et al., 2015).

In the present study, we used farm-bred raccoon dogs to evaluate the effects of wintertime fasting and seasonal adaptation on the expression and phosphorylation of AMPK and ACC in the hypothalamus, liver and WAT, essential tissues in the body energy metabolism. In addition, the circulating levels of several hormones regulating energy homeostasis (leptin, ghrelin, insulin and adiponectin) were analyzed. Based on previous studies with hibernating species, we hypothesized that (*i*) hypothalamic AMPK and ACC phosphorylation would decrease in the fasted raccoon dogs. Since the raccoon dogs are able to maintain their plasma glucose levels during prolonged fasting and AMPK activation is known to inhibit liver gluconeogenesis, we expected (*ii*) fasting to cause a decrease in the phosphorylation of AMPK in the liver. Furthermore, we predicted (*iii*) higher AMPK and concomitant ACC phosphorylation in WAT of the fasted raccoon dogs. We also hypothesized that (*iv*) the levels of AMPK and ACC would follow a seasonal pattern, observed as differences between the winter fed and autumn fed animals.

2. Material and methods

The experiment was conducted at the Kannus Research Farm Luova Ltd. (63°54′30″N, 23°56′26″E). The experimental protocol was approved by the national Animal Experiment Board (license no. ESLH-2009-08219/Ym-23) and complied with the current laws of Finland. In total, 20 farm-bred raccoon dogs were used in this study. All animals were housed in cages (150 × 107 × 70 cm) in a roofed enclosure at natural ambient temperature and photoperiod. The animals were fed according to common farming practices and the feeding regimen varied depending on the season. In late summer–autumn (from July), the animals were fed *ad libitum* with a commercial fur animal energy-rich diet (5120 kcal/kg dry matter) to maximize the body size before skinning. All animals used in the present study received similar diet until sampled. They also received water/ice *ad libitum*. Twelve raccoon dogs (average age 7–8 months, eleven females and one male) were used for the wintertime fasting experiment (December 22, 2009–March 1, 2010). The animals were housed individually in cages with nest boxes. Half of the animals were food-deprived for ten weeks ($N = 6$; winter fasted) and the other half were fed throughout the experiment ($N = 6$; winter fed). Raccoon dogs were weighed once before the fasting period (December 7) and three times (January 19, February 23, March 1) during the experiment. The average BM of the winter fasted and fed raccoon dogs were 13.9 ± 0.7 and 14.0 ± 0.5 kg before the experiment. The third experimental group (autumn fed) included eight farm-bred adult (age 19–69 months, average BM 12.0 ± 0.5 kg) female raccoon dogs housed in pairs in cages. The autumn fed animals were sampled in late autumn (December), when the fat stores are the most abundant.

Both winter and autumn fed groups were fasted overnight before sampling. The autumn fed animals were used for commercial fur farming purposes and sampled after euthanasia and skinning. For this reason, no license from the Animal Experiment Board was necessary for the sampling of this group. The winter fasted and fed groups were sampled at the end of the experiment (March 1, 2010) and the autumn