Chapter 4

Bovine Intestinal Alkaline Phosphatase (BIAP) reduces inflammation after induction of acute myocardial infarction (AMI) in mice

submitted

Daniëlle Fiechter, Suzanne Kats, Ruud Brands, Ben van Middelaar, Gerard Pasterkamp, Dominique de Kleijn, and Willem Seinen

Abstract

Objective

There has been increasing evidence suggesting that lipopolysaccharide (LPS) may be an important activator of the innate immune system after acute myocardial infarction (AMI). Bovine intestinal alkaline phosphatase (BIAP) reduces inflammation in several LPS-mediated diseases by dephosphorylation of the lipid A moiety of LPS. The aim of this study was to investigate the effect of BIAP on reducing inflammation after AMI in Balb/c mice.

Methods

Just before permanent ligation of the left anterior descending (LAD) coronary artery to induce AMI, BIAP was administrated i.v. as a prophylaxis. After 4 hours, mice were sacrificed and the inflammatory response was assessed.

Results

AMI induced the production of IL-6, IL-1β, IL-10 and mMCP-1. BIAP treatment resulted in a significantly reduction of the pro-inflammatory cytokines IL-6, IL-1β and the chymase mMCP-1. No difference in the production of the anti-inflammatory cytokine IL-10 was observed between the control group and the BIAP-treated group.

Conclusion

In a mouse model of permanent LAD coronary artery ligation, BIAP diminishes the pro-inflammatory responses but does not have an effect on the IL-10 anti-inflammatory response in the acute phase after AMI.

Introduction

Lipopolysaccharide (LPS), an endotoxin present in the outer cell membrane of Gram-negative bacteria, is a potent stimulator of the innate immune response. When entering the circulation, LPS binds to the lipopolysaccharide-binding protein (LBP) which interacts with CD14, MD2, and Toll-like receptor 4 (TLR4) to start a signaling cascade leading to cytokine production [1-4].

Cardiogenic shock (CS), a severe form of heart failure (HF), is the leading cause of death among patients hospitalized with acute myocardial infarction (AMI). It is well known that AMI is associated with an increased inflammatory reaction [5]. There has been growing evidence suggesting that endotoxin is an important stimulus for this phenomenon. Decreased cardiac function reduces bowel perfusion, leading to hypoperfusion and ischemia of the intestinal mucosa. This results in increase of gut permeability, and subsequent translocation of endotoxin into the circulation [6, 7].

Several studies with HF patients, irrespective of etiology, have shown an increase of soluble CD14 (sCD14) in plasma, TLR4 expression on monocytes and increased levels of bacteria or endotoxin when compared to control groups [6, 8-12]. Taken together, these data lead to suggest that LPS is an important mediator in the observed inflammatory response after AMI.

There is increasing evidence that alkaline phosphatase (AP) is able to remove one phosphate group from the lipid A moiety of LPS, thereby dephosphorylating and detoxifying LPS [13, 14]. In mice, infected with a lethal dose of Gram-negative bacteria, mortality was reduced after injection of human placental alkaline phosphatase (HPLAP) or bovine intestinal alkaline phosphatase (BIAP) [15, 16]. A reduction in the inflammatory response induced by LPS could be observed in mice and piglets after treatment with HPLAP or BIAP [15, 17]. Oral treatment of rats with LPS resulted in a prolonged endotoxemia after inhibition of endogenous intestinal alkaline phosphatase (IAP) [18]. In addition, the potential effects of AP on LPS-mediated diseases have been demonstrated in animal studies with polymicrobial sepsis. Cytokine response and neutrophil influx in secondary peritonitis in mice was attenuated by BIAP [19]. Hepatic and pulmonary injury after liver ischemia-reperfusion with partial resection was

reduced in rats treated with BIAP when compared to control animals [20]. Studies performed by the group of J.-L. Vincent (Brussels, Belgium) with BIAP administration to sheep, injected with feces to mimic severe endotoxemia conditions, showed a decrease in IL-6 concentrations and a prolonged survival time [21].

In this study, the left anterior descending (LAD) coronary artery ligation was used as a model to induce an AMI in mice. The objective was to examine the potential effect of BIAP on reducing the pro-inflammatory response in the acute phase after AMI by its ability to detoxify LPS. Prior to LAD ligation, BIAP was used as a prophylaxis by i.v. administration. The resulting systemic inflammatory response was investigated.

Materials and methods

Bovine intestinal alkaline phosphatase

Clinical grade bovine intestinal alkaline phosphatase was obtained from Biozyme (Blaenavon, UK). One unit is defined as that amount of BIAP able to hydrolyse 1 µmole of p-nitrophenyl phosphate per minute using a Tris-glycin buffer at pH 9.6 at 25 ˚C.

Myocardial infarction induction

Specific pathogen free (SPF) female BALB/c mice (23-27 gram) were purchased from Charles River (Sulzfeld, Germany) and were acclimatized for 1 week under barrier conditions in filter-topped macrolon cages with drinking water and standard food pellets *ad libitum*.

Myocardial infarction was induced as described previously [22]. Briefly, mice were anaesthetized by inhalation of a mixture of O_2 air and 4% isoflurane, endotracheally intubated, and mechanically ventilated. The LAD coronary artery was exposed via a left thoractomy and double ligated with an 8.0 prolene suture.

First, it had to be determined at which time point after AMI induction pro-inflammatory cytokine production could be detected. Therefore, mice were sacrificed 4, 6, and 24 hours after AMI induction and blood was collected (n=3 per time point). To examine the effect of BIAP, mice were divided into two groups: an AMI group treated with BIAP (n=4) and an AMI control group treated with vehicle alone (n=4). BIAP was injected into the tail vein just before anaesthezation as a single intravenous dose of 5 IU in 100 µl PBS (approximately 100 times above plasma levels). Control mice were injected with an equal volume of PBS. Mice were sacrificed and blood was collected. Heart, lung, liver and kidneys were removed and fixed in 4% para-formaldehyde in PBS.

The study was approved by the animal ethics committee of the Faculty of Veterinary Medicine, Utrecht University.

Determination of alkaline phosphatase activity

Five µl of serum was incubated for 60 minutes at 37 ˚C with 200 µl assay mix containing incubation buffer (0.025 M glycin/NaOH, pH 9.6), p-nitrophenyl phosphate and $MgCl_2$ at final concentrations of 1.25 and 2 mM respectively. The end product p-nitrophenol was quantatively determined by measuring the extinction at 405 nm.

Enzyme-linked Immunosorbent Assay (ELISA)

Blood samples were centrifuged and serum was collected for determination of mouse IL-6, TNF-α, IL-1β and IL-10 protein concentrations by commercially available ELISA kits according to the manufacturers' protocol (IL-6 and TNF-α from Biosource, Etten-Leur, The Netherlands; IL-1β from R&D Systems, Abingdon, UK; and IL-10 from BD Biosciences, Erembodegem, Belgium). Mouse mast cell protease-1 (mMCP-1) ELISA was from Moredun (Midlothian, Scotland, UK) and performed according to the manufacturer's instructions.

Statistics

All data presented are mean ± SEM. Statistical analysis was performed using Student's *t*-test for unpaired data (GraphPad Prism). Values were considered significant when $P < 0.05$.

Results

Determination of the IL-6 response

One of the major pro-inflammatory cytokines produced in AMI patients is IL-6. First, it had to be determined at which time point after AMI in Balb/c mice IL-6 production could be detected. Therefore, mice underwent permanent ligation of the LAD coronary artery to induce AMI and were sacrificed at three different time points. Before operation, IL-6 concentration was below detection limit (< 4 pg/ml) (Fig. 1). Peak IL-6 serum levels were observed 4 hours after AMI. Elevated serum levels of IL-6 could still be detected 6 and 24 hours after AMI. Based on these results, mice were sacrificed 4 hours after AMI in the BIAP treatment experiments.

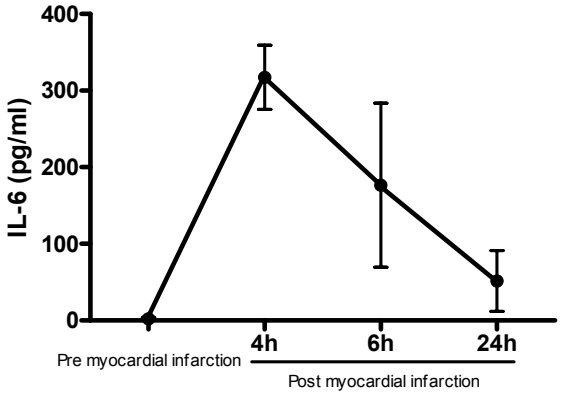

Figure 1: Production of the pro-inflammatory cytokine IL-6 after acute myocardial infarction. Mice were sacrificed at different time points and IL-6 production was determined (n = 3 per time point). Values are depicted as mean ± SEM.

AP activity

AP activity was determined in serum samples by measuring hydrolysis of p-nitrophenyl phosphate by AP. All mice that received BIAP had slightly elevated serum levels of AP activity 4 hours after AMI compared to control mice ($P < 0.05$), indicating that BIAP was still present in the circulation (Fig. 2).

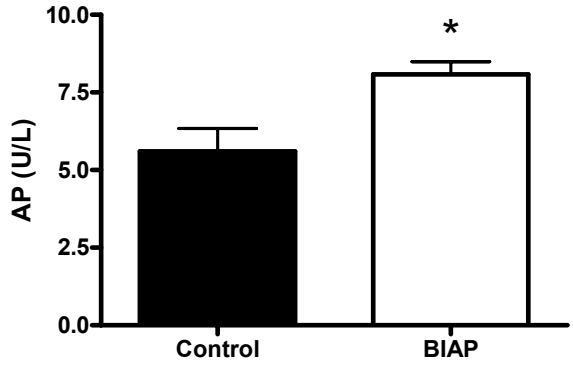

Figure 2: Alkaline phosphatase activity 4 hours after acute myocardial infarction. Values are depicted as mean ± SEM (n = 4 per treatment group). * $P < 0.05$ versus control.

Cytokine response

Systemically elevated concentrations of IL-6, IL-1β, TNF-α, and IL-10 are observed in patients with AMI. Therefore, presence of these cytokines was determined. Before LAD coronary artery ligation, concentrations of the different cytokines were below detection levels. In contrast, 4 hours after AMI IL-6, IL-1β and IL-10 were excessively produced (Fig. 3). TNF-α production could not be determined at this time-point. Administration of BIAP resulted in a significant reduction of the pro-inflammatory cytokines IL-6 and IL-1β when compared to controls. IL-6 levels were reduced by approximately 40% and IL-1β by approximately 30%. No difference in the anti-inflammatory cytokine IL-10 production could be observed between the control group and the BIAP-treated group.

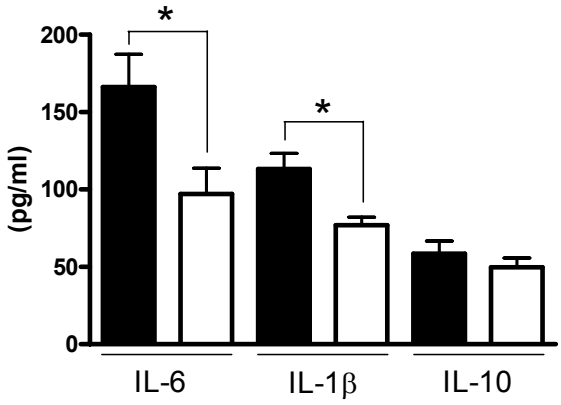

Figure 3: Effect of BIAP on the production of the pro-inflammatory cytokines IL-6 and IL-1β and on the anti-inflammatory cytokine IL-10 4 hours after acute myocardial infarction. Levels of IL-6, IL-1β, and IL-10 were determined using specific ELISA; (■) control mice and (□) BIAP-treated mice. Values are depicted as mean ± SEM (n = 4 per treatment group). * $P < 0.05$ versus control.

Mast cell activation

After AMI in mammals, mast cells are activated to release chymases. Activation of mast cells in mice can be measured by the release of the mouse mast cell protease-1 (mMCP-1) chymase. It was therefore interesting to see what the effect of BIAP administration would be on mast cell behavior. Serum levels of mMCP-1 were 14.7 ng/ml 4 hours after LAD coronary artery ligation. BIAP treatment reduced mMCP-1 levels in serum to 8.4 ng/ml (approximately 40%), implying a significant reduction in mast cell activation ($P < 0.05$) (Fig. 4).

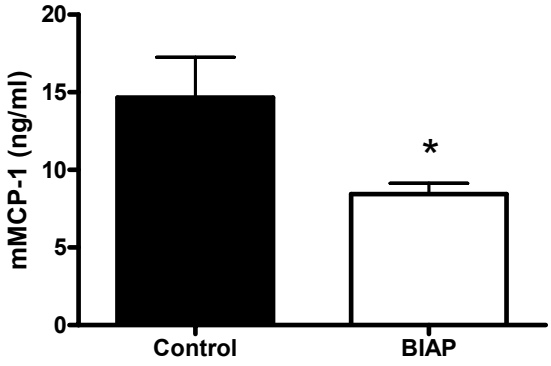

Figure 4: Effect of BIAP on the production of mMCP-1, 4 hours after acute myocardial infarction. Values are depicted as mean ± SEM (n = 4 per treatment group). * $P < 0.05$ versus control.

Discussion

Cardiogenic shock (CS) is the major cause of death in patients hospitalized with acute myocardial infarction (AMI) [23]. AMI results in intestinal hypoperfusion, which leads to increased gut permeability. Consequently, translocation of endotoxin into the circulation occurs. There has been growing evidence that presence of endotoxin is responsible for the observed systemic inflammatory response after AMI and that this may play an important role in the onset of CS [6, 7, 12]. Reducing inflammation after AMI has received little attention in research, and a specific pharmacologic treatment to reduce the inflammatory response after AMI has yet to be introduced.

To date, several studies have demonstrated the potential therapeutic effect of BIAP on LPS-mediated diseases, and it was therefore interesting to examine the ability of BIAP on reducing inflammation after AMI [19-21]. Therefore, Balb/c mice received an i.v. injection of BIAP just before AMI induction by a permanent ligation of the LAD coronary artery.

Four hours after AMI, a significant reduction in the concentrations of the two most prominent pro-inflammatory cytokines present in serum in the acute phase after AMI, IL-6 and IL-1β, was observed when compared to vehicle controls. TNF-α concentration in serum, generally believed to be an early-onset proinflammatory cytokine, was below detection limit, suggesting that the chosen time point is not relevant to detect this cytokine in Balb/c mice after LAD coronary artery ligation. A reduction in pro-inflammatory cytokine production indicates a diminished systemic innate immune response, which may decrease myocardial dysfunction and reduce the development of CS after AMI [8]. BIAP treatment had no effect on IL-10 production. Since IL-10 is a potent anti-inflammatory cytokine, and several *in vivo* studies have shown its protective role in a variety of pathological states (e.g. colitis, hepatic ischemia/reperfusion (I/R), and myocardial I/R), a reduced production due to BIAP treatment would not be favorable [24-26].

Chymases are abundantly produced after AMI in mammals, and are known to be involved in the cleaving of angiotensin I (Ang I) to form angiotensin II (Ang II) [27, 28]. The excessive formation of Ang II, which is observed in the acute phase after AMI,

is arrhythmogenic, and several studies in different animal models have shown that decreasing Ang II formation by a specific chymase inhibitor contributes to a reduction in the mortality rate in the acute phase after AMI [29, 30]. Studies in rats revealed that production of the rat chymase MCP-2 (rMCP-2) is increased after stimulation of mast cells with LPS [31, 32]. Given that BIAP has an effect on decreasing LPS toxicity, the influence of BIAP on the formation of the mouse chymase mMCP-1 was determined. In BIAP-treated mice, mMCP-1 production was significantly reduced by approximately 40% when compared to vehicle-treated mice. This result indicates a reduction of Ang II formation and consequently a decrease in arrhythmias, which may improve cardiac function and reduce CS complication.

Direct effects of BIAP on LPS detoxification could not be determined in this study. Since it is believed that BIAP is able to detoxify LPS through dephosphorylation of the lipid A moiety, the *Limulus* amoebocyte lysate (LAL) assay cannot be used as it is unable to make a discrimination between lipid A and monophosphoryl lipid A (MPLA) [33]. Therefore, decreased activation of the innate immune response because of BIAP administration could not be linked to decreased LPS levels in this study, and thus the direct effect of BIAP on LPS could not be assessed. Nonetheless, the specific activity of HPLAP and BIAP against an LPS insult has been undoubtedly demonstrated *in vivo* [15, 17].

In conclusion, a single i.v. dose of BIAP reduced the production of the chymase mMCP-1 by mast cells and diminished the systemic pro-inflammatory cytokine response in the acute phase after AMI. Therefore, it is proposed that BIAP represents a novel therapeutic drug in attenuating the pro-inflammatory response after AMI thereby reducing the incidence of CS complication.

References

1 **Schumann, R. R., Leong, S., Flaggs, G., Gray, P., Wright, S., Mathison, J., Tobias, P. and Ulevitch, R. J.,** Structure and function of lipopolysaccharide binding protein. *Science* 1990. **21**: 1429-1431.

2 **Wright, S., Ramos, R., Tobias, P. S., Ulevitch, R. J. and Mathison, J.,** CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990. **21**: 1431-1433.

3 **Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. and Kimoto, M.,** MD-2, a molecule that confers lipopolysaccharide responsiveness on toll-like receptor 4. *J Exp Med* 1999. **189**: 1777-1782.

4 **Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J. and Gusovsky, F.,** Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* 1999. **274**: 10689-10692.

5 **Frangogiannis, N. G., Smith, C. W. and Entman, M. L.,** The inflammatory response in myocardial infarction. *Cardiovascular Research* 2002. **53**: 31-47.

6 **Anker, S. D., Egerer, K. R., Volk, H.-D., Kox, W. J., Poole-Wilson, P. A. and Coats, A. J. S.,** Elevated soluble CD14 receptors and altered cytokines in chronic heart failure. *The American Journal of Cardiology* 1997. **79**: 1426-1430.

7 **Krack, A., Richartz, B. M., Gastmann, A., Greim, K., Lotze, U., Anker, S. D. and Figulla, H. R.,** Studies on intragastric PCO2 at rest and during exercise as a marker of intestinal perfusion in patients with chronic heart failure. *European Journal of Heart Failure* 2004. **6**: 403-407.

8 **Satoh, M., Shimoda, Y., Maesawa, C., Akatsu, T., Ishikawa, Y., Minami, Y., Hiramori, K. and Nakamura, M.,** Activated toll-like receptor 4 in monocytes is associated with heart failure after acute myocardial infarction. *International Journal of Cardiology* 2006. **109**: 226-234.

9 **Brunkhorst, F. M., Clark, A. L., Forycki, Z. F. and Anker, S. D.,** Pyrexia, procalcitonin, immune activation and survival in cardiogenic shock: the potential importance of bacterial translocation. *International Journal of Cardiology* 1999. **72**: 3-10.

10 **Niebauer, J., Volk, H.-D., Kemp, M., Dominguez, M., Schumann, R. R., Rauchhaus, M., Poole-Wilson, P. A., Coats, A. J. and Anker, S. D.,** Endotoxin and immune activation in chronic heart failure: a prospective cohort study. *The Lancet* 1999. **353**: 1838-1842.

11 **Peschel, T., Schonauer, M., Thiele, H., Anker, S., Schuler, G. and Niebauer, J.,** Invasive assessment of bacterial endotoxin and inflammatory cytokines in patients with acute heart failure. *European Journal of Heart Failure* 2003. **5**: 609-614.

12 **Kohsaka, S., Menon, V., Lowe, A. M., Lange, M., Dzavik, V., Sleeper, L. A., Hochman, J. S. and for the SHOCK Investigators,** Systemic inflammatory response syndrome after acute myocardial infarction complicated by cardiogenic shock. *Arch Intern Med* 2005. **165**: 1643-1650.

13 **Poelstra, K., Bakker, W., Klok, P., Hardonk, M. and Meijer, D. K. F.,** A physiologic function for alkaline phosphatase: endotoxin detoxification. *Lab Investig* 1997. **76**: 319-327.

14 **Poelstra, K., Bakker, W., Klok, P., Kamps, J., Hardonk, M. and Meijer, D.,** Dephosphorylation of endotoxin by alkaline phosphatase in vivo. *Am J Pathol* 1997. **151**: 1163-1169.

15 **Beumer, C., Wulferink, M., Raaben, W., Fiechter, D., Brands, R. and Seinen, W.,** Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. *J Pharmacol Exp Ther* 2003. **307**: 737-744.

16 **Verweij, W. R., Bentala, H., Huizinga-van der Vlag, A., van Loenen-Weemaes, A. M., Kooi, K., Meijer, D. K. F. and Poelstra, K.,** Protection against an escherichia coli-induced sepsis by alkaline phosphatase in mice. *Shock* 2004. **22**: 174-179.

17 **Bentala, H., Verweij, W. R., der Vlag, A. H.-V., van Loenen-Weemaes, A. M., Meijer, D. K. F. and Poelstra, K.,** Removal of phosphate from lipid A as a strategy to detoxify lipopolysaccharide. *Shock* 2002. **18**: 561-566.

18 **Koyama, I., Matsunaga, T., Harada, T., Hokari, S. and Komoda, T.,** Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. *Clin Biochem* 2002. **35**: 455-461.

19 **van Veen, S. Q., van Vliet, A. K., Wulferink, M., Brands, R., Boermeester, M. A. and van Gulik, T. M.,** Bovine intestinal alkaline phosphatase attenuates the inflammatory response in secondary peritonitis in mice. *Infect Immun* 2005. **73**: 4309-4314.

20 **van Veen, S. Q., Dinant, S., van Vliet, A. K. and van Gulik, T. M.,** Alkaline phosphatase reduces hepatic and pulmonary injury in liver ischaemia-reperfusion combined with partial resection. *British Journal of Surgery* 2006. **93**: 448-456.

21 **Su, F., Brands, R., Wang, Z., Verdant, C., Bruhn, A., Cai, Y., Raaben, W., Wulferink, M. and Vincent, J.,** Benificial effects of alkaline phosphatase in septic shock. *Crit Care Med* 2006. **34**: 2182-2187.

22 **Salto-Tellez, M., Yung Lim, S., El Oakley, R. M., Tang, T. P. L., Almsherqi, Z. A. M. and Lim, S.-K.,** Myocardial infarction in the C57BL/6J mouse: A quantifiable and highly reproducible experimental model. *Cardiovascular Pathology* 2004. **13**: 91-97.

23 **Goldberg, R., Samad, N., Yarzebski, J., Gurwitz, J., Bigelow, C. and Gore, J.,** Temporal trends in cardiogenic shock complicating acute myocardial infarction. *N Engl J Med* 1999. **340**: 1162-1168.

24 **Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. and Muller, W.,** Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993. **75**: 263-274.

25 **Yoshidome, H., Kato, A., Edwards, M. and Lentsch, A.,** Interleukin-10 suppresses hepatic ischemia/reperfusion injury in mice: Implications of a central role for nuclear factor kappa B. *Hepatology* 1999. **30**: 203-208.

26 **Jones, S. P., Trocha, S. D. and Lefer, D. J.,** Cardioprotective actions of endogenous IL-10 are independent of iNOS. *Am J Physiol Heart Circ Physiol* 2001. **281**: H48-52.

27 **Reilly, C., Tewksbury, D., Schechter, N. and Travis, J.,** Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases. *J Biol Chem* 1982. **257**: 8619-8622.

28 **Saito, K., Muto, T., Tomimori, Y., Imajo, S., Maruoka, H., Tanaka, T., Yamashiro, K. and Fukuda, Y.,** Mouse mast cell protease-1 cleaves angiotensin I to form angiotensin II. *Biochem Biophys Res Commun* 2003. **302**: 773-777.

29 **Jin, D., Takai, S., Yamada, M., Sakaguchi, M., Kamoshita, K., Ishida, K., Sukenaga, Y. and Miyazaki, M.,** Impact of chymase inhibitor on cardiac function and survival after myocardial infarction. *Cardiovascular Research* 2003. **60**: 413-420.

30 **Jin, D., Takai, S., Sakaguchi, M., Okamoto, Y., Muramatsu, M. and Miyazaki, M.,** An antiarrhythmic effect of a chymase inhibitor after myocardial nfarction. *J Pharmacol Exp Ther* 2004. **309**: 490-497.

31 **Brown, J., Chafee, K. and Tepperman, B.,** Role of mast cells, neutrophils and nitric oxide in endotoxin-induced damage to the neonatal rat colon. *Br J Pharmacol* 1998. **123**: 31-38.

32 **Tore, F., Reynier-Rebuffel, A., Tuncel, N., Callebert, J. and Aubineau, P.,** Effects of sepsis on mast cells in rat dura mater: influence of L-NAME and VIP. *Br J Pharmacol* 2001. **134**: 1367-1374.

33 **Takayama, K., Qureshi, N., Raetz, C., Ribi, E., Peterson, J., Cantrell, J., Pearson, F., Wiggins, J. and Johnson, A.,** Influence of fine structure of lipid A on Limulus amebocyte lysate clotting and toxic activities. *Infect Immun* 1984. **45**: 350-355.