

SHORT COMMUNICATION

Effects of a caspase and a calpain inhibitor on resting energy expenditures in normal and hypermetabolic rats: a pilot study

Short title: **Effects of caspase/calpain inhibitors on energy expenditures**

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Summary (200 words):

Several diseases induce hypermetabolism, which is characterized by increases in resting energy expenditures (REE) and whole body protein loss. Exaggerated protein degradation is thought to be the driving force underlying this response. The effects of caspase and calpain inhibitors on REE in physiological and hypermetabolic conditions, however, are unknown. Thus, we studied whether MDL28170 (calpain inhibitor) or z-VAD-fmk (caspase inhibitor) affect REE under physiological conditions and during hypermetabolism post-burn. Rats were treated 5xweekly and observed for 6 weeks. Treatment was started 2h (Early) or 48h (Late) after burn. In normal rats, MDL28170 transiently increased REE to 130% of normal during week 2-4. z-VAD-fmk reduced REE by 20-25% throughout the observation period. Within 14 days after burns, REE increased to $130\pm 5\%$. Whereas MDL28170/Early treatment did not affect REE, MDL28170/Late transiently increased REE to $180\pm 10\%$ of normal by week 4 post-burn. In contrast, with z-VAD-fmk/Early REE remained between 90-110% of normal post-burn. z-VAD-fmk/Late did not affect burn-induced increases in REE. These data suggest that caspase cascades contribute to the development of hypermetabolism and that burn-induced hypermetabolism can be pharmacologically modulated. Our data point towards caspase cascades as possible therapeutic targets to attenuate hypermetabolism after burns, and possibly in other catabolic disease processes.

Keywords: Resting energy expenditure; burn, hypermetabolism, calpain, caspase

Main body of the text:

Multiple disease processes, such as cancer, sepsis, trauma or burns, induce hypermetabolism, which is characterized by increases in resting energy expenditures (REE) and total body protein loss (Dev 2014, Madeddu *et al.* 2015, Cohen *et al.* 2015). The clinical consequences of hypermetabolism are profound and include muscle cachexia, increased morbidity and mortality and prolonged hospitalization and recovery periods (Pereira *et al.* 2005, Dev 2014, Madeddu *et al.* 2015, Cohen *et al.* 2015). Drugs that may attenuate the hypermetabolic response are currently not available. Such drugs, however, are highly desirable as they have the potential to improve outcomes.

In patients with burn injuries, increases in REE are proportional to the size and degree of the burn injury and persist for month to years, even after complete wound closure (Matsuda *et al.* 1987, Jeschke *et al.* 2011). Studies of muscle protein turnover after burn injuries in patients showed that both protein synthesis and degradation are significantly increased. As increases in protein breakdown exceeded increases in protein synthesis, exaggerated protein degradation appears to be the driving force underlying the development of hypermetabolism (Biolo *et al.* 2002, Chao *et al.* 2015). Thus, drugs that inhibit protein degradation could potentially attenuate net protein loss and increases in REE in hypermetabolic conditions. The specific contributions of major non-lysosomal proteolytic systems, such as the ubiquitin-proteasome pathway of protein degradation, the calpain or caspase system, to the development of post-burn hypermetabolism, however, are not well understood. We have shown previously that bortezomib, a FDA approved proteasome inhibitor, delayed increases in REE in a rat scald burn model when treatment was initiated 48h after injury and reduced burn-induced REE when treatment

was started within 2h after injury (Vana *et al.* 2015). The latter, however, was associated with significantly increased mortality after burns (Vana *et al.* 2015). The effects of calpain and caspase inhibitors on REE after burns are unknown. Thus, we performed a pilot study to assess whether the calpain inhibitor MDL28170 and the pan-caspase inhibitor Z-Valine-Alanine-Aspartate-fluoromethylketone (z-VAD-fmk) influence REE and body weight within a clinically relevant time frame in a rat scald burn model. MDL28170 is a cell-permeable calpain inhibitor that has been reported to possess neuroprotective effects in vivo (Kawamura *et al.* 2005, Li *et al.* 1998). Z-VAD-fmk is an irreversible cell-permeable pan-caspase inhibitor with in vivo anti-apoptotic and anti-inflammatory effects (Graczyk 2002, Neviere *et al.* 2001).

All procedures were performed according to National Institutes of Health Guidelines for Use of Laboratory Animals and approved by the Loyola Institutional Animal Care and Use Committee and the Department of Defense Animal Care and Use Review Office. Male Sprague Dawley rats (325-375 g, Harlan, Indianapolis, IN) were anesthetized with 2.5% isoflurane, shaved and placed into a template that exposes a dorsal body area corresponding to 30% of their total body surface area (TBSA). A full thickness burn was then induced by immersion of the dorsal skin into boiling water as previously described (Wong *et al.* 2014, Vana *et al.* 2015). Sham animals were treated as described, except that their dorsal surface was immersed in tepid water. Burned animals were then resuscitated with crystalloid solution i.p. as per the Parkland formula (4 mL/kg per percent TBSA) over the first 48h after burn injury. Animals were treated with vehicle (0.4 ml normal saline), 10 mg/kg MDL28170 (Enzo Life Sciences, Farmingdale, NY and Cayman Chemical, Ann Arbor, MI) or 1 mg/kg z-VAD-fmk (Enzo Life Sciences, Santa Cruz

Biotechnology, Dallas, TX) in 0.4 ml of normal saline i.p. five times weekly. Treatment was started 48h after sham injury, and 2h (early) or 48h (late) after burn. The doses of MDL28170 and z-VAD-fmk were selected based on previous animal studies in which these drugs were found to be safe and effective (Rodriguez *et al.* 1996, Equils *et al.* 2009, Yu and Geddes 2007). Body weight and REE were measured weekly throughout a six week observation period. REE is expressed as % of normal (day 0) and was measured with indirect calorimetry, as described previously (Wong *et al.* 2014, Vana *et al.* 2015). At the end of the observation period, animals were euthanized. Data, presented as mean \pm SEM, were analyzed with two-way ANOVA with Holm-Sidak's multiple comparisons test. Survival between groups was compared with the Log-rank test. Statistical analyses were calculated with GraphPad Prism program (GraphPad Software). A two-tailed $p < 0.05$ was considered significant.

The effects of MDL28170 (n=5) and z-VAD-fmk (n=5) treatment on body weight and REE in animals after sham procedure (vehicle treatment – n=9) are shown in Fig. 1. All except one animal after sham procedure survived the observation period and showed normal behavior and food intake. The single animal that died after sham procedure was vehicle treated. Post mortem necropsy did not reveal any macroscopic abnormalities. There were no statistically significant differences in body weights between groups before sham injury (day 0). Body weights increased continuously and were indistinguishable among the groups (Fig. 1A). In vehicle treated animals, REE remained constant throughout the observation period (105 \pm 3 % - 114 \pm 7%; Fig. 1B). With MDL28170 treatment, however, REE increased transiently to 131 \pm 4% on day 14 and normalized by

day 42 (Fig. 1B). In contrast, REE were significantly reduced on day 7 ($76\pm 3\%$) and on days 21 ($84\pm 2\%$) – 42 ($83\pm 3\%$; Fig. 1B) with z-VAD-fmk treatment.

After burn injury and vehicle treatment ($n=10$), body weights slightly increased within the observation period (Fig. 2A). As compared with vehicle treated animals after sham procedure (Fig. 1A), body weights were significantly reduced on days 7-42 after burn injury and vehicle treatment. While early ($n=6$) and late ($n=5$) z-VAD-fmk treatment did not affect body weight after burns, MDL28170 early ($n =6$; $p<0.5$ vs. vehicle on day 14) and late ($n = 5$; $p<0.05$ vs. vehicle on days 7, 14 and 28) treatment transiently reduced body weights after burns, as compared with vehicle treated animals (Fig. 2A).

REE increased within 14 days after burn injury and vehicle treatment to $130\pm 5\%$ and remained elevated thereafter (Fig. 2B). This increase in REE after burns is consistent with our previous observations in this model (Wong *et al.* 2014, Vana *et al.* 2015) and with the expected REE of 129% after a comparable burn injury in humans (Jones 1993, Carlson *et al.* 1992). While early treatment with MDL28170 did not affect REE after burns, late MDL28170 treatment transiently enhanced burn-induced increases in REE, which peaked at $184\pm 10\%$ on day 21 ($p<0.05$ vs. vehicle on days 7 and 21-35) and decreased to $155\pm 9\%$ on day 42 ($p>0.05$ vs. vehicle). In contrast, with early z-VAD-fmk treatment, REE remained between $82\pm 4\%$ - $109\pm 9\%$ throughout the six week observation period. As compared with vehicle treated animals, REE were significantly reduced on days 7, 21, 28 and 42 after burns with early z-VAD-fmk treatment. After burn injury, all animals in the vehicle and MDL28170 treatment groups survived the observation period. One animal after burn and early z-VAD-fmk treatment and one animal after burn and late z-VAD-fmk

treatment died during the observation period ($p > 0.05$ vs. vehicle and MDL28170 treatment).

Several lines of evidence suggest that caspase activities are significantly increased in skeletal muscle after burns, which may contribute to metabolic protein turnover (Duan *et al.* 2009, Yasuhara *et al.* 2000, Lee *et al.* 2011, Wong *et al.* 2014). Thus, the finding of the present pilot study that z-VAD-fmk prevented burn-induced increases in REE supports the concept that caspase activation contributes to the development of post-burn hypermetabolism. Furthermore, our observation points towards caspase cascades as potential therapeutic target after burns, and possibly in other muscle wasting disease processes.

Similar to our previous observation that proteasome inhibition resulted in distinct effects when treatment was initiated early and late after burns, z-VAK-fmk prevented increases in REE after burns only when treatment was initiated early (Vana *et al.* 2015). This further supports the notion that events during the initial hypodynamic-hypometabolic phase after burns (ebb or shock phase (Wolfe 1981, Tredget and Yu 1992) initiate and determine the hypermetabolic response weeks after injury.

Interestingly, MDL28170 and z-VAD-fmk treatment resulted in similar effects on REE in animals with burn-induced hypermetabolism and in normal (sham injured) animals. In contrast, we could not observe any changes in REE after proteasome inhibition in normal animals previously (Vana *et al.* 2015). As caspase and calpain mediated protein cleavage are known to alter substrate availability of the proteasome (Pasiakos and Carbone 2014), these data may suggest an important role of the calpain

and caspase systems in the regulation of normal and pathological protein and energy metabolism.

In context of the existing information on the roles of calpains in muscle wasting diseases, the observation that MDL28170 increased REE after burns appears paradoxical and requires further investigation (Costelli *et al.* 2005, Smith *et al.* 2008). As for any pharmacological intervention, however, we cannot exclude that off-target effects of MDL28170 could explain the observed transient increases in REE. Furthermore, as we tested only a single dosing regimen for MDL28170 and z-VAD-fmk and terminated the experiments at 6 weeks after injury, we can currently not comment on their dose-effect relationships and on possible effects of these drugs beyond this time period.

In conclusion, the findings from this pilot study further suggest that caspase cascades may contribute to the development of hypermetabolism. Our observations indicate that burn-induced hypermetabolism can be pharmacologically modulated and point towards caspase cascades as possible therapeutic targets to attenuate hypermetabolism after burns, and possibly in other catabolic disease processes.

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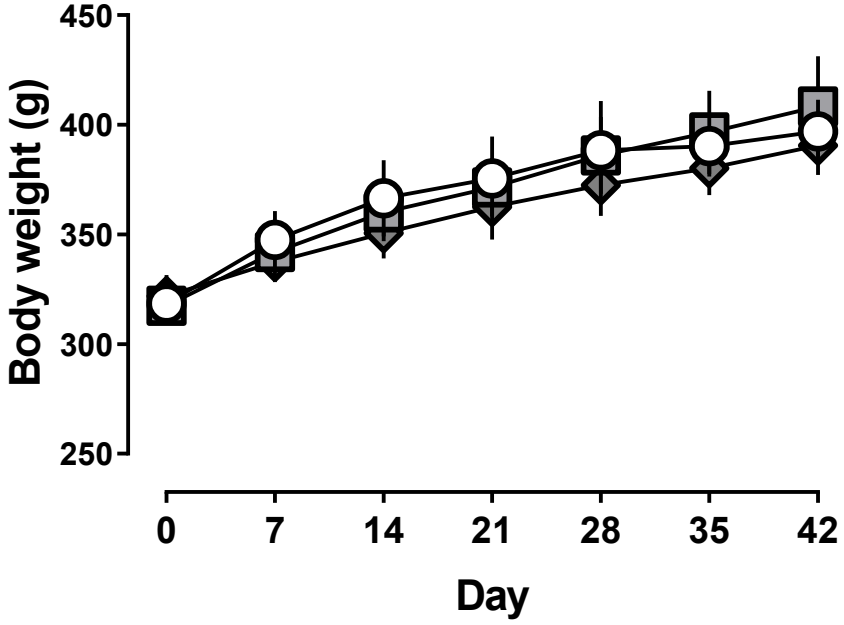
Figure Legends

Figure 1: Effects of MDL28170 and z-VAD-fmk on body weights (A) and resting energy expenditures (B) in normal (sham) rats. **A.** Body weight (g). **B.** Resting energy expenditure (REE; kcal/h/g) in % of normal (day 0). Open circles: vehicle treatment (n=9-10). Grey squares: MDL28170 treatment (n=5). Grey diamonds: z-VAD-fmk treatment (n=5). Data are mean±SEM. *: p<0.05 vs. vehicle treated animals.

Figure 2: Effects of MDL28170 and z-VAD-fmk on body weights (A) and resting energy expenditures (B) after burn injury. **A.** Body weight (g). Significant differences between vehicle treated animals after sham procedure (please see Fig. 1A) and after burn are shown. **B.** Resting energy expenditure (REE; kcal/h/g) in % of normal (day 0). Open circles: vehicle treatment (n=10). Open squares: MDL28170 Early treatment (n=6). Grey squares: MDL28170 Late treatment (n=5). Open diamonds: z-VAD-fmk Early treatment (n=5-6). Grey diamonds: z-VAD-fmk Late treatment (n=4-5). Data are mean±SEM. *: p<0.05 vs. vehicle treated animals after burn injury.

Fig. 1

A



B

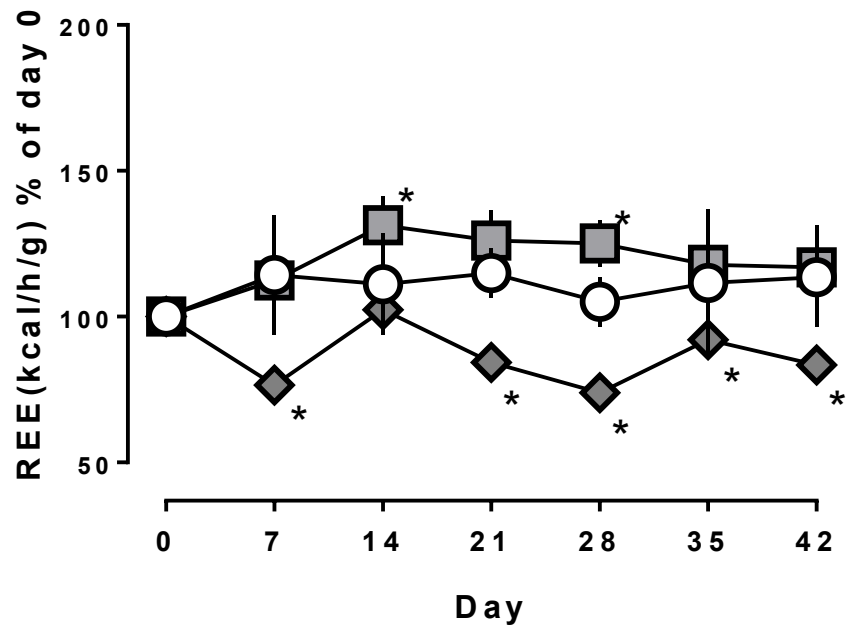
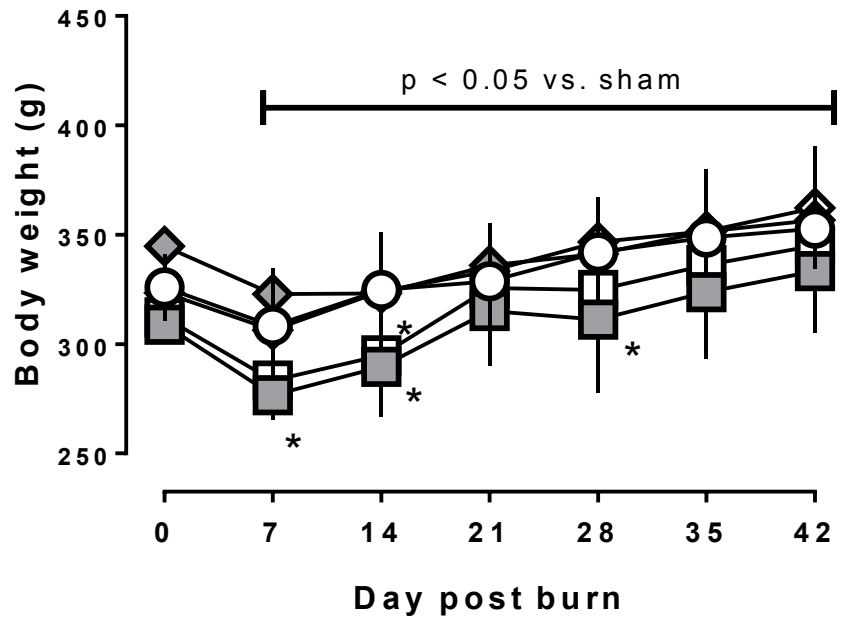


Fig. 2

A



B

