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Role of AMP-activated protein kinase in α-lipoic-acid-induced vasodilatation in spontaneously hypertensive rats

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ABSTRACT

BACKGROUND AMP-activated protein kinase (AMPK) has recently emerged as an attractive and novel target for the regulation of vascular smooth muscle contraction. The present study investigated the vasodilatory effects of α-lipoic acid (α-LA) and the possible mechanism of its action on aortic rings from Wistar–Kyoto (WKY) rats and spontaneously hypertensive rats (SHR).

METHODS Aortae were removed from WKY and SHR, and contractile responses to acetylcholine and α-LA studied in organ chamber. Phosphorylated AMPK (pAMPK), phosphorylated Peutz-Jeghers syndrome kinase LKB1 (pLKB1) and calcium/calmodulin-dependent protein kinase (CaMKK) protein level were measured in SHR, WKY and aortic smooth muscle (A10) cells.

RESULTS α-LA (1-500 μmol/l) produced a concentration-dependent relaxation of precontracted aortic rings from eight- and 16-week-old SHR, but not in those from WKY rats. This vasodilatory effect of α-LA did not change after preincubation with N^G-nitro-L-arginine methyl ester (100 μmol/l), but significantly suppressed by an AMPK inhibitor, compound C (40 μmol/l). The expression of pAMPKα, pLKB1 and CaMKK were also significantly reduced in endothelium-denuded arteries from 16-week-old SHR compared with those from younger SHR or age-matched WKY rats. After incubation with α-LA (100 μmol/l), the expression of pAMPKα and pLKB1

was significantly increased in the endothelium-denuded aortas from 16-week-old SHR, the expression of CaMKK was more reduced in the endothelium-denuded aortas of eight-week-old SHR, but this was not observed in WKY rats. α-LA also activated AMPKα phosphorylation in A10 cells.

CONCLUSION The effects of α-LA on vascular relaxation in SHR result from the enhanced phosphorylation of LKB1-AMPK in aortic smooth muscle.

INTRODUCTION

AMP-activated protein kinase (AMPK) is a ubiquitous serine/threonine protein kinase activated by pathological stimuli, such as oxidative damage, by physiological stimuli, such as exercise and muscle contraction, and by hormones, including leptin and adiponectin.[1] AMPK is activated in response to reduced cellular energy charge (high AMP/ATP ratio) and is involved in regulating carbohydrate and fat metabolism.[1,2] AMPK exists as a heterotrimeric enzyme consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). Isoforms of each subunit exist (α1, α2, β1, β2, γ1, γ2, and γ3), with multiple combinations possible. AMP binds to the γ-subunit of AMPK and facilitates the phosphorylation of threonine 172 (Thr^{172}) of the α-subunit by an upstream kinase, AMPK kinase (AMPKK), increasing the enzyme activity of AMPK.[3] Recent data suggest that the tumor suppressor protein, Peutz-Jeghers syndrome kinase LKB1 (LKB1) functions as an AMPKK in several cell types and that calcium/calmodulin-dependent protein kinase 1 (CaMKK) also phosphorylates Thr^{172} and activates AMPK.[4-7]

A target of AMPK is endothelial nitric oxide synthase (eNOS), an important modulator of vascular tone. It has been clearly established that AMPK can associate with and phosphorylate eNOS in cardiomyocytes and endothelial cells,[8] thus increasing eNOS activity and NO production. The direct activation of AMPK with

5-aminoimidizole-4-carboxamide riboside (AICAR) stimulates NO synthesis in human aortic endothelial cells.[9] Furthermore, metabolically challenged endothelium-denuded porcine carotid artery segments exhibit a rapid increase in AMPK activity after metabolic stress, associated with the recruitment of signaling pathways that may regulate smooth muscle contraction.[10] However, AICAR failed to relax endothelin-1-precontracted carotid artery rings in this species.[10] These data suggest that AMPK plays a complex role in vascular function and remodeling.

α-Lipoic acid (α-LA) is a naturally occurring, essential cofactor for mitochondrial respiratory enzymes.[11] It has been used as a safe and potent antioxidant for the treatment of diabetic neuropathy. This potent free-radical scavenger has been considered for the treatment or prevention of conditions such as diabetes,[12] Alzheimer's disease,[13] and atherogenic dyslipidemia.[14] Because this versatile agent has recently been found to exert an antihypertensive effect in rodents,[15] we hypothesized that α-LA improves the vasoreactivity by the activation of AMPK in the hypertensive vasculature. Therefore, the goal of this study was to investigate the effects of α-LA on the involvement of AMPK phosphorylation and the vasoreactivity using aortas excised from normotensive Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR).

METHODS

Animals

Male SHR and WKY rats, from a stock that originated from the Charles River Breeding Laboratories (Tokyo, Japan), were purchased from the National Laboratory Animal Breeding and Research Center of the National Science Council, Taiwan. The animals were housed individually in clear plastic cages and kept in an environmentally controlled room maintained at room temperature (23 ± 1 °C), relative humidity of 55 ± 5%, and a 12 h–12 h light–dark cycle. The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 85-23, revised 1996). This study was approved by the National Defense Medical Center Institutional Animal Care and Use Committee, Taiwan.

Vascular ring preparation and organ bath experiments

Male WKY rats or SHR were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and their thoracic aortas were isolated and placed in Kreb's solution. The intact thoracic aortas were cleared of adhering periadventitial fat and cut into sections (3–4 mm long). The detail process was as described previously.[16] Concentration– response curves for α-LA were constructed by adding norepinephrine (NE, 1 μmol/l; Sigma, St Louis, MO, USA) to produce the maximum contraction, after which α-LA (1–500

μmol/l; Sigma) was added cumulatively and the relaxation recorded. To examine the roles of endothelium-derived NO and AMPK activation in the vascular response to α-LA, the segments were incubated for 30 min with 100 μmol/l N^G-nitro-L-arginine methyl ester (L-NAME; Sigma) or 40 μmol/l (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl])-3-pyridin-4-yl-pyyrazolo[1,5-a]-pyrimidine (Compound C; an AMPK inhibitor; Merck, Whitehouse Station, NJ, USA), respectively, before they were contracted with NE (1 μmol/l).

Cell culture

Rat aortic smooth muscle A10 cells (Bioresource Collection and Research Center) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies) at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were fed every 2–3 days and were subcultured when they reached 90%–100% confluence.

Cytotoxicity assay

The reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) was used to measure cell survival in a quantitative colorimetric assay.[17] This assay is based on the capacity of mitochondrial enzymes (succinate dehydrogenase) in the cells to reduce MTT, forming the insoluble

product formazan, as described.[18]

Western blot analysis

Aortas from WKY rats and SHR were isolated in Kreb's buffer and cleaned of any connective tissue. The endothelial layer was mechanically disrupted by gently rubbing the luminal surface of the artery. The endothelium-denuded aortic rings were then incubated with α-LA (100 μmol/l) for 30 min. After incubation, the endothelium-denuded aortic rings were rapidly frozen in liquid nitrogen and stored at –80 °C until processed. The endothelium-denuded aortas were ground in a mortar containing liquid nitrogen. The powdered tissue was suspended in 1 ml of lysis buffer containing protease inhibitors, as described.[16] In addition, the cellular proteins were extracted from the control and treated A10 cells. The washed cell pellets were resuspended in extraction lysis buffer, as described previously.[18]

Samples containing equal amounts of protein were electrophoresed in 10% sodium dodecyl sulphate-polyacrylamide gels and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membranes were incubated with antibodies against rabbit anti-phosphorylated AMPKα, rabbit anti-AMPKα, rabbit anti-phosphorylated LKB1 (all 1:1000 dilution; Cell Signaling Technology, MA, USA), rabbit anti-CaMKK (1:1000 dilution; BD Transduction Laboratories, Lexington, KY, USA), and mouse anti-β-actin (1:2000 dilution; Sigma-Aldrich). The

membranes were then incubated with horseradish-peroxidase-conjugated secondary antibodies (1:1000 dilution; Cell Signaling Technology). Immunodetection was performed using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). Protein quantities were measured by densitometric scanning of the blots using Image-Pro software (Media CyberMetrics, Inc., Phoenix, AZ, USA).

Statistical analysis

The results are presented as means ± s.e.m. Statistical evaluation was performed with two-factor analysis of variance followed by the Holm-Sidak test. A *P* value of less than 0.05 was deemed statistically significant.

Results

Vasorelaxation induced by ACh in SHR and WKY rats

The addition of ACh to all the aortic rings with intact endothelium resulted in the concentration-dependent relaxation of the rings that had been precontracted with NE (1 μmol/l). Figure 1 shows dose-response curves for ACh, and NO-dependent relaxation to ACh did not differ between aortic rings from four- and eight-week-old SHR and WKY rats. However, ACh-induced vasorelaxation was significantly reduced in both 16-week-old WKY rats and SHR, and the vasorelaxant response to ACh was significantly lower in SHR than in the age-matched WKY rats (Figure 1).

Vasorelaxation induced by α-LA in SHR and WKY rats

Figure 2A shows that the aortic rings from four-week-old SHR and four- and eight-week-old WKY rats were not significantly affected by α-LA (1–500 μmol/l). However, α-LA induced a dose-dependent relaxation of aortic rings from eight-, 16-week-old SHR and 16-week-old WKY rats. The relaxation was greater in rings from 16-week-old SHR compared with age-matched WKY rats and eight-week-old SHR. Significant differences were seen at 30-500 μmol/l α-LA. Meanwhile, no significant differences were seen in the responses of aortic rings from 16-week-old SHR in either the presence or absence of L-NAME (100 μmol/l) and similar pattern were also found from 16-week-old WKY rats (Figure 2B). The vehicle (0.1% ethanol)

had no significantly effect in the responses of aortic rings from either strain (data are not shown).

Effects of the AMPK inhibitor, compound C, on α-LA-induced vasorelaxation

To investigate the role of AMPK in α-LA-induced vasorelaxation, aortic rings obtained from 16-week-old SHR were treated with the AMPK inhibitor compound C. The results are shown in Figure 2C. After preincubation with compound C (40 μmol/l), α-LA-induced vasorelaxation was reduced in the aortic rings from 16-week-old SHR (α-LA plus compound C, 26.6 ± 1.5% versus α-LA, 44.6 ± 3.7%, $P < 0.05$). Vehicle (DMSO) had no significant effect on the vascular response to α-LA (DMSO, 46.8 ± 2.1% versus α-LA, 44.6 ± 3.7%).

Effects of α-LA on the phosphorylation of AMPK in aortic rings

To demonstrate the physiological relevance of the findings described above, the effect of α-LA on AMPK phosphorylation in isolated rat aortic rings was investigated. The expression of pAMPKα, Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK), and pLKB1 were not significantly different in the isolated aortic rings obtained from four-week-old and eight-week-old WKY rats, but were significantly reduced in 16-week-old WKY rats (Figures 3A, 3C, 3E, 3G). The expression of pAMPKα, CaMKK, and pLKB1 were not altered by preincubation of the aortas with α-LA (100 μmol/l; Figures 3A, 3C, 3E, 3G). In contrast, the expression of pAMPKα and pLKB1

were significantly reduced in the isolated aortic rings obtained from eight- and 16-week-old SHR compared with their expression in four-week-old SHR (Figures 3B, 3D, 3H). AMPKα and LKB1 were clearly phosphorylated after the aortas of 16-week-old SHR were preincubated with α-LA (Figures 3B, 3D, 3H). In addition, the expression of CaMKK were not significantly different in the isolated aortic rings obtained from four-week-old and eight-week-old SHR rats, but was significantly reduced in the isolated aortic rings obtained from 16-week-old SHR. The expression of CaMKK was significantly reduced after the aortas of eight-week-old SHR were preincubated with α-LA (Figure 3F).

Effects of α-LA on cultured cell viability

To exclude any possible artefactual interference, the effects of α-LA (5–100 μmol/l) on cell viability were tested in A10 cells. Cell survival was not affected by exposure to α-LA (5–100 μmol/l) or vehicle (ethanol, < 0.01%) for 24 h (Figure 4A).

Activation of AMPKα by α-LA in A10 cells

To determine whether α-LA induces the activation of AMPKα, A10 cells were treated with various concentrations of α-LA (5–100 μmol/l) for 24 h. The expression of phosphorylated AMPKα protein in the cell lysate was detected by western blotting. As shown in Figure 4B, treatment with α-LA (10–50 μmol/l) significantly increased the phosphorylation of AMPKα, and pAMPKα expression reached maximum levels

206 when the cells were treated with 10 μmol/l α-LA, progressively decreasing at higher
207 α-LA concentrations (30–50 μmol/l). Treatment of the cells with α-LA (10 μmol/l)
208 resulted in the time-dependent activation of AMPK, beginning after incubation for 3 h
209 and reaching a maximum after 6 h (Figure 4C). Ethanol (< 0.01%; the vehicle for
210 α-LA) alone had no significant effect on the phosphorylation of AMPKα in A10 cells
211 (Figure 4B).

212 **CaMKK-mediated AMPKα activation with α-LA stimulation**

213 To determine whether α-LA activates LKB1 and/or CaMKK in A10 cells, the
214 cells were exposed to α-LA (10 μmol/l) for 6 h, and LKB1 and CaMKK protein
215 expression was analyzed by western blotting. There was no significant difference in
216 LKB1 protein expression between the α-LA-treated and control cells (Figure 4D).
217 However, A10 cells exposed to α-LA exhibited a relative increase (2.5-fold) in
218 CaMKK protein expression compared with that in the control cells (Figure 4E).
219 Ethanol (< 0.01%; the vehicle for α-LA) alone had no significant effect on the
220 expression of LKB1 or CaMKK in A10 cells. The inhibition of CaMKK by STO-609
221 (10 μmol/l)[19] abolished the phosphorylation of AMPKα induced by α-LA (Figure 4F).

DISCUSSION

This study was to investigate that the vasodilatory effects of α-LA and the possible mechanism of its action on aortic rings from SHR and WKY rats of different ages. Our results show that α-LA produced a concentration-dependent vasodilatory effect on the aortic rings from SHR. This concentration-dependent effect was greater than that observed in age-matched WKY rats. Furthermore, the vasodilatory effect of α-LA was almost identical in endothelium-intact and L-NAME-preincubated arteries from SHR and WKY rats. These results suggest that α-LA induced vasorelaxation is not associated with NO pathway in either WKY rats or SHR. Instead, α-LA may act directly on the vascular smooth muscle. In addition, both of LKB1 and AMPK phosphorylation were also lower in the endothelium-denuded aortas of 16-week-old SHR compared with that in younger SHR or in age-matched WKY rats. Preincubation with α-LA markedly enhanced the LKB1 and AMPK phosphorylation in the endothelium-denuded aortas of 16-week-old SHR. The effects of α-LA on vascular reactivity were markedly reduced by the AMPK inhibitor, compound C. These results suggest that the effects of α-LA on vascular relaxation in SHR result from the enhanced phosphorylation of LKB1-AMPK in the aortic smooth muscle.

Endothelial dysfunction, generally considered a reduction in endothelium-mediated vascular relaxation, has been reported in hypertensive and diabetic

241 arteries.[20,21] Similar to those reports, the NO-dependent relaxation in response to ACh
242 did n ot differ between the aortic rings from four- and eight-week-old rats of either
243 strain in the present study (Figure 1). However, there was much less ACh-induced
244 relaxation of the aortas from 16-week-old SHR than of those from younger SHR or
245 age-matched WKY rats (Figure 1). α-LA dose-dependently induced vasorelaxation in
246 the preconstricted aortas from eight- and 16-week-old SHR, but not in those from
247 WKY rats (Figures 2A). Preincubation of the aortas with L-NAME, an inhibitor of
248 endothelial NOS, failed to block α-LA-induced vasorelaxation (Figure 2B),
249 suggesting that endothelial NOS is not involved in the vasorelaxation effects of α-LA.
250 Although AMPK is traditionally considered a major regulator of cellular
251 anabolic and catabolic pathways that conserve and synthesize ATP, several studies
252 have suggested that endothelial AMPK also has important physiological functions,
253 such as the modulation of the endothelial cell energy supply,[22] the protection of cells
254 from apoptosis,[23] the mediation of endothelial NOS activation in response to shear
255 stress,[24] the regulation of inflammation and angiogenesis, and the maintenance of
256 perfusion.[25,26] A recent study has also shown that the treatment of aortic smooth
257 muscle cells with AICAR leads to the activation of AMPK.[27] In the present study, the
258 expression of phosphorylated LKB1 and AMPKα was much lower in the
259 endothelium-denuded aortas from 16-week-old rats of both strains compared with that

260 in four- and eight-week-old rats and suggested that this could be causally related to
261 the vascular dysfunction in hypertensive rats (Figure 3B, 3D, 3F). After incubation
262 with α-LA, the expression of pLKB1 and pAMPKα were significantly increased in
263 the endothelium-denuded aortas from 16-week-old SHR, but this phenomenon was
264 not observed in WKY rats (Figure 3A, 3C, 3E). Moreover, the α-LA-induced
265 relaxation in the aortas from 16-week-old SHR was also significantly suppressed by
266 preincubation with an AMPK inhibitor (Figure 2C). These data confirm the report by
267 Goirand et al.[28] and suggest that the activation of smooth-muscle LKB1-AMPK
268 induces the vasorelaxation of the aorta and independently of NO.

269 In additional to LKB1, in certain cell types CaMKK has also been shown to
270 phosphorylate and activate AMPK in response to increase in intracellular Ca^{2+}.[19,29,30]
271 Previous studies had shown that the activation of AMPK by thrombin or α-LA in
272 endothelial cells and C2C12 myotubes, respectively, was mediated by increasing of
273 the intracellular Ca^{2+} concentration, which were abolished by STO-609 or siRNAs
274 targeting CaMKK.[31,32] However, in this study, the expression of CaMKK was
275 significantly reduced in isolated aortas of eight-week-old SHR after preincubated with
276 α-LA (Figures 3F). This may be explained by the fact that the antihypertensive effect
277 of α-LA is produced by increasing free sulfhydryl groups of calcium channels, leading
278 to a decrease in cytosolic free calcium.[15,33,34] Thus, this study suggested that the

279 elevation of AMPK phosphorylation may mainly contributed to the relaxation of
280 α-LA in aortas from 8- and 16-week-old rats of SHR. Although we provide evidence
281 that α-LA mediated the AMPK signaling in cultured aortic smooth muscle cells (Fig 4)
282 and in aortic tissue of SHR (Fig 3). Some limitations to our study need to be
283 addressed; for example, α-LA activates AMPK via CaMKK in normal cultured aortic
284 smooth muscle cells (A10 cells) but via LKB1 in the endothelium-denuded aortas of
285 SHR. These divergent results may simply be due to the intrinsic differences in the two
286 experimental model systems (ie, cultured cells represent a normal state versus aortas
287 tissue represent a hypertensive state). Further study need to clarify the upstream
288 kinase involve in the regulation of AMPK phosphorylation in primary culture of
289 aortae both from SHR and WKY rats.

290 It is noteworthy that incubation with α-LA produced significant vasorelaxation
291 in the aortic rings of SHR, which was not observed in those of WKY rats. These
292 differences in the vascular activity of α-LA in SHR and WKY rats may be
293 attributable to the structural and functional modifications present in the hypertensive
294 state, such as the changes in superoxide levels,[35] and reduced pAMPKα expression
295 (Figure 3B, 3F). α-LA is well known as an antioxidant, and it directly scavenges
296 free radicals. This suggests that the observed effects of α-LA on the vascular
297 responses in isolated SHR rat aortic rings could be attributable to the inhibition of

298 superoxide anions and/or their reactive oxygen metabolites. In SHR, the action of
299 α-LA on AMPK phosphorylation may also contribute to the vasorelaxation induced
300 by α-LA. Further studies are required to clarify the mechanism of α-LA on the
301 AMPK activation in hypertensive rats.

302 In conclusion, our results suggest that reduced pLKB1 and pAMPKα expression
303 in the endothelium-denuded aorta plays an important role in the genesis of vascular
304 dysfunction in hypertensive rats, and that α-LA ameliorates vascular dysfunction in
305 SHR by activating LKB1-AMPK in aortic smooth muscle. This study provides a
306 rationale for the therapeutic use of α-LA for vascular dysfunction in hypertension.

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Figure legend

Figure 1 Relaxation responses to acetylcholine (ACh) in aortic rings from WKY rats and SHR. Symbols represent means ± s.e.m. (n = 6). *P < 0.05 vs. same strain four-week-old rats. $^{\#}P$ < 0.05 vs. age-matched WKY rats. SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats.

Figure 2 Relaxation responses to α-LA in aortic rings precontracted with norepinephrine from (A) four-, eight- and 16-week-old WKY rats and SHR. Symbols represent means ± s.e.m. (n = 6). *P < 0.05 vs. same strain four-week-old rats. $^{\#}P$ < 0.05 vs. age-matched WKY rats. (B) Effects of L-NAME (100 μmol/l) on the relaxation induced by α-LA (100 μmol/l) in aortic rings from 16-week-old SHR and WKY rats. Data are means ± s.e.m. (n = 6 in each group). *P < 0.05 vs. age-matched WKY. $^{\#}P$ < 0.05 vs. treated with L-NAME. (C) Effects of the AMPK inhibitor, compound C (40 μmol/l), on the relaxation induced by α-LA (100 μmol/l) in aortic rings from 16-week-old SHR. Data are means ± s.e.m. (n = 6 in each group). *P < 0.05 vs. α-LA alone. SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats; AMPK, AMP-activated protein kinase.

Figure 3 Effects of α-LA on AMPK phosphorylation and protein level, CaMKK

expression, and LKB1 phosphorylation in endothelium-denuded aortic tissues from WKY rats and SHR. In (A, B), representative western blot data are shown. (C, D), (E, F) and (G, H) are bar graphs for LKB1 phosphorylation, CaMKK expression and AMPK phosphorylation levels, respectively. Data are means ± s.e.m. (n = 6 in each group). *$P < 0.05$ vs. same treatment four-week-old rats. $^{\#}P < 0.05$ α-LA-treated vs. age-matched-untreated rats. SHR, spontaneously hypertensive rats; WKY, Wistar–Kyoto rats; AMPK, AMP-activated protein kinase; CaMKK, calcium/calmodulin-dependent protein kinase.

Figure 4 (A) Effects of α-LA on cells viability. (B) The effect of α-LA (5–100 μmol/l) on AMPKα phosphorylation in A10 cells. (C) The effect of α-LA (10 μmol/l) on the time course of changes in the phosphorylation of AMPKα in A10 cells. (D, E) The effect of α-LA (10 μmol/l) on the expression of pLKB1 and CaMKK in A10 cells, respectively. (F) The modulation of α-LA (10 μmol/l)-induced AMPKα phosphorylation by STO-609 (10 μmol/l), a CaMKK inhibitor, in A10 cells. Data shown are means ± s.e.m. (n = 6). *$P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. α-LA. AMPK, AMP-activated protein kinase; CaMKK, calcium/calmodulin-dependent protein kinase.