

# Natural therapeutic magnesium lithospermate B potently protects the endothelium from hyperglycaemia-induced dysfunction

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## Aims

We have investigated the effects of magnesium lithospermate B (MLB), the active compound of the Oriental herbal remedy, *Salvia miltiorrhizae*, on endothelial dysfunction associated with diabetes mellitus using cultured endothelial cells and an animal model of type 2 diabetes mellitus.

## Methods and results

The effect of MLB on vasodilatory function in Otsuka Long-Evans Tokushima Fatty (OLETF) rats was assessed. MLB treatment for 20 weeks starting at 12 weeks attenuated the decrease in endothelium-dependent vasodilation in OLETF rats. MLB treatment also increased serum nitrite level and reduced serum advanced glycation end products concentration. The effect of MLB was greater than an equivalent dose of  $\alpha$ -lipoic acid ( $\alpha$ LA), a popular antioxidant treatment. MLB rescued the inhibition of endothelial nitric oxide synthase (eNOS) activity and eNOS phosphorylation in endothelial cells cultured in hyperglycaemia. This effect was dependent on Akt phosphorylation and associated with decreased O-linked N-acetylglucosamine protein modification of eNOS. MLB also increased nuclear factor erythroid 2-related factor-2 (Nrf-2) activation in a phosphoinositide 3-kinase/Akt pathway dependent manner. MLB treatment induced the expression of the Nrf-2-regulated antioxidant enzyme, heme oxygenase-1. The antioxidant  $\alpha$ LA could not produce this effect. Moreover, MLB decreased oxidative stress and endothelial cell apoptosis caused by hyperglycaemia.

## Conclusion

MLB is a naturally occurring, new generation antioxidant that activates eNOS and ameliorates endothelial dysfunction in diabetes by enhancing vasodilation in addition to reducing oxidative stress. The relative strong performance of MLB makes it an ideal candidate for further, expanded trials as a new generation of antioxidant to treat diabetes-related complications.

## Keywords

Diabetes • Endothelial function • Nitric oxide • Antioxidant • Magnesium lithospermate B

## 1. Introduction

The vascular endothelium is of key importance in regulating blood flow and tissue homeostasis. It is also the common target for all risk factors linked to atherosclerosis and endothelial dysfunction predicts future vascular problems. In diabetes mellitus, endothelial vasodilator function becomes compromised and produces a proatherosclerotic state. There is a strong correlation between alterations in glucose metabolism and the development of coronary artery

disease, suggesting that atherosclerosis and diabetes possess similar precursory events.<sup>1</sup>

Antioxidants have been tested as potential therapeutics for treating vascular dysfunction in diabetes. However, despite a large number of promising animal studies, the performance of antioxidants in large-scale clinical trials has generally been disappointing.<sup>2</sup> However, it is still too early to discount the potential of antioxidant therapy as a treatment for diabetes. Increases in our understanding of how oxidative stress is generated in diabetes suggest that new antioxidants which suppress

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the generation of reactive species (in addition to merely scavenging free radicals) can be important new therapeutic agents.

Traditional Chinese medicine can be a valuable source of new antioxidants. Over 80 Chinese natural medicines have been documented for the treatment of the secondary complications associated with diabetes.<sup>3</sup> Most of these originate from plants and a small number originate from animals or insects. An attractive candidate antioxidant to treat diabetes is magnesium lithospermate B (MLB). MLB is the active component of the water soluble fraction of the Chinese medicine, Danshen, which is a root preparation of the plant Red Sage (*salvia miltiorrhizae* radix, 丹参; related to lavender and oregano).<sup>4</sup> MLB is an interesting antioxidant for further study because it has interesting secondary effects in cells. MLB inhibits the enzyme aldose reductase, which is a key component of the polyol biochemical pathway involved in the pathogenesis of diabetic complications.<sup>5</sup> Also, the global market value of Danshen, an already popular herbal remedy in North America, exceeds 2 billion US dollars.<sup>4</sup>

Previous research has shown that MLB has antifibrotic, myocardial salvage, and neuroprotective effects.<sup>6</sup> MLB prevents hepatitis, uraemia, and improves blood circulation, arrhythmia, and renal function.<sup>7,8</sup> Recent work in our laboratory showed that MLB can prevent the development of neointimal hyperplasia in animal models of diabetes (H.C.L., unpublished data) and after balloon-induced injury.<sup>9</sup> However, the potential benefit of MLB on endothelial dysfunction in diabetes is unknown. Thus, we assessed the effect of MLB on the consequences of endothelial cell oxidative stress caused by hyperglycaemia [reactive oxygen species (ROS) generation, apoptosis, glutathione (GSH) reduction, increased leucocyte adhesion and advanced glycation end products (AGE) accumulation]. We compared MLB with the widely studied antioxidant,  $\alpha$ -lipoic acid ( $\alpha$ LA).<sup>10</sup> In addition, we assessed the effect of MLB on hyperglycaemia-induced inactivation of the enzyme endothelial nitric oxide synthase (eNOS). eNOS catalyses the production of nitric oxide (NO) from L-arginine. NO is widely considered to be the 'antiatherogenic' molecule.<sup>11</sup> The effect of MLB on hyperglycaemia-induced endothelial dysfunction *in vivo* was measured in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, an animal model of diabetes.<sup>12</sup>

## 2. Methods

Expanded experimental detail and information concerning the purchase of reagents and methods are described in the Supplementary material online.

### 2.1 Cell culture

Human umbilical endothelial cells (HUVEC) and human aortic endothelial cells (HAEC) were supplied by Cambrex (NJ, USA) and cultured in EBM-2 growth media supplemented with the EGM-2 bullet kit. THP-1 human monocytic cells were cultured in RPMI-1640 medium supplemented with 0.05 nM 2-mercaptoethanol, 10% foetal bovine serum and antibiotics. Cells were cultured in growth media containing 5 mM glucose for the normoglycaemia condition or 30 mM glucose for the hyperglycaemia condition.

### 2.2 Measurement of cell proliferation

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay, as previously described.<sup>13</sup>

### 2.3 Measurement of ROS

The generation of ROS was determined analysing the oxidation of the non-fluorescent dichlorofluorescein diacetate (DCF-DA) to the

fluorescent DCF. DCF emission was recorded using a fluorescence microplate reader (SpectraMax GeminiEM, Molecular Devices) as previously described.<sup>14</sup>

### 2.4 Glutathione assay

Glutathione and the disulfide dimer, glutathione disulfide, were measured using the Glutathione Assay Kit II (Merck, NJ, USA). Cell lysate was harvested and a deproteination step was carried out using metaphosphoric acid and triethanolamine (in accordance with the kit instructions).

### 2.5 Adhesion assay

The adhesion of mouse inflammatory peritoneal (IP) exudate macrophages to endothelial cell monolayers was carried out as previously described.<sup>15</sup> Briefly, endothelial cells were cultured to confluence in six-well culture plates and treated with drug with or without high glucose (30 mM) culture media. IP exudates macrophages ( $2 \times 10^4$  cells/mL) were added to the monolayers and incubated for 20 min at 37°C. The unbound cells were washed three times and the total number of adherent cells was counted in four randomly selected optical fields per well.

### 2.6 Detection of apoptosis

Cells were cultured to confluence in 24-well plates and incubated with according treatments. Cells were lysed and centrifuged at 200 g for 10 min. Apoptosis was detected using the 'Cell Death Detection ELISA Kit' (Roche, CA, USA), which is based on a quantitative sandwich-enzyme-immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones released into the cytoplasm of cells that die from apoptosis.

### 2.7 Nitrite assay

Cells were cultured to confluence in 24-well plates. Total nitrite was measured using the Parameter-TM Total NO/Nitrate/Nitrite assay kit (R&D Systems, MN, USA), in accordance with the manufacturer's instructions.

### 2.8 Measurement of AGE

Cells were cultured in 12-well plates. AGE was measured by an ELISA based on the method of Araki et al.<sup>16</sup> 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma-Aldrich, SL, USA) was used as the detection reagent. Purified human AGE was used to generate the standard curve.

### 2.9 Immunoprecipitation and western blot analysis

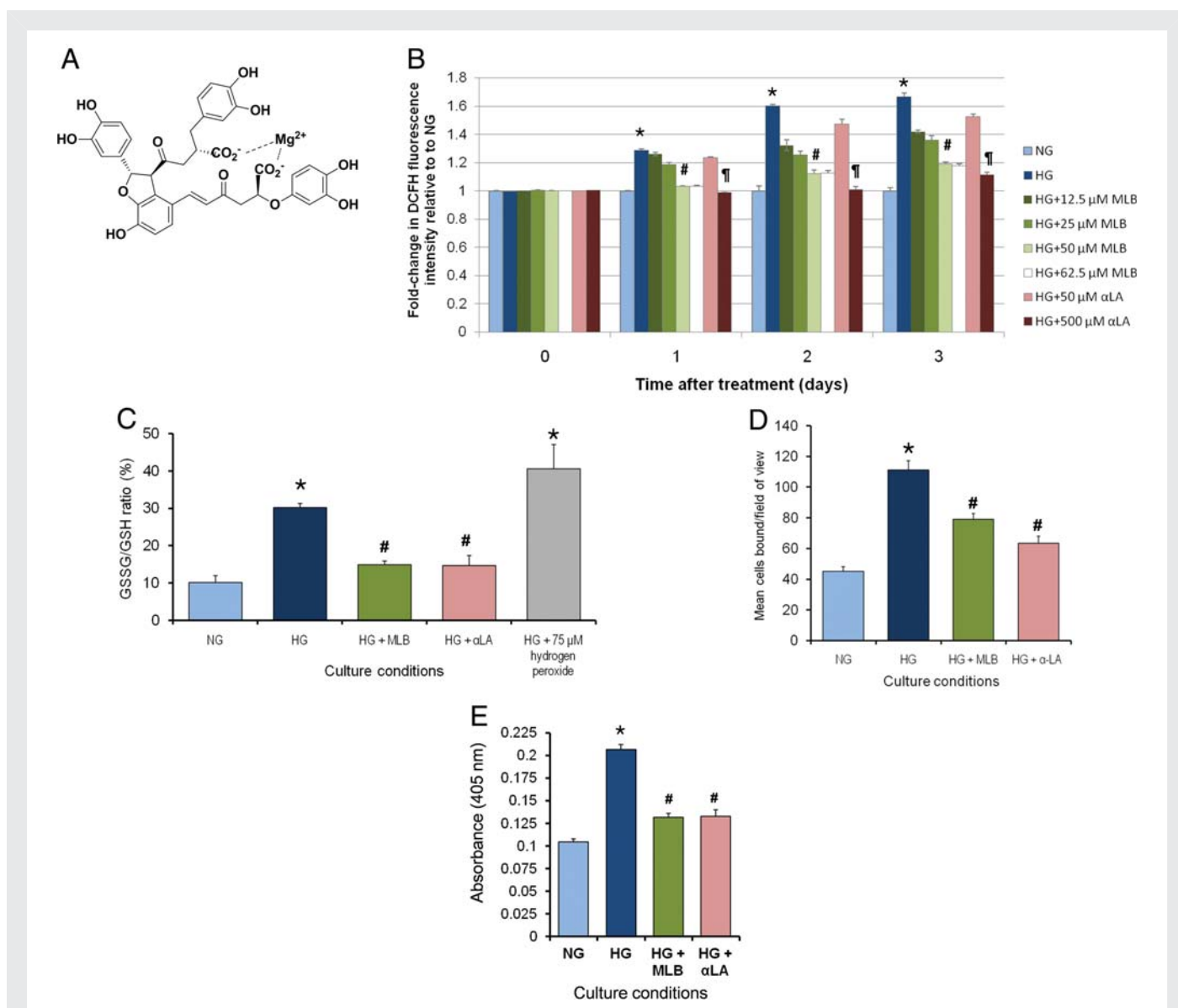
For immunoprecipitation, the antibody/antigen complex was precipitated using GammaBind Plus Sepharose (GE Healthcare, WI, USA.) in accordance with the online protocol provided by Abcam, MA, USA. Proteins were separated by 7.5% or 10% SDS-PAGE and transferred onto PVDF membranes (Roth, Karlsruhe, Germany).

### 2.10 Immunocytochemical analysis of nuclear factor erythroid 2-related factor-2

HAEC ( $1 \times 10^5$  cells/600  $\mu$ L in a four-well chamber slide) were immunostained with anti-nuclear factor erythroid 2-related factor-2 (Nrf-2) primary antibody (Santa Cruz, CA, USA) followed by treatment with FITC-goat anti-rabbit IgG secondary antibody. Stained cells were analysed using a confocal microscope (Leica, NJ, USA).

### 2.11 Animals

OLETF rats and non-diabetic control Long-Evans Tokushima Otsuka (LETO) rats were obtained from the Otsuka Pharmaceutical Company (Tokushima, Japan). Animal studies were carried out under the guidelines for the care and use of laboratory animals established by Yonsei University College of Medicine; on the basis of the Guide for the Care and Use of



**Figure 1** (A) Chemical structure of MLB (molecular weight = 739). (B) Effect of MLB or  $\alpha$ LA treatment on ROS generation in HAEC exposed to hyperglycaemia (error = SD;  $P < 0.05$  for HG compared with NG (\*), HG + 50  $\mu$ M MLB compared with HG (#), and HG + 500  $\mu$ M  $\alpha$ LA compared with HG (¶)). Passage 5 HAEC; data are representative of three independent experiments. (C) MLB treatment decreased the GSSG/GSH ratio in HAEC exposed to hyperglycaemia for 48 h (error = SD;  $*P < 0.05$  compared with NG;  $\#P < 0.05$  compared with HG). Treatment with 75  $\mu$ M hydrogen peroxide for 3 h was used as a positive control to induce oxidative stress. Data are representative of three independent experiments. (D) MLB treatment decreased leucocyte adhesion to HAEC exposed to hyperglycaemia for 48 h (Error = SD;  $*P < 0.05$  compared with NG;  $\#P < 0.05$  compared with HG). Data are representative of three independent experiments. (E) MLB inhibited apoptosis in HAEC exposed to hyperglycaemia for 48 h in a similar manner to  $\alpha$ LA. (Error = SD;  $*P < 0.05$  compared with NG;  $\#P < 0.05$  compared with HG). Data are representative of five independent experiments. Abbreviations: NG, 5 mM glucose; HG, 30 mM glucose; MLB, 50  $\mu$ M magnesium lithospermate B;  $\alpha$ LA, 500  $\mu$ M alpha lipoic acid.

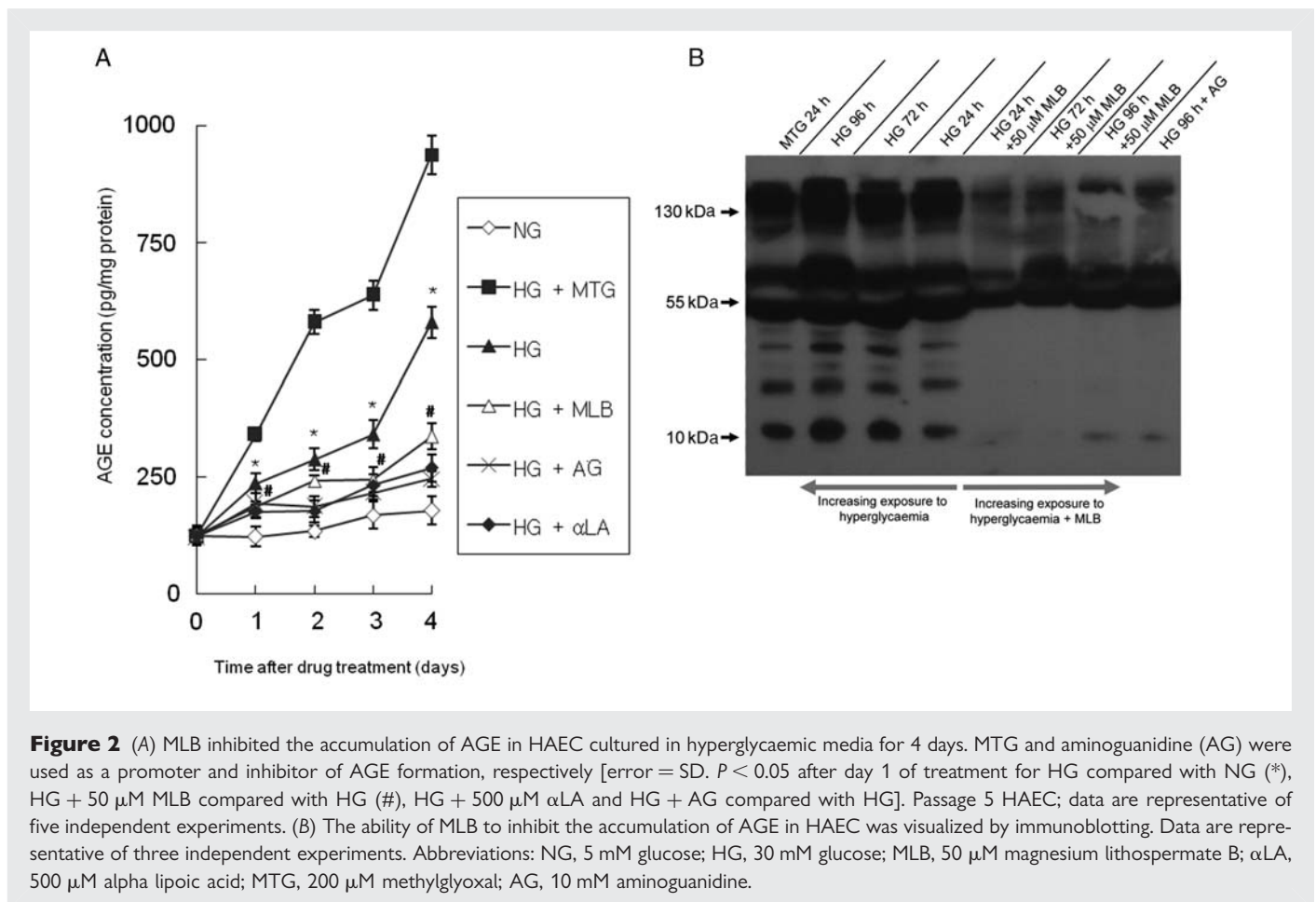
Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996, PHS Approved Animal Welfare Assurance code A5645-01).

## 2.12 Animal study

For studies of the effect of MLB on serum concentration of nitrite and AGE, animals were divided into four groups of five rats at 12 weeks of age: a control group (LETO), untreated diabetes group (OLETF), vehicle-treated diabetes group (OLETF + vehicle), and MLB-treated diabetes group (OLETF + MLB). MLB was dissolved in water and administered orally to OLETF rats from 12 weeks of age, at a daily dose of 20 mg/kg,

via oral gavage which was shown to have beneficial systemic effects without toxicity in previous studies.<sup>17,18</sup> Vehicle-treated animals were administered an identical volume of distilled water. Blood was collected at baseline and at 4 and 8 weeks after commencing MLB treatment.

For vascular function studies, animals were divided into three groups of eight rats at 12 weeks of age: a control group (LETO), vehicle-treated diabetes group (OLETF + Vehicle), and MLB-treated diabetes group (OLETF + MLB). MLB was administered from 12 weeks of age, at a daily dose of 20 mg/kg, via oral gavage. Oral glucose tolerance tests were performed at the age of 29 weeks. At 32 weeks, the thoracic aorta was isolated for vascular function studies.



**Figure 2** (A) MLB inhibited the accumulation of AGE in HAEC cultured in hyperglycaemic media for 4 days. MTG and aminoguanidine (AG) were used as a promoter and inhibitor of AGE formation, respectively [error = SD.  $P < 0.05$  after day 1 of treatment for HG compared with NG (\*), HG + 50  $\mu$ M MLB compared with HG (#), HG + 500  $\mu$ M  $\alpha$ LA and HG + AG compared with HG]. Passage 5 HAEC; data are representative of five independent experiments. (B) The ability of MLB to inhibit the accumulation of AGE in HAEC was visualized by immunoblotting. Data are representative of three independent experiments. Abbreviations: NG, 5 mM glucose; HG, 30 mM glucose; MLB, 50  $\mu$ M magnesium lithospermate B;  $\alpha$ LA, 500  $\mu$ M alpha lipoic acid; MTG, 200  $\mu$ M methylglyoxal; AG, 10 mM aminoguanidine.

## 2.13 Vascular function study

Endothelium-dependent and -independent vasorelaxation were measured using an isometric force displacement transducer as described in the online supplementary methods section.

## 2.14 Statistical analysis of data

For data sets with  $n \leq 5$ , the Mann–Whitney  $U$ -test was used to determine significance. For data sets with  $n \geq 5$ , one-way ANOVA (SPSS 12.0K software, IL, USA) was used to determine statistical significance, employing Bonferonni corrections for multiple comparisons. A  $P$ -value of  $<0.05$  was considered to be significant.

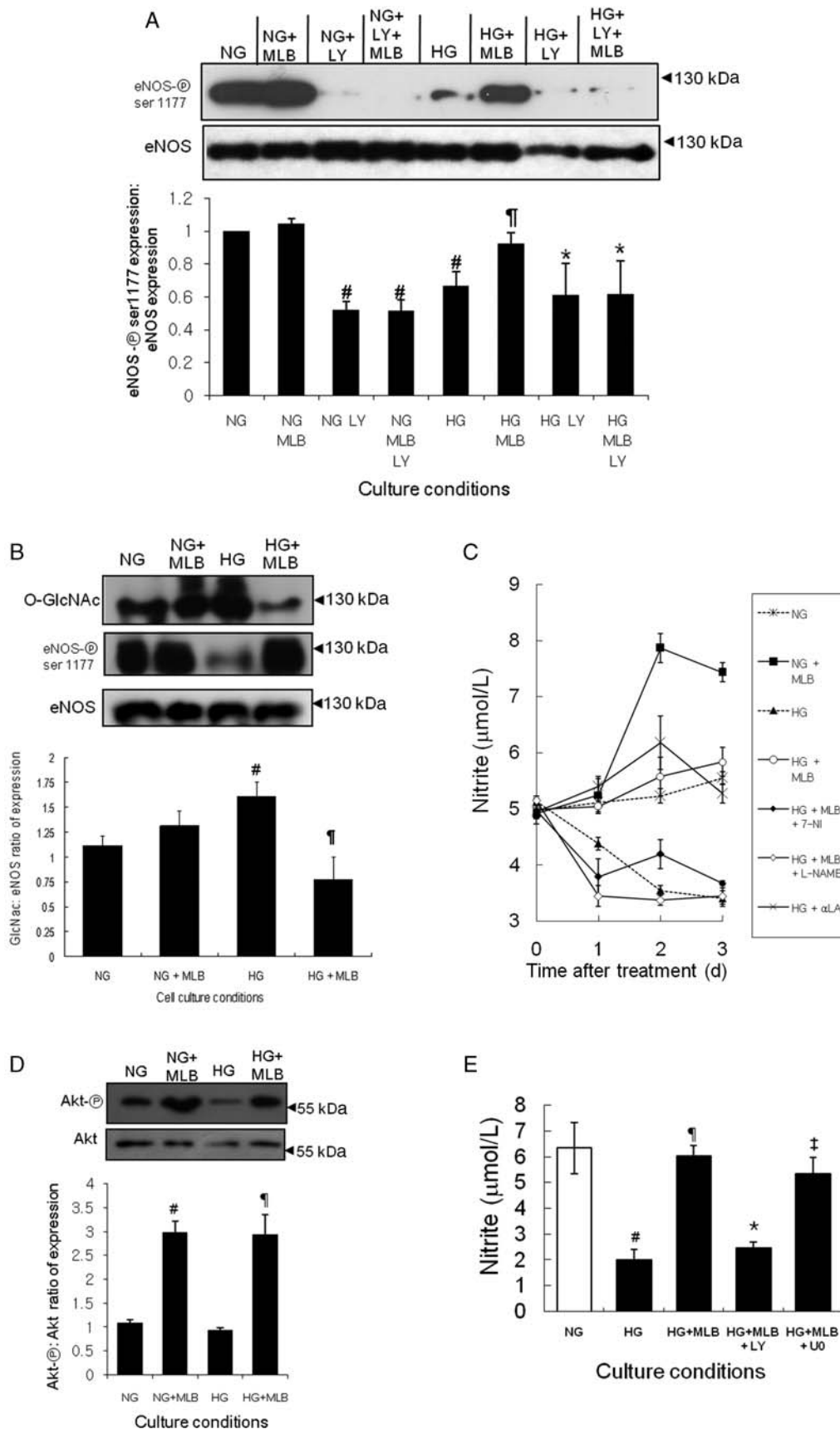
## 3. Results

### 3.1 MLB reduces ROS generation, oxidative stress, apoptosis, and leucocyte adhesion in human endothelial cells exposed to hyperglycaemia

The structure of MLB is shown in Figure 1A. There are little published data concerning the effects of MLB in endothelial cells. Therefore, we tested the effect of MLB on cell proliferation in HAEC and HUVEC, and compared MLB with  $\alpha$ LA (see Supplementary material online, Figure S1A and B). Treatment with MLB at a dose between 25 and 50  $\mu$ M reduced proliferation in HAEC and HUVEC, in contrast to a single published study.<sup>19</sup> The effect of 50  $\mu$ M MLB on cell proliferation

was similar to treatment with 500  $\mu$ M  $\alpha$ LA. However, treatment of HAEC with 50  $\mu$ M MLB for 48 h did not induce a significant increase in cell death, as determined by flow cytometry (see Supplementary material online, Figure S1C).

MLB reduced ROS generation in HAEC exposed to hyperglycaemia after 1 day of treatment and continued to reduce ROS over a period of 3 days (Figure 1B). The ability of MLB to reduce ROS generation was concentration dependent. The effect of 50  $\mu$ M MLB on ROS generation was similar to 62.5  $\mu$ M MLB. Thus, 50  $\mu$ M MLB treatment was chosen for further study. The effect of 50  $\mu$ M MLB on ROS generation was similar to treatment with 500  $\mu$ M  $\alpha$ LA. MLB also reduced ROS generation in HUVEC exposed to hyperglycaemia for 3 days (see Supplementary material online, Figure S2). HAEC exposed to hyperglycaemia showed increased molar ratio of GSSG/GSH, indicative of increased oxidative stress, and MLB treatment decreased the ratio of GSSG/GSH (GSSG = the oxidized form of GSH; Figure 1C). The ability of 50  $\mu$ M MLB to decrease the GSSG/GSH ratio in endothelial cells exposed to hyperglycaemia was similar to 500  $\mu$ M  $\alpha$ LA. Treatment with hydrogen peroxide was used as a positive control for the induction of oxidative stress. Increased adhesion of leucocytes to HAEC exposed to hyperglycaemia was inhibited by MLB treatment (Figure 1D). A similar result was achieved in HUVEC (see Supplementary material online, Figure S3). The reduced adhesion was associated with reduction in the expression of vascular adhesion molecule-1 (VCAM-1) after MLB treatment (see Supplementary material online, Figure S4). VCAM-1 is a major intracellular binding molecule involved



**Figure 3** (A) MLB rescued the decreased phosphorylation of eNOS at residue serine 1177 in HAEC exposed to hyperglycaemia for 48 h. PI 3-K signaling pathway inhibitor LY-294,002 inhibited the effect of MLB on eNOS phosphorylation. Data are representative of three independent immunoblots.



in monocyte binding to the vascular endothelium.<sup>20</sup> The decrease in VCAM-1 expression was partially linked to NO production, because the eNOS inhibitor, L-NAME, could partially block this effect. Moreover, apoptosis in HAEC exposed to hyperglycaemia for 48 h was inhibited by MLB (*Figure 1E*).  $\alpha$ LA treatment produced a similar decrease in apoptosis.

### 3.2 MLB reduces AGE accumulation in human endothelial cells exposed to hyperglycaemia

AGE accumulation in vascular endothelial cells is an irreversible complication of hyperglycaemia and is linked to 'metabolic memory' in diabetic patients.<sup>21</sup> MLB reduced AGE accumulation in HAEC exposed to hyperglycaemia after 1 day of treatment and continued to reduce AGE accumulation for 4 days (*Figure 2A*).  $\alpha$ LA treatment produced a similar effect. Methylglyoxal (MTG) and aminoguanidine (AG) were used as an inducer and inhibitor of AGE accumulation, respectively. The ability of MLB to inhibit the AGE accumulation could be visualized by western blotting (*Figure 2B*). MLB also inhibited AGE accumulation in HUVEC (see Supplementary material online, *Figure S5*).

### 3.3 MLB increases eNOS phosphorylation and activity in endothelial cells exposed to hyperglycaemia: MLB acts through the phosphoinositide 3-kinase/Akt intracellular signalling pathway

Phosphorylation of residue serine 1177 is important in inducing eNOS enzyme activity.<sup>22</sup> MLB could inhibit the decrease in eNOS phosphorylation at residue serine 1177 in HAEC exposed to hyperglycaemia (*Figure 3A*). In addition, the phosphoinositide 3-kinase (PI 3-K)/Akt intracellular signalling pathway inhibitor LY-294,002 inhibited the effect of MLB on eNOS phosphorylation. O-linked N-acetylglucosamine (O-GlcNAc) modification of eNOS in diabetic subjects has also been linked with reduced eNOS activity.<sup>23</sup> MLB reduced the O-GlcNAc modification of eNOS in HAEC exposed to hyperglycaemia (*Figure 3B*). MLB increased NOS activity in HAEC exposed to hyperglycaemia after 1 day of treatment and continuously over the 3 day time course of the experiment (*Figure 3C*).  $\alpha$ LA treatment produced a similar effect. Two NOS specific inhibitors (L-NAME and 7-nitroindazole) could inhibit MLB-induced increase in NOS activity in HAEC exposed to hyperglycaemia. MLB also increased NOS activity

in HUVEC exposed to hyperglycaemia (see Supplementary material online, *Figure S6*).

MLB treatment of HAEC increased the phosphorylation of Akt in both normoglycaemic and hyperglycaemic conditions (*Figure 3D*). Inhibition of the PI 3-K intracellular signalling pathway by LY-294,002 inhibited the effect of MLB on NOS activity in HAEC (*Figure 3E*), confirming the role of the PI 3-K/Akt intracellular signalling pathway in mediating the effect of MLB. Previous data from our laboratory have shown that treatment with MLB has no significant effect on the mitogen-activated protein kinase (MAPK) pathway (H.C.L., unpublished observations). Thus, we employed MAP kinase inhibitors as a negative control when assessing the effect of MLB on other intracellular signalling pathways. Accordingly, the MAPK pathway inhibitor UO126 had no significant effect on NOS activity (*Figure 3E*).

### 3.4 MLB acts through the PI 3-K/Akt intracellular signalling pathway and the Nrf-2 antioxidant response element pathway in a sequential manner

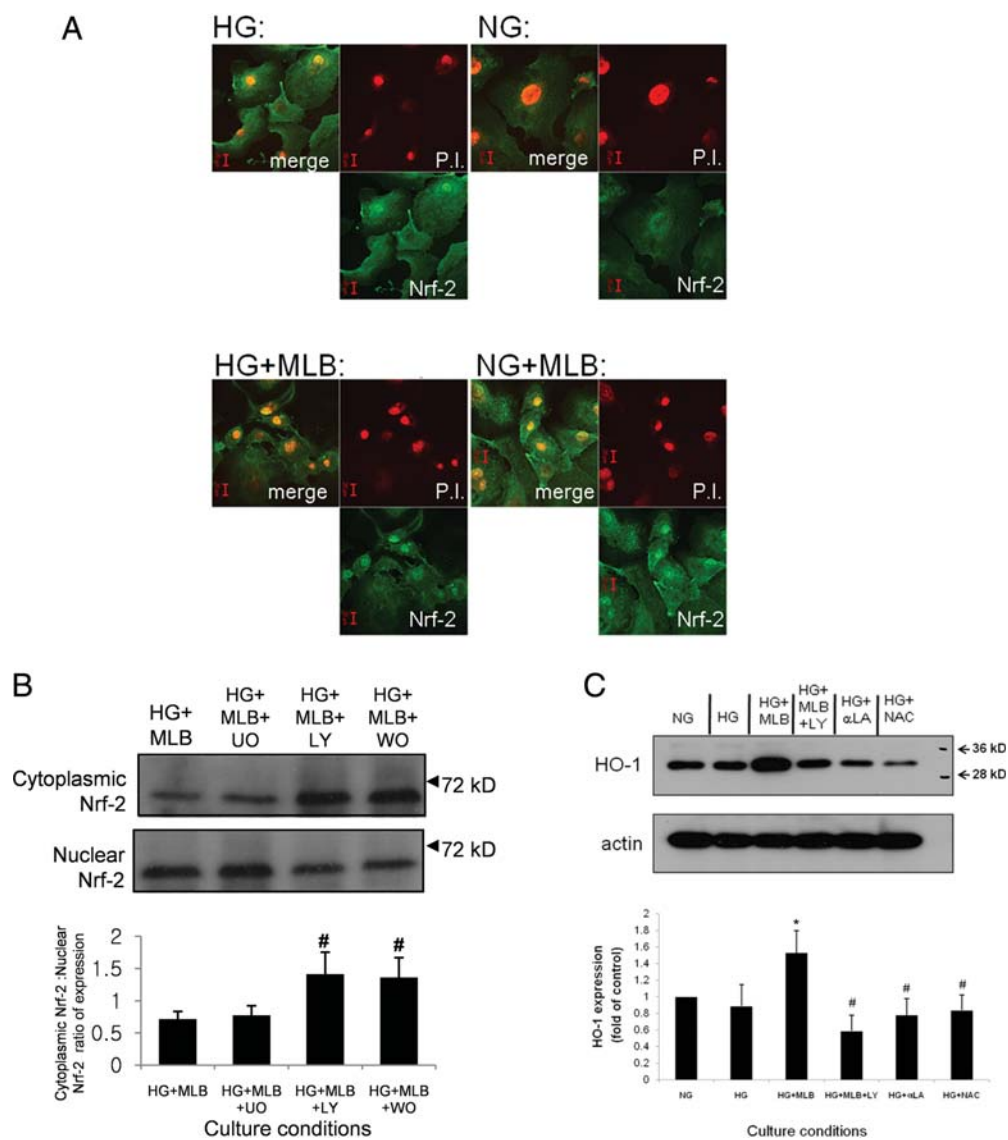
Confocal microscopic analysis of HAEC showed that MLB promoted Nrf-2 nuclear translocation (*Figure 4A*). This is a key step in the activation of the antioxidant response element (ARE) pathway. In addition, inhibition of the PI 3-K intracellular signalling pathway by LY-294,002 and wortmannin reduced the nuclear accumulation of Nrf-2 in MLB-treated HAEC exposed to hyperglycaemia (*Figure 4B*). As a negative control, the MAPK pathway inhibitor UO126 had no significant effect on Nrf-2 nuclear accumulation. MLB treatment could induce the expression of a key target of the ARE pathway, heme oxygenase-1 (HO-1) (*Figure 4C*). Treatment with other antioxidants ( $\alpha$ LA and N-acetyl-L-cysteine) could not produce this effect, confirming that activation of the ARE pathway is an important feature of MLB treatment.

### 3.5 MLB decreases AGE serum concentration, increases serum nitrite, and improves endothelium-dependent vasodilatory function in OLETF diabetic rats

Daily treatment with MLB for 8 weeks lowered serum AGE concentration in OLETF diabetic rats (*Figure 5A*). In addition, daily treatment

#### Figure 3 continued

Densitometric intensity ratios are means SD ( $n = 3$ ) (<sup>#</sup> $P < 0.05$  compared with NG; <sup>¶</sup> $P < 0.05$  compared with HG; <sup>\*</sup> $P < 0.05$  compared with HG + MLB). (B) MLB inhibited the increased O-GlcNAc glycosylation of eNOS in HAEC exposed to hyperglycaemia for 48 h ( $n = 3$ ) (<sup>#</sup> $P < 0.05$  compared with NG; <sup>¶</sup> $P < 0.05$  compared with HG). (C) MLB increased total NO production in HAEC cultured in normoglycaemia. MLB also inhibited the decreased NO production in HAEC exposed to hyperglycaemia for 48 h. Two NOS inhibitors, L-NAME and 7-NI, could inhibit the effect of MLB on NO production (error = SD; after day 1 of treatment  $P < 0.05$  for HG compared with NG, HG + 50  $\mu$ M MLB compared with HG, and HG + 500  $\mu$ M  $\alpha$ LA compared with HG). Passage 5 HAEC; data are representative of three independent experiments. (D) MLB increased phosphorylation of Akt in HAEC exposed to hyperglycaemia for 48 h ( $n = 3$ ) (<sup>#</sup> $P < 0.05$  compared with NG; <sup>¶</sup> $P < 0.05$  compared with HG). (E) The PI 3-K signalling pathway inhibitor LY-294,002 reduced the ability of MLB to increase NOS activity in HAEC exposed to hyperglycaemia. Treatment with the MAPK signalling pathway inhibitor UO126 had no significant effect on NOS activity. Data are representative of three independent experiments (error = SD; <sup>#</sup> $P < 0.05$  compared with NG; <sup>¶</sup> $P < 0.05$  compared with HG; <sup>\*</sup> $P < 0.05$  compared with HG + MLB; <sup>‡</sup> $P < 0.05$  compared with HG + MLB + LY). Abbreviations: NG, 5 mM glucose; HG, 30 mM glucose; MLB, 50  $\mu$ M magnesium lithospermate B; eNOS-(p) ser 1177, eNOS phosphorylated at residue serine 1177; O-GlcNAc, O-linked N-acetylglucosamine protein modification; L-NAME, 300  $\mu$ M G-nitro-L-arginine-methyl ester; 7-NI, 900 nM 7-nitroindazole; UO, 10  $\mu$ M UO126; LY, 10  $\mu$ M LY-294,002.

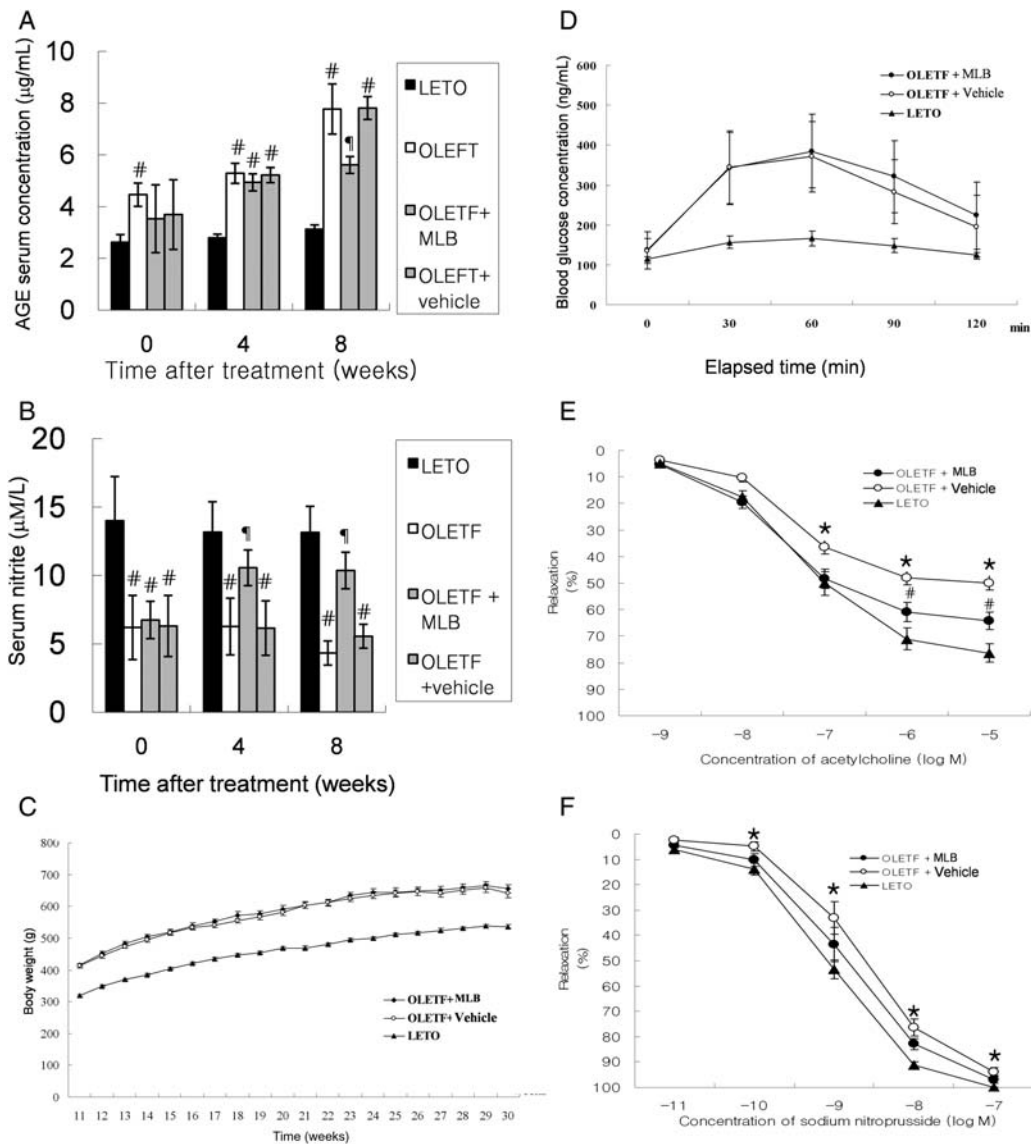


**Figure 4** (A) 48 h MLB treatment of HAEC cultured in normoglycaemia induced the nuclear translocation of Nrf-2. HAEC cultured in hyperglycaemia or HG + 50  $\mu$ M MLB also showed increased nuclear translocation of Nrf-2 (Bar = 20  $\mu$ m). (B) Nuclear translocation of Nrf-2 in HAEC treated with MLB for 48 h was inhibited by treatment with the PI 3-K signalling pathway inhibitors LY and WO. The MAPK signalling pathway inhibitor UO126 had no significant effect on nuclear translocation. Passage 5 HAEC. Data are representative of three independent immunoblots. Densitometric intensity ratios are means  $\pm$  SD ( $n = 3$ ; <sup>#</sup> $P < 0.05$  compared with HG + MLB). (C) Treatment with MLB can induce the expression of the antioxidant enzyme, HO-1. The antioxidants  $\alpha$ LA and *N*-acetyl-L-cysteine could not produce this effect ( $n = 3$ ; <sup>\*</sup> $P < 0.05$  compared with HG; <sup>#</sup> $P < 0.05$  compared with HG + MLB). Abbreviations: NG, 5 mM glucose; HG, 30 mM glucose; MLB, 50  $\mu$ M magnesium lithospermate B; UO, 10  $\mu$ M UO126; WO, 100 nM wortmannin; LY, 10  $\mu$ M LY-294,002;  $\alpha$ LA, 500  $\mu$ M alpha lipoic acid; NAC, 1 mM *N*-acetyl-L-cysteine; PI, propidium iodide nuclear marker.

with MLB increased serum nitrite levels (Figure 5B). MLB treatment had no significant effect on body weight (Figure 5C) or glucose intolerance in OLETF rats compared with vehicle-treated rats (Figure 5D). There was no difference in lipid profile or mean blood pressure in MLB-treated OLETF rats compared with vehicle-treated rats (see Supplementary material online, Table S1). MLB treatment significantly improved endothelium-dependent vasodilatory function in OLETF rats (Figure 5E). Endothelium-independent vasodilation was slightly reduced in OLETF rats, but the MLB-treated rats showed no significant difference compared with those treated with vehicle (Figure 5F).

## 4. Discussion

The research study presented herein aimed to investigate the effect of MLB treatment on hyperglycaemia-induced endothelial dysfunction *in vitro* and in an animal model of diabetes. We measured the effect of MLB on a number of consequences of hyperglycaemia-induced oxidative stress and compared its effects with the antioxidant,  $\alpha$ LA.  $\alpha$ LA was chosen for comparative study because: (i)  $\alpha$ LA has been studied extensively, (ii)  $\alpha$ LA exerts its effects in both aqueous and lipid environments, and (iii) it is an approved treatment for complications in diabetes.<sup>10</sup> The effect of MLB on eNOS activity was



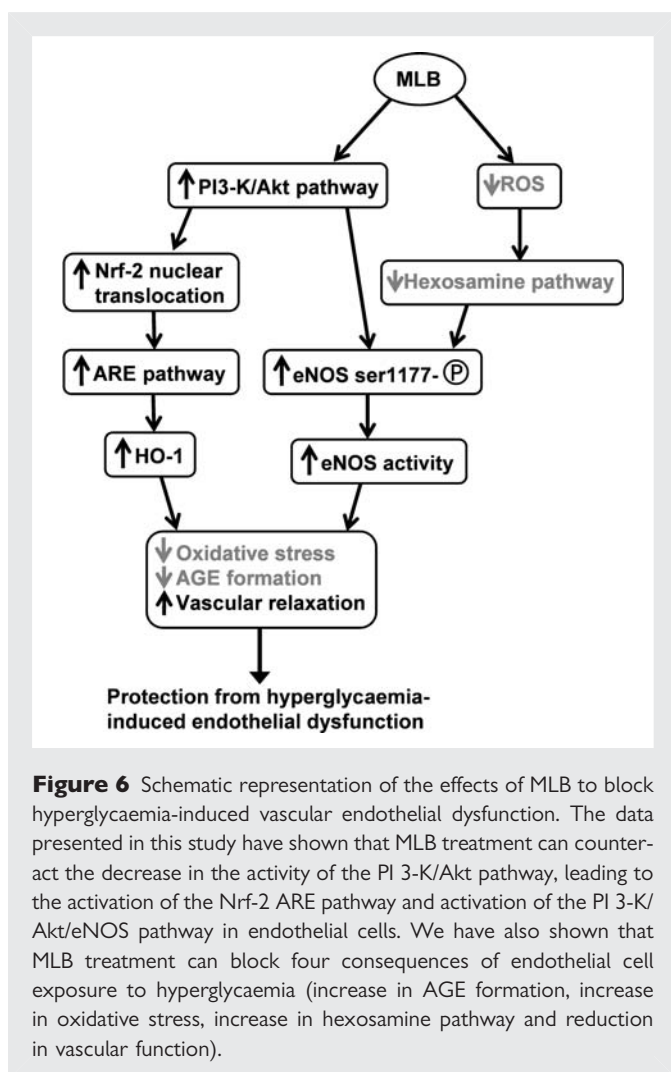
**Figure 5** (A) MLB treatment lowered serum AGE concentration in OLETf diabetic rats. Rats were administered MLB or vehicle (distilled water) daily from 12 weeks of age (day 0 of treatment). Serum AGE accumulation was significantly reduced after 8 weeks of treatment with MLB (error = SD; # $P < 0.05$  compared with LETO non-diabetic rats; \* $P < 0.05$  compared with OLETf treated with vehicle). (B) MLB treatment increased serum nitrite levels in OLETf diabetic rats. Rats were administered MLB or vehicle (distilled water) daily from 12 weeks of age (day 0 of treatment). Serum nitrite level was significantly increased at both 4 and 8 weeks of treatment with MLB (error = SD; # $P < 0.05$  compared with LETO non-diabetic rats; \* $P < 0.05$  compared with OLETf treated with vehicle). (C) Mean change in body weight over 17 weeks of treatment with MLB. Body weight was measured weekly. (D) Blood glucose concentration at 29 weeks; after 17 weeks of MLB treatment. (E) Effect of MLB treatment on endothelium-dependent vasodilatory function in OLETf diabetic rats, in response to acetylcholine after phenylephrine precontraction of aortic segments. (F) Effect of MLB treatment on endothelium-independent vasodilatory function in OLETf diabetic rats, in response to sodium nitroprusside in phenylephrine-precontracted vessels. Data are expressed as mean  $\pm$  SD ( $n = 8$  per group). \* $P < 0.05$  vs. LETO group; # $P < 0.05$  vs. vehicle-treated OLETf group.

tested because this enzyme is an important producer of NO; a pivotal inducer of vasodilation that has a large number of beneficial effects on endothelial integrity.<sup>11</sup> The OLETf type II diabetic rat was chosen as an animal model to study the effect of MLB treatment because the most prevalent form of diabetes mellitus is type II and these rats display hyperinsulinaemia, hyperglycaemia, insulin resistance, hypertriglycaemia, and mild obesity.<sup>12</sup>

This is the first study to show that MLB treatment has beneficial effects on endothelial cell dysfunction induced by hyperglycaemia. MLB performed as well as  $\alpha$ LA at a 10-fold lower dose. We

showed that MLB produced its beneficial effect by stimulating the PI 3-K signalling pathway, which phosphorylates Akt. Akt phosphorylates eNOS and increases the production of NO. MLB treatment of diabetic rats increased serum NO and decreased serum accumulation of AGE. These data are valuable because there are relatively little published data concerning serum NO and, especially, serum AGE, in diabetic animals treated with antioxidants. It should be noted that MLB also increased NOS activity in cells cultured in normoglycaemia, suggesting that constitutively expressed eNOS mediates the beneficial effect of MLB. Moreover, this study showed that MLB increased





eNOS phosphorylation at residue serine 1177. Previous reports suggest that serine 1177 phosphorylation alone is the major regulator of eNOS activity in hyperglycaemic conditions.<sup>24</sup> MLB-induced eNOS phosphorylation was associated with decreased O-GlcNAc protein modification of eNOS. Hyperglycaemia induces overproduction of superoxide by the mitochondria and results in hexosamine pathway activation. It has been shown that hyperglycaemia inhibited eNOS activity in cultured bovine aortic endothelial cells by activating the hexosamine pathway via mitochondrial overproduction of superoxide, which increases eNOS modification by O-GlcNAc and decreases eNOS serine phosphorylation in a reciprocal manner.<sup>24</sup> The reciprocal modification was reported to occur specifically at Ser1177, the Akt phosphorylation site responsible for the activation of eNOS.<sup>22</sup>

MLB was shown to activate the PI 3-K/Akt and ARE pathways in endothelial cells. This activity is similar to that reported for the antioxidant, tert-butylhydroquinone (tBHQ). A previous study reported a beneficial effect of tBHQ treatment in a model of diabetes.<sup>25</sup> However, in contrast to MLB, tBHQ treatment has also been linked to DNA damage and carcinogenesis. Moreover, MLB-treated rats had favourable blood pressure measurements, which would be consistent with an absence of endothelial cytotoxicity associated with long-term ingestion of MLB. MLB-treated OLETF rats also had improved endothelium-dependent vasodilation response to acetylcholine

compared with the placebo treated OLETF rats, suggesting a low probability of cytotoxicity and an intact endothelium. In addition, a previous study on the effect of MLB on the development of diabetic in OLETF rats<sup>17</sup> employed a dose of 20 mg/kg LAB for 28 weeks, starting at 10 weeks, and there were no toxicity observed in the animals. However, in-depth investigation of potential MLB-mediated endothelial toxicity in higher doses *in vivo* could be an area of future study.

There is a pressing need to develop a new generation of antioxidant based therapies for treating complications in diabetes.<sup>26</sup> Treatment with 'traditional' antioxidants, such as vitamin C and vitamin E, has recently been suggested to be detrimental by blocking exercise induced decreases in insulin resistance.<sup>27</sup> MLB represents a new generation of antioxidants that possess additional beneficial effects in addition to ROS scavenging activity. MLB can inhibit the enzyme aldose reductase, which is a key component of the polyol pathway.<sup>5</sup> In addition, this study has shown that MLB can also activate the PI 3-K signalling pathway and ARE pathway in endothelial cells, producing an increased expression of the antioxidant enzyme, HO-1. The antioxidants  $\alpha$ LA and NAC could not produce this effect. This is an important finding, because targeting the Nrf-2 defence pathway in cardiovascular disease is currently an important focus of research.<sup>28</sup> Moreover, this study has shown that MLB enhanced endothelium-dependent relaxation in diabetic rats to a similar degree as reported for  $\alpha$ LA.<sup>10</sup> These additional features make MLB an attractive candidate agent for future clinical trials in diabetic subjects. A schematic diagram showing the proposed mechanisms by which MLB can prevent endothelial dysfunction resulting from hyperglycaemia is shown in Figure 6.

Recent diabetes research has developed the concept of 'molecular memory', which is the long-term influence of early metabolic control on clinical outcomes such as diabetic complications. Specific long-lasting epigenetic changes induced by ROS and AGE formation (irreversible, non-enzymatic protein modifications that generate inflammatory cytokines and growth factors) are both implicated in the mechanism of molecular memory.<sup>21</sup> Therefore, MLB may become an ideal therapeutic for preventing or delaying the onset of diabetic complications.

Nevertheless, a number of questions remain unanswered about the biochemical action of MLB. The chemical structure of MLB comprises a repeating motif that makes modification of this molecule, such as biotinylation, difficult without abrogating activity (H.C.L., unpublished data). Moreover, microarray analysis of the effect of MLB in cells susceptible to hyperglycaemia-induced damage only confirmed the involvement of the ARE pathway, without giving clues about the actual molecular target(s) (H.C.L., unpublished data). Thus, further study is required to provide further clues about the molecular target(s) of MLB. Moreover, extracts from the plant *salvia miltiorrhizae* radix contain other active compounds in addition to MLB.<sup>29</sup> Combination therapy with these additional active compounds may further enhance the therapeutic effect of MLB. Thus, MLB possesses many attractive characteristics for future study.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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