



# Anti-ischaemic effects of bilobalide on neonatal rat cardiomyocytes and the involvement of the platelet-activating factor receptor

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## Synopsis

Terpene trilactones from *Ginkgo biloba* have been investigated extensively for their antioxidant and anti-ischaemic activities on the brain and the heart, but the mechanisms of these effects remain unclear. For the present study, a terpenoid constituent from *G. biloba*, bilobalide, was screened for protective effects on the ischaemic heart and the involvement of the PAFR [PAF (platelet-activating factor) receptor] and the enzyme that degrades PAF, PAF-AH (PAF acetylhydrolase) during hypoxia. The PAF pathway is supposed to play a role in hypoxia and its regulation may prevent or alleviate MI (myocardial infarction). Cardiomyocytes from neonatal rat hearts were cultured and treated with different concentrations of bilobalide (500–0.5 ng/ml). After being subjected to a hypoxic environment, the cells' viability was evaluated and proteins as well as RNA were extracted for analysis by Western blotting and RT-PCR (reverse transcription PCR) respectively. With the MI model we tested for bilobalide's cardioprotective effects and the involvement of PAFR and PAF-AH. Bilobalide (5 ng/ml) significantly decreased the mortality of cells in a concentration-dependent way. mRNA expression of PAFR was up-regulated in hypoxic cells but in the groups treated with bilobalide, its expression was down-regulated to the level of the normal control. In hypoxic tissue, PAFR protein expression was also up-regulated, but was reduced in the bilobalide (10 mg/kg of body weight) treated group. Our results indicate that PAF and its receptor may be involved in the cellular response of cardiomyocytes to hypoxia and that bilobalide may interact with this receptor to exert its cardioprotective effects.

**Key words:** bilobalide, *Ginkgo biloba*, hypoxia, myocardial infarction (MI), platelet-activating factor acetylhydrolase (PAF-AH), platelet-activating factor receptor (PAFR)

## INTRODUCTION

*Ginkgo biloba*, a genetically unique tree, native to East Asia, is planted as a decorative tree around the world. The leaves and fruits are traditionally used in Chinese medicine for treatment of respiratory diseases such as asthma, wheezing or coughing and many other illnesses. The terpene trilactones, bilobalide and ginkgolide A, B, C, M and J are, besides the flavonoids, the pharmacologically active components of the *G. biloba* leaves. The standardized extract [EGb (extract *G. biloba*) 761] of *G. biloba* leaves contains 24–27% flavonoid glycosides, 5–7% terpene lactones and 5 ppm of ginkgolic acids.

The main interest of *G. biloba* research has been in its memory enhancing effects, which have recently been challenged [1,2] as well as antioxidant, anti-ischaemic and anti-cancer effects [3–7].

Other focus of research has been on the prevention or treatment of ischaemia and reperfusion injury by the extract and its terpenoid constituents, especially in stroke and MI (myocardial infarction) [8–11].

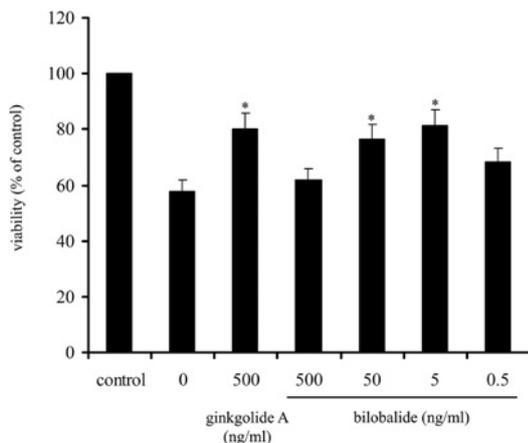
In an effort to purify the effective components and the pharmacological pathway that leads to the herb's many protective effects, it was found that the terpenoids, especially ginkgolide B, are antagonists of PAFR [PAF (platelet-activating factor) receptor] [12]. Blocking the PAFR signalling could possibly inhibit inflammation or ischaemic injury. Ginkgolide B is widely used as an antagonist for the G-protein-coupled PAFR that is present on key target cells of the inflammatory, immune and haemostatic systems. Its ligands, the PAF and PAF-like lipids, on binding to PAFR, trigger a variety of intracellular signalling cascades and induce functional responses of PAFR-bearing cells that then

**Abbreviations used:** GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MI, myocardial infarction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NMDA, N-methyl-D-aspartate; PAF, platelet-activating factor; PAF-AH, PAF acetylhydrolase; PAFR, PAF receptor; RT-PCR, reverse transcription PCR; SD, Sprague-Dawley; TTC, 2,3,5-triphenyltetrazolium chloride.

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**Table 1 Primers used in the PCR experiments**

Gene	GenBank® accession no.	Forward primer (5'→3')	Reverse primer (5'→3')
GAPDH	NM_017008.3	TTCAACGGCACAGTCAAGG	CGGCATGTCAGATCCACAA
PAFR	NM_053321.2	GGCAGTAGCCTATCCCATCA	TCTGGTAGCCCAACTCTGCT
PAF-AH II	AY225592.1	ATCAGGAAGGGGAGAAGGA	AAGGAGTGTCCCATCACAG



**Figure 1 Cell viability assessed by MTT**

Cardiomyocytes were exposed to hypoxia for 6 h with or without bilobalide pre-treatment. Results are represented as means ± S.E.M., *n* = 6. \**P* < 0.01 versus 0 (model).

initiate or amplify inflammatory, thrombotic or apoptotic events. Intra- and extracellular phospholipases A2 control the degrading of PAF selectively and thereby determine the degree of stimulation [13]. The tightly regulated PAF system becomes involved in pathological conditions like ischaemia, where PAF is one of the main damaging factors accumulating in the hypoxic tissue [14]. PAF has been shown to significantly participate in myocardial reperfusion injury [15] and it exerts direct apoptotic effect on H9c2 cardiomyocytes via a Ca<sup>2+</sup>-dependent pathway [16].

Weichel et al. [17] have shown that bilobalide inhibits NMDA (*N*-methyl-D-aspartate)-induced phospholipase A2 activation and phospholipid breakdown in the rat hippocampus. The phospholipase A2, PAF-AH (PAF acetylhydrolase), catalyses the removal of the acyl group at the sn-2 position of PAF and truncated phospholipids, thereby inactivating the lipids. The enzyme exists in intracellular (PAF-AH I and II) and extracellular (plasma PAF-AH) isotypes and is mainly expressed by cells of the immune system. Other cells may also express and release PAF-AH, but it is not known to what extent. In the present study, we show for the first time that the intracellular subtype PAF-AH II [18] is expressed by neonatal cardiomyocytes and we tested its translational expression levels during hypoxia. PAF-AH has been shown to prevent myocardial ischaemia-reperfusion injury [19], and so we were interested in whether bilobalide's possible inhibition of phospholipase A2 has any effect on the enzyme.

Bilobalide was the most active compound in studies on the consequences of hypoxia in hippocampal neurons [20] and en-

dothelial cells [21] and on apoptotic cell death in neuronal cultures [22]. The anti-apoptotic effects of bilobalide on the heart have not been researched much when compared with those on the brain. Besides all the attention that ginkgolide B received as an antagonist of PAFR, bilobalide's reaction with the PAF pathway has not been studied so far. In the present study, we investigated the cardioprotective effects of bilobalide in neonatal rat cardiomyocytes and in an MI model in rats. We tested for the involvement of PAFR and PAF-AH. For the first time it is shown that bilobalide has anti-ischaemic effects on neonatal rat cardiomyocytes and that the expression of PAFR is linked to hypoxia-induced apoptosis in the heart *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Animal care

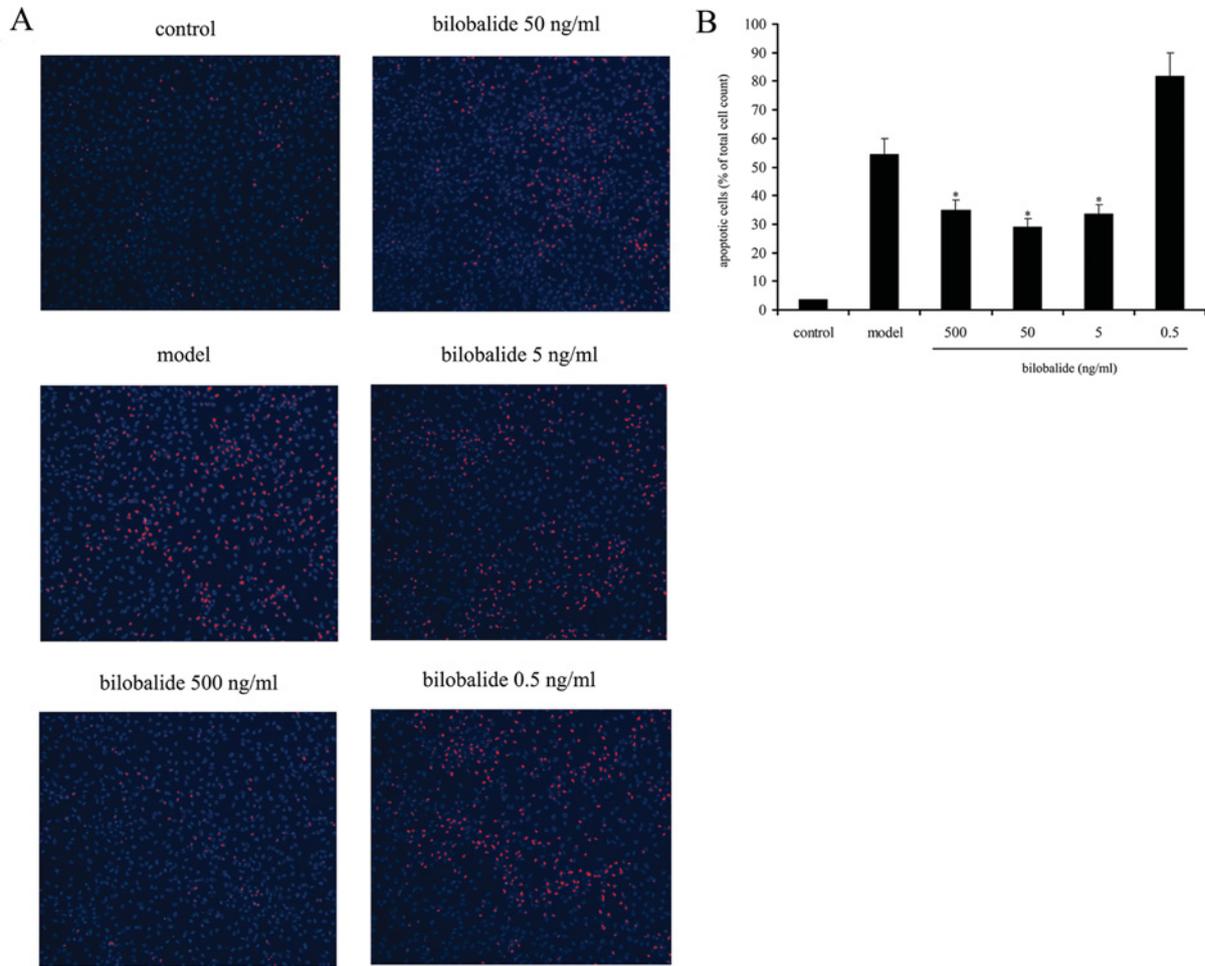
Male SD (Sprague–Dawley) rats weighing 250–300 g were used for this experiment. The animals were housed under diurnal lighting conditions and fed standard rat chow and water *ad libitum*. All animal experiments were performed in accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China and approved by the Animal Research Ethics Committee, School of Pharmacy, Fudan University.

### Drugs

Ginkgolide A and bilobalide (>98 % purity) were purchased from Shanghai Winherb Medical Science Co. Ltd. Because of their hydrophobic character, ginkgolide A and bilobalide were first dissolved in DMSO and then in PBS and saline for cell culture and animal experiments respectively. The final concentration of DMSO in medium or saline was below 0.1 %.

### *In vitro*: neonatal rat cardiomyocyte culture

Primary cultures of cardiomyocytes were obtained from the ventricles of 1–3-day-old SD rats according to the method described by Sadoshima et al. [23]. Isolated cardiomyocytes were seeded at a density of 1000 cells/ml in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % FBS (fetal bovine serum), 100 units/ml penicillin and 100 mg/ml streptomycin. Cultures were maintained at 37°C in a humidified incubator with 95 % air and 5 % CO<sub>2</sub> for at least 48 h before being subjected to treatment. Twelve hours and immediately before hypoxia, different concentrations of bilobalide and ginkgolide A, as positive control, were added. Hypoxia was then induced based on the technique described by Rakhit et al. [24]. All culture plates, excluding the normoxic group, were placed in a hypoxic



**Figure 2** Hoechst staining

(A) Nuclear morphology (blue) in control, hypoxia, hypoxia + bilobalide at concentrations 500, 50, 5 and 0.5 ng/ml group was analysed using fluorescence microscopy  $\times 100$ . Red dots indicate condensed or crescent-shaped nuclei. Representative images from four independent experiments in triplicate are shown here. (B) Apoptotic cells expressed as a percentage of total number of cardiomyocytes in treatment groups. Results are expressed as means  $\pm$  S.E.M.,  $n = 4$ ,  $*P < 0.05$  versus model.

solution (116 mM NaCl, 50 mM KCl, 1.8 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 26 mM NaHCO<sub>3</sub> and 1 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O) in an anaerobic chamber (BD Diagnostics System) and maintained at 37°C with a humidified atmosphere of 5% CO<sub>2</sub>, 10% H<sub>2</sub> and 85% N<sub>2</sub> for 6 h. Control groups were kept in normoxic conditions at 37°C for the same amount of time. After hypoxia, cells were either used for MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] measurements, protein and RNA extraction, immunofluorescence staining or Hoechst staining.

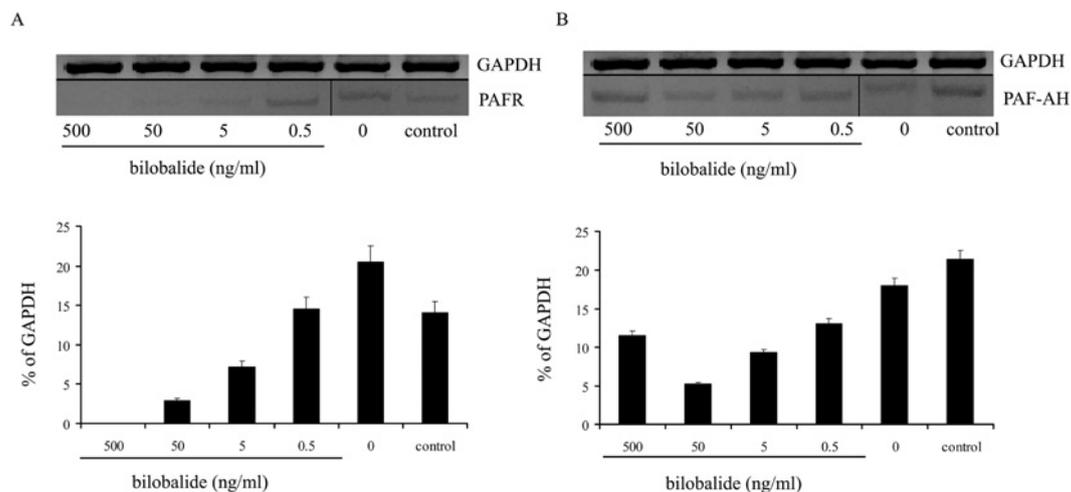
#### **In vivo: MI model**

Adult male SD rats (250–300 g) were randomly assigned to four groups: sham group ( $n = 6$ ); vehicle MI group (saline;  $n = 18$ ); MI treated with ginkgolide A (10 mg·kg<sup>-1</sup>·day<sup>-1</sup>;  $n = 18$ ); MI

treated with bilobalide (10 mg·kg<sup>-1</sup>·day<sup>-1</sup>;  $n = 18$ ). Rats were injected intraperitoneally on a daily basis for 7 days before occlusion of the left descending coronary artery. The MI model was induced as previously described [25]. Rats were anesthetized with 7% chloral hydrate (35 mg/kg of body weight, intraperitoneally). Animals that died within 48 h of the experiment were excluded from subsequent experiments. At 48 h post-surgery, hearts were excised, washed in ice-cold saline and stored at  $-80^{\circ}\text{C}$  for infarct size measurements, and protein and RNA extraction.

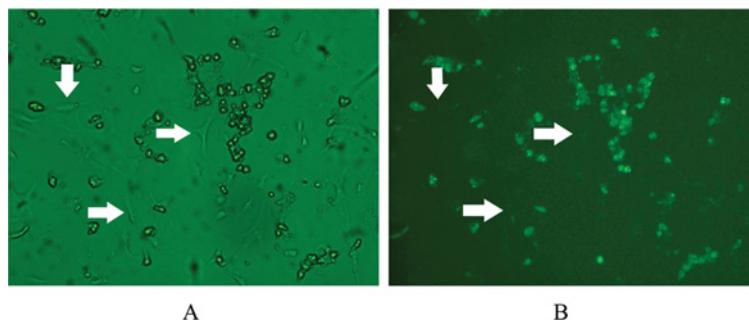
#### **MTT assay**

Cell viability was assessed by measuring the reduction in MTT (0.5 mg/ml) with a microplate spectrophotometric reader at 570 nm as described by Hansen et al. [26].



**Figure 3 mRNA expression of PAFR and PAF-AH**

(A) PAFR electrophoresis blot of reverse-transcribed mRNA of PAFR and internal standard GAPDH. (B) PAF-AH mRNA expression as a percentage of internal standard GAPDH mRNA expression in bilobalide-treated groups, model (0) and control group. Results are means  $\pm$  S.E.M. ( $n = 3$ ). The black lines in the gel photographs of PAFR and PAF-AH indicate the places where the images have been cut and rearranged to represent the same order as the GAPDH bands.



**Figure 4 Detection of PAFR in cardiomyocytes**

(A) Confocal microscopy image of primary culture of cardiomyocytes. The white arrows point to fibroblasts. Small, bright and round cells are cardiomyocytes ( $\times 400$ ). (B) Same as (A) but with fluorescence detection of PAFR. White arrows point to the fibroblasts that were in the same positions as in (A).

### Hoechst staining

Cardiomyocytes were analysed for the appearance of pyknotic or shrink nuclei, which is a hallmark of apoptosis. After removal of culture media, cells were fixed with  $-10^{\circ}\text{C}$  cold methanol. After fixation, cells were incubated with  $10\ \mu\text{M}$  Hoechst 33258 (Beyotime Biotech). Following three washes, cells were examined with a fluorescence microscope (Leica Microsystems) at  $\times 100$ . Using Image Pro Plus imaging software, apoptotic cells (cells with shrink nuclei that showed higher fluorescence intensity) were counted and marked in red using the segmentation tool.

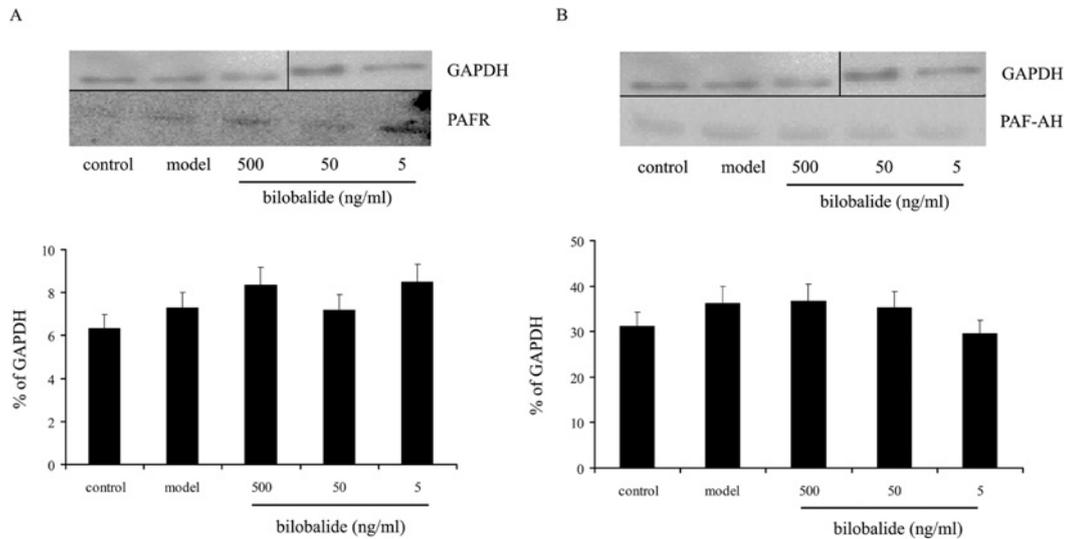
### Infarct size

The entire ventricular tissue was dissected and cut into five horizontal slices ( $n = 4$  per group). After incubation in  $0.1\%$  TTC (2,3,5-triphenyltetrazolium chloride) solution for 15 min ( $37^{\circ}\text{C}$ ),

the sections were immersed in  $4\%$  (w/v) formalin for another 30 min [27]. Ischaemic myocardium remained pale, whereas the non-ischaemic zone was stained deep purple. Total area and the infarct area were determined by computerized planimetry (Scion Image). Infarct size was expressed as a percentage of the infarct area with respect to the entire left ventricular area.

### RNA isolation and RT-PCR (reverse transcription-PCR)

The total RNA was isolated from cells and infarcted left ventricular tissue using TRIzol<sup>®</sup> reagent (Invitrogen), and its purity and concentration was assessed by the absorption at 260 nm and the ratio of 260/280 nm respectively. RNA was then reverse transcribed and amplified using a RT-PCR Kit (Takara). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was considered as an internal standard gene. RT-PCR was performed in MyCycler



**Figure 5** PAFR and PAF-AH protein expression

Western blot of PAFR (A) and PAF-AH (B) proteins compared with internal standard GAPDH. Protein expression is represented as a percentage of internal standard protein GAPDH expression in bilobalide-treated groups, model and control group. Results are means  $\pm$  S.E.M.,  $n = 3$ . The black lines in the gel photographs of GAPDH indicate the places where the images have been cut and rearranged to represent the same order as the PAFR and PAF-AH bands.

(Bio-Rad Laboratories). The PCR products were run on an agarose gel (1.5%), imaged by AlphaEaseFC system (Alpha Innotech) and analysed by Image-Pro Plus (Media Cybernetics). Primers (designed by Primer3 online software; constructed by Sangon) and GenBank® numbers are listed in Table 1.

### Western blotting

Cardiomyocytes from cell culture and from around the infarcted left ventricular tissue were scraped and homogenized respectively and then centrifuged to obtain lysis buffer soluble proteins as previously described [28]. Samples were boiled with loading buffer and then separated by SDS/PAGE (10% gel) prior to being transferred to a nitrocellulose membrane (Millipore). Subsequently, membranes were immersed in PAFR (1:200) and PAF-AH (1:200) antibody dilution (Santa Cruz Biotechnology) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (ICL Laboratory) at 1:1000 dilution. GAPDH (1:1000) and alpha tubulin (1:1000, Santa Cruz Biotechnology) was run as a loading control to normalize samples. Chemiluminescent signals were generated by the addition of the enhanced chemiluminescent detection reagent (ECL, Applygen Corporation) and detected on a FluorChem SP laser densitometer (Alpha Innotech). The band density was analysed using Image-Pro Plus (Media Cybernetics).

### Immunofluorescence

Cardiomyocytes in 6-cm-diameter cell culture dishes were washed three times with PBS, blocked with 10% FBS in PBS for 20 min and then incubated with the primary antibody PAFR (1:100) for 60 min. Subsequently, cells were washed three times

before being incubated with the secondary antibody, FITC-goat anti-rabbit (1:100, Beyotime) with 3% FBS in PBS. After three more washes with PBS, cells were observed under a confocal fluorescence microscope. Images were processed using Image Pro Plus (Media Cybernetics).

### Statistical analysis

Results were expressed as means  $\pm$  S.E.M. Differences between individual groups were analysed using the Student's *t* test. A difference with  $P < 0.05$  was considered statistically significant.

## RESULTS

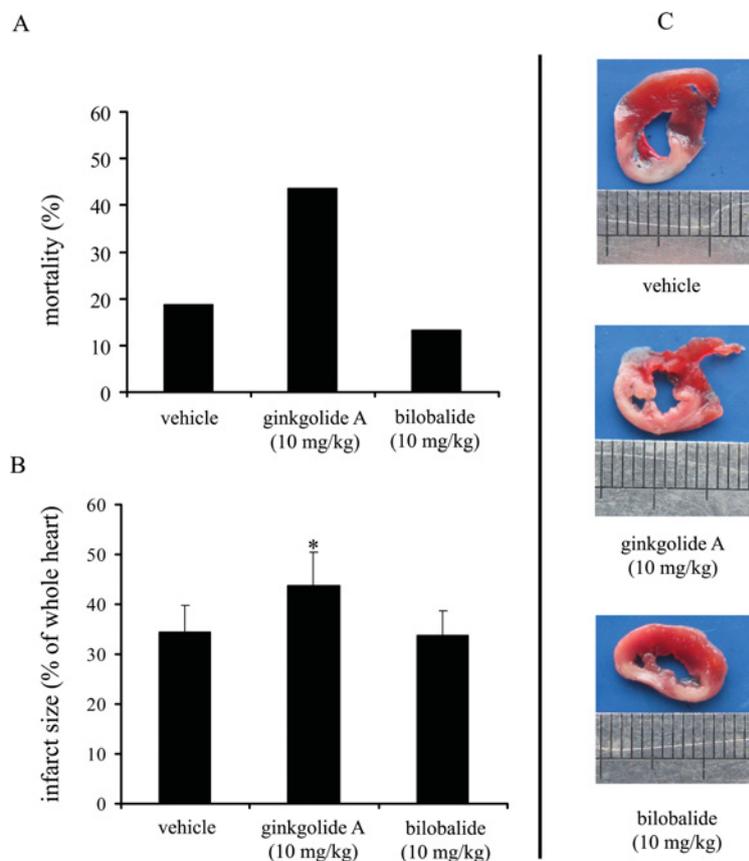
### Cell viability

#### MTT

After 6 h of hypoxia neonatal cardiomyocytes' viability was assayed using MTT. MTT assay is a conventional method to assess cell survival rate. Consistent with previous reports [29], exposure to hypoxia for 6 h incurred a 40–50% decrease in cardiomyocytes' viability. However, bilobalide reversed cardiomyocyte loss in a dose-dependent manner. Bilobalide (50 and 5 ng/ml) significantly prevented cell death (Figure 1). Bilobalide at each of the concentrations used alone did not cause any apparent cytotoxicity (results not shown).

#### Hoechst staining

Using Image Pro Plus imaging software, apoptotic cells (cells with shrink nuclei that showed higher fluorescence intensity) were counted and marked in red using the segmentation tool



**Figure 6 Mortality and infarct size**

(A) Mortality after MI in vehicle, ginkgolide A (10 mg/kg of body weight) and bilobalide 10 mg/kg of body weight. (B) Histograms represent the percentage of infarcted area (as made visible by TTC stain) from the whole heart. Results are means  $\pm$  S.E.M.,  $n = 3$ , \* $P < 0.05$  versus vehicle. (C) Pictures of comparable slices of rat heart stained with TTC solution, infarcted tissue is white.

(Figure 2A). Bilobalide could reduce programmed cell death in neonatal cardiomyocytes in a concentration-dependent manner compared with the model group (Figure 2B).

**PAFR and PAF-AH mRNA expression (RT-PCR)**

PAFR mRNA expression was up-regulated in the hypoxic model, but bilobalide at concentrations of 5 and 50 ng/ml reversed this and at the highest concentration even blocked expression completely (Figure 3A). PAF-AH was down-regulated in the hypoxic group but bilobalide showed no significant effect on increasing PAF-AH expression at the concentrations effective in the cell viability tests. Only at 500 ng/ml could a slight rise in expression levels be observed.

**Immunofluorescence microscopy of PAFR on neonatal myocardial cells**

The pictures presented here are representative of four independent experiments of the cells that were incubated with PAFR. They

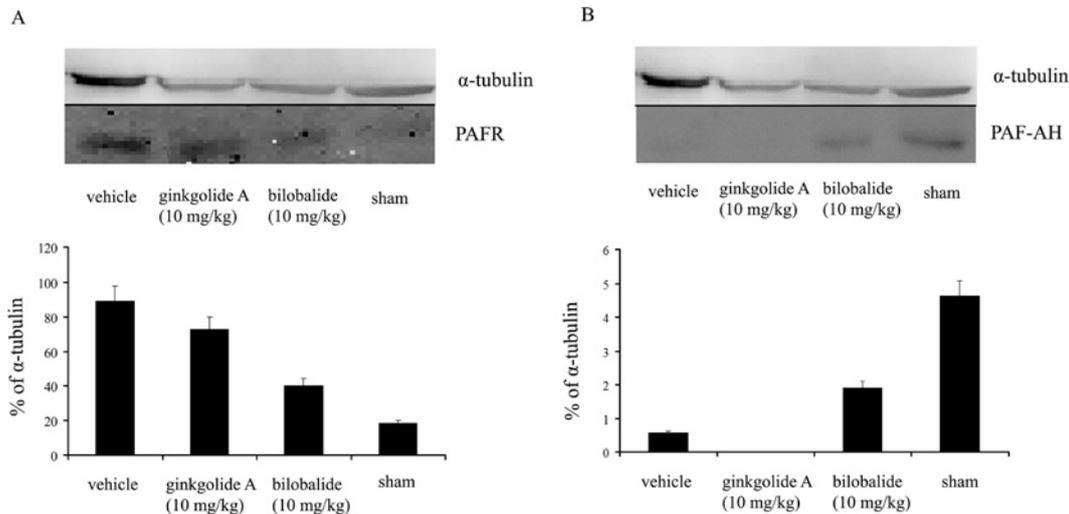
clearly show that PAFR is expressed by cardiomyocytes, whereas not by fibroblasts (Figures 4A and 4B). In further experiments, where cell cultures were exposed to hypoxia with addition of bilobalide, no significant changes in fluorescence were found between the groups (results not shown). Nevertheless, we show that PAFR is expressed on the surface of the cells. The results also correspond to the bands of the Western blotting. However, protein expression levels between the groups were not significantly different.

**Western blotting of PAFR and PAF-AH in neonatal cardiomyocytes**

PAFR showed higher expression in the model group and in all bilobalide-treated groups (Figure 5A), while PAF-AH was more or less evenly expressed in all groups (Figure 5B).

**In vivo mortality and infarct size**

Mortality was calculated by dividing the number of rats that had died within 48 h after MI by the total number of rats per group. After TTC staining, ischaemic myocardium remained



**Figure 7 PAFR and PAF-AH protein expression in tissue**

Western blot of PAFR (A) and PAF-AH (B) proteins compared with internal standard  $\alpha$ -tubulin. Protein expression is represented as a percentage of internal standard protein expression in vehicle, ginkgolide A-treated, bilobalide-treated and sham groups. Results are means  $\pm$  S.E.M.

pale, whereas the non-ischaemic zone was stained deep purple. Total area and the infarct area were determined by computerized planimetry (Scion Image). Infarct size was expressed as a percentage of the infarct area with respect to the entire left ventricular area. Surprisingly, rats treated with ginkgolide A suffered more severely from the surgery and the infarcted tissue area was observed to be significantly larger than in the other groups (Figures 5A–5C). Bilobalide showed no significant difference to the vehicle group in this trial (Figure 6).

### Western blotting of PAFR and PAF-AH in myocardial tissue

PAFR was overexpressed in the myocardial tissue of vehicle and ginkgolide A-treated groups, whereas, bilobalide-treated rats showed a reduced expression that was also observed in the sham group. PAF-AH protein expression similar to the mRNA expression levels was lowered in hypoxic tissue and raised in the bilobalide group (Figure 7).

## DISCUSSION

In the present study, we show the anti-ischaemic effects of bilobalide in neonatal cardiomyocytes by MTT viability assays and Hoechst staining of cells. In previous experiments of our group, the MTT test has already been used successfully on neonatal cardiomyocytes to test for toxicity or efficacy of Chinese herbs. Cells treated with bilobalide prior to hypoxia have a significantly higher probability of survival than untreated cells (20%). We used the Hoechst staining to visualize this effect. Cardiomyocytes that had undergone apoptosis were coloured in a brighter

fluorescent green than healthy cells and could therefore be distinguished and counted with a software tool. Also, we found a concentration-dependent decrease in mortality, consistent with the MTT test.

For determination of the gene and protein expression of PAFR and PAF-AH, RT-PCR and Western blotting were employed. In the literature, no reference that links bilobalide with this receptor can be found. Nevertheless, the receptor has been shown to play an important role in ischaemia of the brain and the heart [15]. Hypoxia might induce stimuli for PAF release [14], which in turn could exert a direct apoptotic effect on the cells [16]. We hypothesize that *G. biloba*'s anti-ischaemic effect on neonatal cardiomyocytes may partly be because of bilobalide's down-regulation of PAFR. Through the down-regulation, PAF signalling is decreased and apoptosis is slowed down consecutively. The gene expression of PAFR in cardiomyocytes that were subjected to hypoxia increased significantly and was reversed in a concentration-dependent manner in cells that had been treated with bilobalide before exposure. However, protein expression of PAFR remained unchanged. This might be because the hypoxia time was only 6 h, maybe not enough time for the receptor to become expressed after translation.

Also, we found that mRNA expression of PAF-AH, the enzyme that degrades PAF, was reduced in cardiomyocytes during hypoxia but not influenced by bilobalide treatment. This phenomenon is consistent with previous findings in which PAF is overexpressed in apoptosis [30] or as a response to inflammatory stimuli [31]. A down-regulation of PAF-AH would result in a reduced degradation of PAF and therefore an accumulation thereof.

*In vivo* experiments on rats with MI failed to show a significantly decreased mortality in bilobalide-treated rats. The size of the apoptotic area also did not differ between the vehicle



and the bilobalide-treated groups. However, we found that the PAFR protein is overexpressed in vehicle and ginkgolide A-treated groups compared with the sham and bilobalide-treated groups. Bilobalide-treated rat hearts show a reduced expression of the receptor. This is consistent with the findings from the cell culture experiments. Also, expression of the enzyme PAF-AH was down-regulated in hearts that had developed MI.

In our study, we have shown that bilobalide has cardioprotective effects on neonatal cardiomyocytes and that this could partly be mediated by the down-regulation of PAFR expression. PAFR and PAF-AH levels are influenced in a similar way in the *in vivo* model of MI. Although bilobalide could not show a significant improvement of mortality *in vivo*, the altered levels of PAFR and PAF-AH may give clues of the involvement of the PAF pathway in the development of MI. To further understand the mechanism behind the findings, the PAF accumulation in the hypoxic tissue should be monitored and bilobalide should be investigated in further *in vivo* studies with different models and concentrations. If this special ingredient of *G. biloba* received as much attention as the ginkgolides, it would contribute immensely to our so far often contradictory and confusing understanding of the herb.

#### AUTHOR CONTRIBUTION

Stefanie Maerz conducted all of the experiments and prepared the manuscript. Chun-Hua Liu assisted with the organization of the animal trial. Wei Guo supervised the cell culture experiments in the early stage and purchased the drugs and antibodies. Yi-Zhun Zhu supervised and sponsored the entire project.

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