

Format for ANSWERING REVIEWERS



April 20, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 1416-revised.doc).

Title: *Roles of BN52021 in platelet-activating factor pathway in inflammatory MSI cells*" (Ref. No.: 1416).

Author: Shi-hai Xia, Xiao-hui Xiang, Kai Chen, Wei Xu

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 1416

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

(1) We have explained the rationale behind our choice to study the specific signaling molecules in the discussion section as following:

"PAFR is almost ubiquitous in diverse type cells and acts not only on local pancreas including the pancreatic vascular endothelium but on distant organs inducing systemic inflammatory response and multiple organ injury^[1]. PAFR belongs to the G protein coupled receptor subfamily^[2]. By binding to its receptor, PAF activates the associated G protein, which in turn activates phosphoinositide hydrolysis by phosphoinositide specific phospholipase C, arachidonic acid release by phospholipase A₂, increase in intracellular Ca²⁺ concentration, activation of protein kinase C, and protein tyrosine kinase (PTK)^[3]. PAF was also shown to activate mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK)^[4-8], p38 MAPK^[4, 6, 7], and c-Jun N-terminal kinase (JNK)^[9]. Deo *et al.*^[10] reported that PAF activated pertussis toxin-insensitive Gαq protein upon binding to its seven transmembrane receptors and adenylate cyclase, and elevated cAMP levels, thus activating protein kinase A (PKA) in human umbilical vein endothelial cells. GPCR kinase (GRK) plays a key role in homologous desensitization of GPCRs and GRKs phosphorylate activated receptors, promoting high affinity binding of arrestins, thus precluding G protein coupling. Direct binding to active GPCRs activates GRKs, so that they selectively phosphorylate only the activated form of the receptor regardless of the accessibility of the substrate peptides within it and their Ser/Thr-containing sequence^[11]. Most GPCRs display a rapid loss of responsiveness in the continuing presence of chemoattractants in a process of desensitization that involves the phosphorylation of agonist-occupied GPCR by GPCR kinase (GRK)^[12]. Inflammation in pancreatic vascular endothelial cells induced by LPS was suppressed by BN52021, which might contribute to the mechanism underlying microcirculatory disturbance in the pathogenesis of SAP."

(2) We have added a paragraph to discuss the cell wall / PAFR interaction problem as following: "It is known that PAFR is also able to interact with components of the bacterial wall, such as lipopolysaccharides^[13] and phosphorylcholine^[14]. The cell wall exited the vasculature into the heart and brain, accumulating within endothelial cells, cardiomyocytes, and neurons in a PAFR-dependent way. Physiological consequences of the cell wall / PAFR interaction were cell specific, being noninflammatory in endothelial cells and neurons but causing a rapid loss of cardiomyocyte contractility that contributed to death. Thus, PAFR shepherds phosphorylcholine-containing bacterial components such as the cell wall into host cells from where the response ranges from quiescence to

severe pathophysiology^[14]. The explain for the protective effect of BN-52021 can not simply attribute the antagonism of LPS binding to PAFR or the prevention of PAF binding to its receptor. So other potential mechanisms of PAFR antagonist in AP treatment must exist."

(3) It is not very clear why the authors used LPS+DMSO for their experiments. Is DMSO the solvent of BN-52021?

Response: Because DMSO is the solvent of BN-52021, so we set a vehicle group as a control.

(4) Paragraph 3.2: The authors pretreated the cells for 20 min with BN-52021. How did they end with this incubation time? Did they try other incubation times?

Response: BN52021 ($t_{1/2} = 8$ h) is a selective antagonist of PAF-R. Pretreatment with BN52021 results in blockade of PAF receptor^[15]. Pretreatment time with BN52021 before administration LPS varies in a long span from 5 min^[16] to 30 min^[17], even 60 min^[18]. We pretreated cells with BN52021 20 min before administration LPS as a suitable duration according to the previous report^[19].

(5) Paragraph 3.2 and Figure 2: The concentration of 50 mM BN-52021 was used for the experiments. This is a huge concentration. Do the authors test its toxicity to cells? In addition, do the authors believe that such a concentration has clinical significance? Finally, how do they explain reduction of BN-52021 inhibitory activity at higher concentrations (100 mM)?

Response: We checked the original data records; the concentration of BN-52021 should read 50 μ M. We made a mistake when we drafted the manuscript. Reduction of BN-52021 inhibitory activity at higher concentrations (100 mM) may be explained by ceiling effects, and the dose higher than the maximum of the suitable concentration may be toxicity to cells.

(6) Minor comments 1. Figure 1. The authors should add the standard deviation bars in the charts 2. Figures 3 and 4: The legend of the third bar should be LPS+DMSO not DMSO alone.

Response: The legend of the third bar has been changed to "LPS+DMSO".

(7) Major comment: Authors have used MTT colorimetric assay as a tool to analyze apoptosis and necrosis. This method is used for cell survival and is not appropriate for assessing apoptosis as cells with treatments do not always die. If they undergo senescence or cell cycle arrest, they do not proliferate but remain viable for the longer period of time. Authors need to perform caspase-3 activity assay in order to see the effect of BN52021 on apoptosis.

Response: We have used MTT colorimetric assay as a tool to analyze Viability/proliferation. "2. Materials and Methods / MTT Colorimetric Assay" part : "Apoptosis and necrosis of the cells induced by LPS were observed by MTT Colorimetric Assay as previously described (Sun et al, 2010)" should read "Apoptosis and necrosis of the cells induced by LPS were observed by MTT Colorimetric Assay as previously described (Sun et al, 2010)". We have checked this mistake and correct it in the manuscript. We have used Hoechst 33342 / propidium iodide (PI) staining as a tool to analyze apoptosis and necrosis.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Yours sincerely,

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