Author's response to reviews

Title: Hypothyroidism attenuates protein tyrosine nitration, oxidative stress and renal damage induced by ischemia and reperfusion: effect unrelated to antioxidant enzymes activity

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Author's response to reviews: see over
September 12th, 2005

Editor
BMC Nephrology

Dear Editor:

I am attaching our point by-point response and the revised version of our manuscript. All the points were carefully considered and pertinent amendments were performed. We are very grateful to both reviewers for the constructive and valuable comments. We feel that the manuscript was significantly improved. We hope this revised version could be suitable for publication in BMC Nephrology.

Best regards

José Pedraza-Chaverri, PhD

POINT-BY-POINT RESPONE

Reviewer's report
Title: Hypothyroidism attenuates protein tyrosine nitration, oxidative stress and renal damage induced by ischemia and reperfusion: effect unrelated to antioxidant enzymes activity
Version1: Date: 21 July 2005
Reviewer: Devinder Singh
Reviewer's report: General

Major Compulsory

Major Compulsory Revisions:
1. The authors are requested to do an extensive literature search on the Oxidative damage and antioxidant enzyme activities in experimental hypothyroidism and incorporate the same in the introduction and discussion parts.

R1. An extensive literature search on the oxidative damage and antioxidant enzyme activities in experimental hypothyroidism was performed and included in tables 1 and 2 and incorporated in the introduction and discussion parts.

2. The ‘Ischemia reperfusion studies’ suggests that there are only two groups used in the study and the same groups served as CT & HTX at t=0 and IR & HTX+IR at t=24. There is no mentioning of plasma isolation at t=0? The whole paragraph is confusing. If I am getting it right, there should be minimum of four groups: CT, HTX, IR and HTX+IR??

R2. The reviewer is correct; in fact we used four groups of rats: CT, HTX, IR, and HTX+IR. This was clarified along the revised version of the manuscript. Accordingly, the
tables 4-6 were redesigned to indicate clearly the four groups of rats. Following is the modified paragraph in the section “Ischemia and reperfusion studies”

“We studied four groups of rats: Control (CT), sham operated animals; hypothyroid (HTX), rats subjected to thyroidectomy; ischemia and reperfusion (IR), rats submitted to IR; and HTX+IR, rats subjected to HTX plus IR. The experimental protocol was performed 15 days after the thyroidectomy (HTX group) or the simulated surgery (CT). Under anesthesia and heparin administration, blood samples were obtained and the kidneys were reperfused and removed. Additional animals from CT and HTX groups were to right nephrectomy and the left renal artery was occluded with a non-traumatic vascular clamp for 60 minutes. Then, the clamp was released allowing the reestablishment of renal blood flow or reperfusion and 24 hours after the rats were anesthetized, blood samples were obtained and the kidney was reperfused and excised. These groups were named as IR and HTX+IR, respectively. Blood plasma was obtained and stored at -40°C. Kidney was used for histological and immunohistochemical studies and for determination of antioxidant enzymes activity. Activity of antioxidant enzymes was measured in renal cortex, outer medullas and inner medulla.”

3. In the kidney homogenization part of Methods, the authors state that the whole kidney was homogenized and the supernatant was used for the further enzymatic analysis. However, in the results it is said that the antioxidant enzymes were estimated separately in cortex and outer/inner medulla. This thing needs explanation?

   R3. To clarify this point, we performed the following change under the section “Tissue homogenization”: “Kidney was homogenized…” was changed to “Renal cortex, outer medulla and inner medulla were homogenized…”

4. Table 1. The creatinine and BUN levels in HTX+IR animals are still alarmingly high and are rather close to the IR group (5.08 vs 3.83)?

   R4. The reviewer is correct. The increase in creatinine and BUN levels was not completely prevented in the HTX+IR groups. This may be secondary to the fact that the damage induced to the kidney by 60 minutes of ischemia was very high. In spite of this fact, it is clear that creatinine and BUN values in HTX+IR were significantly lower (P<0.001) compared to HTX rats. It is possible that with a lower time of ischemia (e.g. 30 min or less) the protection afforded by hyperthyroidism had been higher.

5. Table 2, 3 & 4. There was no change observed in the antioxidant enzyme levels even after IR alone. However, the role of oxidative stress and depletion of antioxidant enzyme pools in the renal IR is well established. Furthermore the following points need some explanation: a) The fall in CT and HTX cortex catalase activities from 0.22 to 0.17 and 0.22 to 0.14? The similar is the case with outer medulla catalase here, a 50% decrease from 0.08 to 0.04? b) The fall is HTX cortex glutathione peroxidase levels: 0.11 to 0.04?

   c) The fall in HTX medulla superoxide dismutase levels: 10.4 to 5.6?

   All these changes have been shown to be non significant in the manuscript?
R.5 Many thank for this important observation. The statistical analyses were revised carefully and it was found that of the above comparisons indicated by the reviewer were, indeed, significative. My sincere apologies for this error. This is clearly indicated in the respective tables which were redesigned to indicate clearly the four groups of animals. The corresponding changes about these data were added to the text in the following sections “Abstract”, “Results”, and “Discussion”

6. The authors are suggested to include the effect HTX on the renal IR induced oxidative stress, by doing lipid peroxidative studies.

R6. Many thank by the suggestion. We avoided the measurement of this parameter, which is commonly performed by the measurement of malondialdehyde levels, taking into account that in the original study of Paller, he measured malondialdehyde levels as an index of lipid peroxidation. Paller found that hypothyroidism effectively prevented the increase in malondialdehyde levels induced by ischemia and reperfusion. Instead of we measured, by immunohistochemistry, another molecule that is also a product of lipid peroxidation: 4-hydroxy-2-noneal (4-HNE). I hope that this explanation could satisfy the request of the referee.

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Minor Essential Revisions:
Please check the following and any related typographical mistakes:

Introduction:

1. line 9; it should be or in the other circumstances such as….. Instead of circumstance.
   R1. This has been corrected in the revised version.

2. …..[16, 17], previous to renal oxidant insult should be replaced by prior to renal oxidative insult.
   R2. This has been corrected in the revised version.

Method:

1. Induction of hypothyroidism------ line 4, replace anaesthesia with anesthesia, as in the whole of manuscript it is US English that is used by the authors.
   R1. This has been corrected in the revised version

2. Immunohistological localization of 3-NT, DNP, and 4-HNE------ deparaffinized instead of deparaffined.
   R. This has been corrected in the revised version
Reviewer's report

Title: Hypothyroidism attenuates protein tyrosine nitration, oxidative stress and renal damage induced by ischemia and reperfusion: effect unrelated to antioxidant enzymes activity

Version 1: Date: 21 July 2005
Reviewer: Prabal K Chatterjee

Reviewer's report:

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Major Compulsory Revisions

(1) There is now good evidence that hypothyroidism can contribute to the development of acute renal failure by causing rhabdomyolysis (see Sekine N, et al., Intern Med. 1993 32:269-71).

(i) Could the authors comment on the lack of any renal dysfunction or injury in their thyroidectomised rats prior to I-R?

(ii) In view of this potential complication of hypothyroidism, what is the clinical relevance of this study? If there is any, the authors should describe this in their Introduction or Discussion.

R1.1. We used rats with acute hypothyroidism, at this the kidney is characterized by renal vasoconstriction which we have already characterized by a small but significative reduction in total glomerular filtration rate measured by inulin clearance (1.1 ± 0.05 ml/min in normal rats vs 0.8 ± 1.2 ml/min in 15-day thyroidectomized rats, p<0.005), as well as single nephron glomerular filtration rate (32.7± 0.05 nl/min NI vs 21.7± 0.05 nl/min, HTX p<0.005). These alterations were due to an increase in afferent and efferent resistances and a slight decrease in the ultrafiltration coefficient without change in glomerular capillary pressure. We attribute these alterations to a decrease in extracellular concentration and predominant activation of A1 adenosine receptors (Franco et al. Am. J. Physiol 270 (Renal Fluid Electrolyte Physiol 39): F254-F262, 1996) or activation of the renal renin-angiotensin system (Guillum et al Am J. Physiol 253 (Renal Fluid Electrolyte Physiol 22):F170-F179, 1987). In this study, we measured serum creatinine, a GFR marker not as reliable as inulin clearance, and it was similar in both groups. Interestingly, Paller found that hypothyroid rats have decreased glomerular filtration rate clearance (creatinine clearance decreased from 1.3±0.2 to 1.0±0.1 ml/min, NS and inulin clearance decreased from 1.1±0.07 to 0.81±0.08 ml/min, p<0.05) with similar values of serum creatinine (Figs 1 and 2, Kidney Int 1986, 29:1162-1166). These data confirm that serum creatinine is not a reliable marker of renal function when the decrease in glomerular filtration rate is very small. This is happening in our rats. Histological analysis only had shown a decrease in luminal diameter in acute hypothyroidism (Davis RG et al. Am J Pathol. 1983 Oct;113(1):41-9; Capasso G, et al. Kidney Int. 32:443-451, 1987), but as the evolution is longer (chronic hypothyroidism) the alterations mentioned above become irreversible, and after a month it becomes chronic hypothyroidism, in which renal alterations are considered irreversible. In addition cellular dysfunction is a general observation (Capasso G, et al. Kidney Int. 32:443-451, 1987). We could argue that a kidney with the alterations mentioned above should have a highest risk of develop acute renal failure of greater severity than normal rats. On the contrary, the severity of the renal failure is lower. In fact, Paller wrote in the abstract (Kidney Int 1986, 29:1161-1166): “Twenty-four hours after ischemia, inulin clearance was higher in thyroidectomized than control animals (0.40±0.06 vs.
0.17±0.03 mliter/min; P<0.01, despite an initially lower inulin clearance in thyrodecomized animals (0.81±0.08 vs. 1.1±0.07 mliter/min; P<0.05)

We avoided to discuss this information in the manuscript due to we did not measure creatinine clearance in our rats.

R1.2. The observation of the reviewer is correct, however, most of the reports of rhabdomyolysis in hypothyroid patients published isolated cases in which hypothyroidism is severe, or it is associated to factors such as: lipid lowering drugs, alcohol ingestion, chronic renal failure. (Altay M, Nephrol, Dial Transplant 20(4)847-8, 2005; Kar PM, Clin, Nephrol 60(6):428-428; Kisakol G, Endocr. J. 50(2) 221-223, 2003; Barahona MJ, Endocr. J, 49(6) 621-623, 2002; Shek A. Ann. Pharmacother. 35(7-8):908-917, 2001 etc.),

In our study we did not observe macroscopic hemoglobinuria in any of the rats before, or during I-R; since we did not measure myoglobin or creatine phosphokinase, we can not discard the presence of microhemoglobinuria, but if that could be the case, we should expect a higher severity of acute renal, instead of prevention.

Regarding the clinical relevance of rhabdomyolysis in hypothyroidism, since the purpose of these study was to understand the mechanism by which hypothyroidism protect from I-R as it was observed, we think that it is out of the scope of the Discussion Section to mention a mechanism that should worsen I-R.

(2) There is also some evidence that renal I-R can modulate the protein expression and activity of antioxidant enzymes, especially after 90 min ischemia (see Dobashi K, et al. Mol Cell Biochem. 2000 205:1-11). There is some suggestion in the data that CAT and GPx levels were modulated by I-R. Could a significant effect be hidden by the small number of samples used for the determination of CAT and GPx (n=5 or 7)? If further analysis were performed and group sizes matched (n=16) would a significant effect become apparent? The authors should consider this in light of their claim that antioxidant enzyme activities were unaffected by renal I-R in their model.

R2. Thank you for this important observation. We revised again the statistical analysis and we found that, indeed, IR decreased antioxidants enzymes in our study:

Catalase activity was decreased in renal cortex of IR group compared to CT group and in renal cortex and outer medulla of HTX+IR group compared to HTX group.

Glutathione peroxidase activity decrease in renal cortex and outer medulla of IR group compared to CT group and in renal cortex and outer medulla of HTX+IR group compared to HTX group.

Superoxide dismutase activity decreased in outer medulla of HTX+IR group compared to HTX group.

In addition the reference of Dobashi et al was included in the manuscript.

(3) A more robust method of assessment of histological evidence of renal injury is required, e.g. a commonly-used scoring system based on loss of nuclei from tubules could also be used (see Chatterjee PK, Kidney Int. 2000 58:658-73). Measurement of glomerular size is inadequate – even in Figure 1 the glomeruli look similar in size in most of the images.

R3. We eliminated the data about glomerular size from Fig 2B and from the text. Instead of we included the data of severity score which was performed as the referee indicated. The description of the method and the corresponding reference were included.
Minor Essential Revisions

(1) Grammatical and typographical errors are present throughout the manuscript from Abstract (glutathione peroxide?) to References (‘membrana’ in Ref 41?). There are too many to list here but they all need to be corrected.
   R1. Many thanks for this observation. These and other typographical errors were corrected in the revised version.

(2) What was the species of rat used in this study?
   R2. Male Wistar rats were used in this work. This is stated in the revised version under “Induction of hypothyroidism”

(3) How were total protein levels measured?
   R2. Total protein levels were measured by the method of Lowy et al. This is stated in the revised version under “Tissue homogenization”. The corresponding reference was included in the manuscript.

(4) Bobadilla (ref 42) described how hypothyroidism can protect the heart against I-R injury, not Chavez (ref 41) as stated at the end of the Discussion.
   R4. This was corrected in the revised version.

   R5. This was corrected in the revised version.

Discretionary Revisions (which the author can choose to ignore)

(1) Page numbering would have been helpful (not present on my PDF version).
   R1. Page numbering has now included in the revised version.

(2) It would be interesting to see if thyroxine levels change in thyroidectomised rats after I-R.
   R2. Many thanks for this observation. We think that thyroxine levels remains unchanged after IR taking into account that these rats have no thyroid glands. We did not perform this assay in the HTX+IR rats but we are going to consider this point in future studies.