Author's response to reviews

Title: Antiglucocorticoid RU38486 reduces net protein catabolism in experimental acute renal failure

Authors:

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Author's response to reviews: see over
Response to Dr. Bevington:

Thank you for the detailed and very insightful review. The manuscript has benefited greatly from this input.
Please note that, while references cited here are mostly included in the manuscript’s reference list, they bear different citation numbers due to the use of EndNote.

Major compulsory revisions:

1. The animal model.

The animals were, indeed, subjected to a very high stress level. As pointed out in the methods section, procedure received both the internal ethics committee and then the relevant government office’s approval. The reviewer argues that the double stress of starvation and uremia does not immediately reflect a commonly encountered clinical situation. Severe acute renal failure, however, causes clinical symptoms such as lack of appetite and nausea that will account for reduced or no food intake in afflicted patients. While this situation nowadays is not routinely seen in the developed world, it is the natural course of untreated uremia and the fate of most patients in countries that do not provide affordable medical care to the majority of citizens.
From a practical point of view, experience in the laboratory where the experiments were carried out (Institut für Physiologische Chemie, Heinrich Heine Universität, Düsseldorf, Germany) had shown a much reduced survival rate if animals were allowed free access to food (Prof. M. Schwenen, unpublished data).

2. The title of the paper refers to protein synthesis and uremia-specific net protein catabolism but, as far as I can see, protein synthesis rate was not measured directly in this study, nor is it clear how the author has distinguished between the uremia-specific and starvation-induced or stress-induced contributions to the measured net protein catabolism. The basis of these distinctions needs to be explained more clearly.

The distinctions were, indeed, not made very clearly in the original manuscript as the conclusions are inferred indirectly. The text of the discussion has been changed accordingly. Below please find a more detailed explanation.

“Sham-operated animals are catabolic at the time of perfusion, having lost about 36 g BW (see results), which is approximately 16% of initial BW. Rats of this age are still growing, with an increase of approx. 5 g/day (2-3% BW)[1]. The weight loss is due to both lipolysis[2] and protein loss[3], which causes the typical increase of amino acid release in the hindquarter of fasting rats[4]. Compared to data[5] from non-operated rats fasted for 48 hours under otherwise identical conditions, the total amino acid release is increased by 30% in the sham-operated rats described here. While nephrectomy further increases the amino acid release by approximately 15%, the relative decrease of amino acid release following administration of RU 38486 is similar in both nephrectomized and sham-operated animals. This indicates a stress-accentuated adaptation to fasting caused by corticosteron secretion[6] increased beyond the normal range, with a further effect of
uremia. Increased amino acid serum concentrations during fasting are mostly due to an inhibition of protein synthesis[7], although proteolysis mainly of myofibrillary proteins does play a role[8]. However, corticosterone is only one of several effectors at play. RU 38486 affects neither acidosis nor lactate/pyruvate ratio. Both factors may contribute to the continuously increased amino acid release. In the case of acidosis, this may be due to an action on acid inhibitable transporters such as system A which reduce the supply of nutrients to the cells[9]. Another possible mechanism is through inhibition of leptin by acidosis[10], which in neutral pH might counteract muscle wasting[11]. Balancing acidosis in chronically uremic rats with increased corticosterone secretion inhibited protein degradation, but had no effect on the defective protein synthesis[12]. More recently, RU 38486 was shown to be ineffective in blocking acid-mediated protein degradation as its action is only an indirect one, mediated via insuline-like growth factor I (IGF-I)[13, 14]. These findings indicate that RU 38486 acts through inhibition of corticosterone-mediated decrease of protein synthesis without affecting other factors that act predominantly on the level of protein degradation. While all these and more factors contribute to the muscle degradation seen in excess glucocorticoid situations, the mechanisms responsible in ultima causa remain still unclear[15].

3. Most of the measurements in this study concern the release of amino acids into the extracellular compartment of the perfusion medium. In the case of 3-methyl-histidine release, this can be interpreted, as this amino acid is only released from myofibrillar protein and is not re-utilised, so it is an accurate index of myofibrillar protein degradation. The release of the other amino acids however is more difficult to interpret because they can be re-utilised in cell metabolism and, in the case of the non-essential amino acids, can be synthesised in the cells. Consequently their rate of appearance in the extracellular compartment will show a complex dependence on protein synthesis rate, protein degradation rate, amino acid synthesis and catabolism rates, and also on the rate of transport across the plasma membrane. Several of these factors (including amino acid transport rate) can be affected by glucocorticoid in the hind limb perfusion model (Hundal HS. Babij P. Taylor PM. Watt PW. Rennie MJ. Effects of corticosteroid on the transport and metabolism of glutamine in rat skeletal muscle. Biochimica et Biophysica Acta. 1092(3):376-83, 1991). The author should therefore provide evidence or cite references to show how such amino acid data are to be interpreted. For example, was protein synthesis blocked with cycloheximide during the perfusion to eliminate the contribution from protein synthesis?

The following has been added to the discussion section of the revised manuscript:

“In the given experimental setting, it is difficult to account for the in detail contribution of protein degradation, amino acid intermediate metabolism, and protein synthesis. Factors that modify the efflux are transport systems in the cell membrane[9, 16, 17], which can be concentration-dependent (system L) or acting against the concentration gradient (system A), and the intermediary metabolism within the muscle cells[18]. Numerous previous assessments of the metabolic situation observed in the isolated perfused hindlimb demonstrate that these factors are relatively minor contributors, while amino acid efflux is nearly exclusively characterized by the net balance of protein
metabolism both in anabolic and catabolic situations[4, 5, 19-22]. It is mostly due to changes in skeletal muscle, with only minor contributions from other tissues in this preparation[23-25].”

4) To maintain efficient oxygenation during the perfusion, calf erythrocytes were used in the perfusion medium. The author should state (or estimate from data in the literature) the contribution of erythrocyte amino acid efflux to the extracellular amino acid accumulation during the 60 min recirculation perfusions. Has it been shown previously to be negligible?

Perfusion procedure was identical in all groups. RU 38486 was last administered 24 hours before perfusion. As explained in the revised “methods” section, a pre-perfusion using 70 ml of perfusion medium was performed, as described in[26]. These 70 ml were discarded and not re-circulated. In this experimental setting, the metabolism of erythrocytes in the perfusion medium may have contributed to the observed amino acid concentrations, but equally so for all four groups. The conclusions made, therefore, are independent of the erythrocytes’ amino acid metabolism.

The following sentence was altered/added in the “methods” section:
“The hindquarter was linked to the recirculation system after full passage of 70 ml of pre-perfusion medium, as shown in illustration 1. The pre-perfusion medium was discarded and not used for the recirculation experiment.”

5) The dose of RU38486 used in this study is unclear. In the abstract it is stated to be 25 mg/kg body weight/day, whereas elsewhere in the paper it is stated to be 50 mg/kg body weight/day. This is important because RU38486 is difficult to dissolve in water and consequently it is difficult to administer sufficient to rats to achieve efficient blockade of the glucocorticoid receptors. The author should present or cite evidence that the receptors are effectively blocked under the conditions used in this study. For example, even at a dose of 50 mg/kg body weight/day given to rats by gavage, only 80% of the receptors were blocked (Pickering WP. Baker FE. Brown J. Butler HL. Govindji S. Parsons JM. Pawluczyk IZ. Walls J. Bevington A. Glucocorticoid antagonist RU38486 fails to block acid-induced muscle wasting in vivo or in vitro. Nephrology Dialysis Transplantation. 18(8):1475-84, 2003).

Apologies for the erroneous reporting of 25 mg/kg BW in the abstract. The correct dose is, as described in the methods section, 50 mg/ kg BW. As described in the methods section, RU 38486 was not dissolved in water, but suspended in phenylmethanol dissolved in sesame oil. Effectiveness of the antiglucocorticoid depends very much on the mode of application, and the block seems to occur with variable results in the tissues studied. While the reference cited by the reviewer shows 80% receptor blocking in rat muscle following oral application, Kim et al[27] demonstrated effective blocking of glucocorticoid receptors in rat brain following subcutaneous application of 80 mg/ kg.
Schaefer et al.[28], on whose experimental set-up the present study was modeled, had reported significant effects of an oral dose of 20 mg/kg on muscle, as already pointed out in the first version of the manuscript. In view of this, the choice for the experimental procedure seems justified as the present study uses a substantially higher dose. While binding assays would have been ideal to answer the question quantitatively, these experiments were not carried out because the literature cited seemed to indicate that the application and dosage would be sufficient.

In order to make this response to the reviewer’s comment more accessible to the reader, the following passage was included in the “discussion” section: “The degree to which RU 38486 blocks the glucocorticoid receptor depends very much on the mode of application, and the target tissue. While a recent study[13] shows that 80% of glucocorticoid receptors are blocked in rat muscle following oral application of mifepristone of 50 mg/ kg BW, Kim et al[27]. demonstrated effective blocking of glucocorticoid receptors in rat brain following subcutaneous application of 80 mg/ kg over two days. Schaefer et al.[28], on whose experimental set-up the present study was modeled, had reported significant effects of an oral dose of 20 mg/ kg on muscle. In view of this, the choice for the experimental procedure seems justified as the present study uses a substantially higher dose.”

**Minor Essential Revisions:**

6) *Even though many interesting and relevant references are cited, the reference list is out of date, with nothing cited after 2001, and most of the references are before 1992. More recent work in this field should also be cited.*

The reference list now includes more recent literature, where appropriate. The choice for older literature, none of which has been deleted, is based on a preference to go to the most original source of information.

7) *The components of the perfusion diagram in Fig 1 should be labeled in more detail.*

This has been done as requested.

8) *Error bars and statistical analysis should be shown on Fig 2.*

This has been done as requested. As described in the legend to figure 2, the detailed statistical analysis is found in the additional tables (4 & 5) that have been added.

9) *The Conclusion refers to “non-operated animals” which were not included in the study. This should be removed.*

This has been done as requested.

**Discretionary Revisions:**

10) *I suggest that the second sentence of the Abstract (Background) should be altered to “.....the question to what extent does corticoid action specific to uremia cause the*
observed muscle degradation, and does inhibition of glucocorticoid action reduce the protein wasting?"

This has been done as requested.
References:

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