Author's response to reviews

Title: 2, 4-Diamino-6- hydroxy pyrimidine inhibits NSAIDs induced Nitrosyl-complex EPR signals and ulcer in rat jejunum

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Dear Sir,

I would like to resubmit my manuscript and I have carried out the corrections as advised by the reviewers.

I profoundly express my thanks for their time to read my earlier manuscript.

Reviewer 1 : Professor. H Sakurai.

Comment 1: The title of the manuscript does not appear to reflect the results of the investigation. The following title might be a suitable to explain the results of the investigation.
Answer: '2,4-Diamino -6-hydroxy pyrimidine inhibits NSAIDs induced Nitrosyl-complex EPR signals and ulcer in rat jejunum'

Comment 2: When the authors isolated the sub mitochondrial fraction, did they check the purity of the fraction by some enzyme activities such as succinate dehydrogenase and glucose-6-phosphatase?
Answer: The sub mitochondrial fraction was prepared from beef heart mitochondria in order to study the direct effect of inhibition of indomethcin on to the electron transport chain ,since the isolation of mitochondria from rat intestine was not a pure and cumbersome. We did check the purity of the fraction by studying the electron transfer reactions using potassium ferricyanide as an electron acceptor and succinate as a substrate. (Estabrook RW, JBC. 236:3051-3057. 1961) The purity was 96% and inhibited the electron transfer by indomethacin (I.C.50%) at 450uM/mg protein concentration. (Rafi S. PhD Thesis dissertation, University of London, 1998) We also studied the specific mitochondrial succcinate dehydrogenase, cytochrome C oxidase and citrate synthase enzyme activities in rat jejunal homogenates in our earlier article. (Gut, 41: 344-53.1997).

Comment 3: Did the authors test the other types of inhibitors of iNOS in the experimental stages.
Answer: We did use the N(G)-nitro-L-argenine methyl ester (L-NAME-10mg /kg b wt s.c 1 hr prior to indomethacin ) to prevent nitrosoyl signals and ulcers induced by indomethacin. We did not find the inhibition of intestinal ulcers. We conclude that this effect may be due to the nonspecific inhibition of
L-NAME and it is not a specific inhibitor of iNOS cofactor biopterin complex and hence excluded from our study.

This observation is further confirmed by the recent reports stating,' The worsening effect of L-NAME (5-20 mg/kg) on these lesions was dose-dependently observed in association with further enhancement of the bacterial translocation and intestinal hypermotility following indomethacin.' (Tanaka A, Mizoguchi H, Kunikata T, Miyazawa T, Takeuchi K. Protection by constitutively formed nitric oxide of intestinal damage induced by indomethacin in rats. J Physiol Paris. 2001 Jan-Dec;95(1-6):35-41.)

Comment 4: The expression of, for example, 90 uL of sub mitochondrial particles is not suitable, instead, the authors should use the expression such as protein amounts or contents in an EPR tube.

Answer: The amount of protein has been included in the revised manuscript as 1 mg protein for EPR tube instead of 90ul of sub mitochondrial particles and jejunum homogenates.

Comment 5: The measurement conditions for EPR should be described in detail in the Materials and Methods

Answer: The following EPR measurement conditions have been included in Materials and Method section in the revised manuscript.

EPR conditions: temperature 8K or 20K or 30K: microwave power 20mW, microwave frequency 9.35 GHz, modulation frequency 100Khs, modulation amplitude 1 mT, receiver gain 1.25 X105 , time constant 0.33 sec, sweep time 2.4 mT/sec. Spectra displayed are average of 6-8 scans.

Comment 6: In Results, more detail explanations are needed, especially in the figures 3, 4, and 5.

Answer: The following explanation has been included in the revised manuscript.

Figure 3 explains the unique features of nitrosyl signals (g~2.04) at 20K only in indomethacin treated group and not in the DMSO-control or nabumetone group. Figure 4 displays the differential pattern of the nitrosyl signals (i)-(ii) at 30K after 24 hr of indomethacin (i) and DAHP treated indomethacin group(ii) when compare to the DMSO-control (iii) spectra. This prominent nitrosyl signals at 30K is characteristics of the free radical species at this temperature when compare to the signals at 8K, displayed in Figure 5. In indomethacin alone treated group (Figure 4) this broad signal displayed a peak at g~2.04 , a distinct trough at g ~1.98 and complex splitting (triplet) centered at g~2.01 at temperature above 25K, indicative of haem-nitrosyl formation.(21). The triplet hyperfine structure is due to the interaction of unpaired electron on the 14N nucleus of NO' is characterized by A14N ~1.7mT. Similar signals have been observed for the NO-adducts of type II haem proteins such as cytochrome c oxidase and haemoglobin.

Comment 7: It might be natural to consider that the center S3 reacts with NO generated in the system. Why the intensities of EPR signal due to g=2.02 are almost the same between the systems for indomethacin, indomethacin plus DAHP, and control?

Answer: The intensities of EPR signals due to g~2.02 are almost the same between the systems for indomethacin and indomethacin plus DAHP and control only at the temperatures below 30K, i.e 8K and 20K and they are the characteristic signals of iron-sulphur proteins. At 30K the interacting signals are more and displays the triplet hyperfine structure due to the interaction of unpaired electron on the 14N nucleus of NO. at g~ 1.98 and not g~2.02.

Comment 8: Many spell errors are found in the manuscript.
Reviewer 2: Dr BL Tepperman

Comment 1: The data are achieved using only single dosage regimens of indomethacin. If as the authors maintain, doses of indomethacin higher than 500 uM inhibit electron transport, then this should be also shown for the intestine.

Answer: Different doses (1, 3, 5, 10, 20, and 30 mg/kg bwt) of indomethacin were tried to induce intestinal ulcers. 1, 3, and 5 mg/kg doses were needed more than two doses to induce ulcers and it took few days to develop ulcers. Our aim is to study the pathogenesis of NSAID enteropathy at an early stage. Hence we established a dose of 20g/kg (56uM) which itself an eight fold higher doses of human recommendation dose. In addition the 500uM dose was used in invitro rat liver and beef heart mitochondrial respiration study.

Comment 2: The role of NO is inferred from the use of an iNOS inhibitor. NO should be measured or at least the effect confirmed using a standard NO scavenger. The formation of nitrosyl complexes could be confirmed using nitrotyrosine staining.

Answer: We did use the N(G)-nitro-L-arginine methyl ester (L-NAME-10mg /kg b wt s.c 1 hr prior to indomethacin) to prevent nitrosyl signals and ulcers induced by indomethacin. We did not find the inhibition of intestinal ulcers. We conclude that this effect may be due to the nonspecific inhibition of L-NAME and it is not a specific inhibitor of iNOS cofactor biopterin complex. Moreover, Peroxynitrite is a potent oxidant, an intermediate product of nitration that can induce lipid peroxidation and as such may provide important insight. However, endogenous production of peroxynitrite remains controversial. Nitration of tyrosine residues is used as an index of peroxynitrite formation but it is not specific [Cancer Research 1996, 56: 3238-3243]. We hope this might answer clearly the above question.

Comment 3: Is it possible to quantify the EPR spectra. Examination of the spectra in Figures 1-5 is quite difficult especially without any scale and for a non-expert in the field. This is the only way a reader may appreciate the impact of the procedure on electron transport in response to NSAID treatment.

Answer: It is possible to quantify the EPR spectra with caution and it is not as easy as we think due to the following reasons.

If an iron protein is a major constituent of a cell it may sometimes be studied by EPR without purification. This makes it possible to examine and quantify protein in their natural environment. Difficulties may be encountered owing to weak signal strengths and the presence of overlapping signals. Judicious use of signal averaging will alleviate the former problem, and computer-assisted
spectral subtraction the latter. Figure 4 (i) - (ii) illustrates how this can be used to determine the amount of a haem-nitrosyl complex has been expressed. In this case the spectra of the DMSO-control is used as a reference, though in principle simulated spectra could also be used.

For a pure protein iron complex reaction the following method can be applicable and not for the whole tissue homogenates. It is desirable to use standards that are as similar in spectral line shape as possible to the sample being studied, and the two samples should be run under the same measurement conditions. Thus, although Cu(II) is appropriate for Fe-S clusters, it is usually preferable to use a low-spin heme standard for S=1/2 heme proteins. Horse heart metmyoglobin readily forms an azide complex with an optical extinction coefficient of 123 mM-1cm-1 at 420-nm and can thus be used to construct a standard curve to calculate the concentration of the unknown sample.

Since we are dealing with the whole tissue homogenates we are unable to apply the above protocol for quantification of this signals. Hence in our study we quantify the spectra by implementing the judicious use of signal averaging and computer-assisted spectral subtraction with the constant protein concentration. The difference in signal is directly proportional to the amount of nitrosyl complex produced in this system.

I hope the explanation will satisfy the reviewers and we will look forward to accept for publication in BMC-Gastroenterology.

Yours sincerely
S. Somasundaram PhD