ORIGINAL ARTICLE

Olmesartan Alleviates Diabetic-induced Cardiac Apoptosis Partially Through the Reduction of Endoplasmic Reticulum Stress

Flori R. Sari[1,2], Vivian Soetikno[2], Wawaimuli Arozal[2]

[1] Department of Pharmacology, Faculty of Medicine Universitas Islam Negeri Syarif Hidayatullah, Jalan Kertamukti No. 5, Banten, Indonesia, 15412
[2] Department of Pharmacology and Therapeutic, Faculty of Medicine, University of Indonesia, Jalan Salemba Raya No. 6, Jakarta, Indonesia, 10430

ABSTRACT

Introduction: Prolonged hyperglycaemia leads to several disadvantageous effects including hyperglycaemia-induced cardiac apoptosis in the diabetic cardiomyopathy. Previously, we have reported that olmesartan gave benefits in diabetic nephropathy by attenuating endoplasmic reticulum (ER) stress. However, the roles that olmesartan play in diabetic cardiomyopathy are not confirmed yet. **Methods:** The role of 6 weeks olmesartan medoxomil 10 mg/kg BW in the streptozotocin-induced C57/BL-6 mice were evaluated by measuring blood glucose level and body weight. Protein expression of glucose-regulated protein (GRP)-78 –an ER stress sensor-, cleaved caspase12 and C-EBP homologous protein (CHOP) –ER stress apoptosis initiating factors- were analysed using western blot. Cardiac apoptosis was measured by TUNEL staining. **Results:** GRP-78, caspase12 and CHOP protein expressions were significantly increased in the diabetic mice. Daily decoction of olmesartan for 6 weeks significantly reduced not only the ER stress chaperone GRP-78 but also the ER stress key initiating factors for apoptosis, caspase12 and CHOP. Constantly, diabetic mice receiving olmesartan suffered from lesser levels of cardiac apoptosis than diabetic mice receiving vehicle treatment. **Conclusion:** Olmesartan support a beneficial function in the diabetic cardiomyopathy not only by its original properties as an ARB but also as an ER stress inhibitor partly by inhibiting the initiation of pro-apoptotic pathway of GRP78/CHOP/caspase12.

Keywords: Olmesartan, Apoptosis, Diabetic cardiomyopathies, Endoplasmic reticulum, CHOP

Corresponding Author:
Flori R. Sari, PhD
Email: florirsari@uinjkt.ac.id
Tel: +62 21 740 2116

INTRODUCTION

Diabetes mellitus elicited by either absolute insulin reduction or insulin resistance will result in the appearance of hyperglycaemia. It is estimated, 422 million people lived with diabetes mellitus worldwide in 2014 and the prevalence will double to around 800 million in 2022 (1). Additionally, at around 16.9% diabetic patients had cardiac complications of diabetic cardiomyopathy and the cumulative possibility of death was 18% (2). Thus, diabetes management should also focus on the prevention of diabetic cardiomyopathy. Prolonged and persistent hyperglycaemia will activate several detrimental processes including inflammation, cell dysfunction or cell injury by amplifying advanced glycation end (AGE) products and by stimulating stress which plays important roles in the further diabetic complication (3,4). Thus, giving an AGE-blocker drug including aminoguanidine may prevent the progression of diabetic cardiomyopathy in the diabetic rat (5). Additionally, great numbers of evidence have shown that hyperglycaemia directly activates multiple apoptotic pathways both externally and internally (6,7). Hyperglycaemia-induced massive cardiac cell death subsequently contributes to reduced contractility and decreased function of the heart as pump (4). In the external pathway, chronic hyperglycaemia activates cell death ligand in the death receptor pathway through the tumour necrosis factor (TNF)-α and Fas ligand and further invoke the apoptotic cascade by executing caspase-3, 6 and 7. In the internal pathway, high glucose stimuli will activate pro-apoptotic molecules of beta cell lymphoma (Bcl)-2 which further activates several executioner downstream caspases including caspase-9 and 3 (6). Recently, chronic hyperglycaemia has been reported also to elicit ER stress in the early event of diabetic cardiomyopathy by inhibiting calcium entry capacity and reducing protein folding ability (8,9). We have reported that hyperglycaemia activates ER

stress molecules from the upstream chaperone to the downstream key-initiating factor in the diabetic kidney. Decoction of olmesartan, an angiotensin receptor blocker (ARB), improved diabetic kidney by inhibiting Ang-II-stimulated ER stress-related apoptosis (10). Therefore, we continue the work to further validate ARB role in ER stress-induced diabetic cardiomyopathy.

MATERIALS AND METHODS

Diabetes animal model

To create diabetic animal model, 150 mg per kilogram body weight (BW) streptozotocin (STZ, Sigma-Aldrich Inc. USA) were intraperitoneally injected to male pathogen-free 8–10-week-old C57BL6/JAX mice (Charles River Inc. Japan) as described previously (11). Five days after STZ injection, non-fasting, random, blood glucose (BG) levels were measured using BG strips (Terumo Inc. Japan). Diabetic was defined as BG levels higher than 300 mg/dl. Minimum number of mice in each group were estimated with Federer method. Twelve diabetic mice were randomly divided into positive controls of diabetic mice (D group, n=6) and olmesartan treatment group (O group, n=6). Age matched normal mice (N group, n=5), as non-diabetic negative controls, were injected with a buffer. During the treatment protocol, body weight (BW) and BG level were measured every week. Normal (N), diabetic (D) and olmesartan (O) groups were sacrificed 6 weeks after olmesartan treatment. The mice were examined according to the animal experimentation ethical guidelines of Niigata University of Pharmacy and Applied Life Sciences (NUPALS) as stated in our previously published work (10). All mice examined were treated with care, fed with chow and drink with free access water throughout the study period.

Olmesartan treatment

Olmesartan medoxomil was received from Daiichi-Sankyo Pharmaceutical and was dissolved according to the company protocol (10). Olmesartan with the dose of 10 mg/kg BW was given to the O group for 6 weeks meanwhile the N and the D group as negative and positive control, respectively, were given vehicles. Six weeks after the treatment, all mice were sacrificed by using a single intraperitoneal of pentobarbital injection (50 mg/kg BW).

Heart to body weight ratio (HW/BW)

At the sacrifice time, mice hearts were cut from the body. All hearts were weighed, and each mouse HW/BW ratio was determined. Mice hearts are stored in 10% formalin for paraffin sections and in liquid nitrogen for protein analysis.

Cardiac apoptosis by TUNEL assay

Detection of cell death in situ was done by the TUNEL assay kit (Takara Bio Inc. Japan) as described previously (11,12). In brief, paraffin sections were deparaffinized and processed through the enzymatic process as well as staining in accordance with the company protocol. Positive apoptotic cells were defined as cells that were stained with brown color. Cardiac apoptosis was measured by counting positive-stained cells in fifty fields taken consecutively at the 200x magnification from each slide. The results were defined as the percentages of total number of cell deaths to the total fields as described previously (11). All digital images were taken and analysed by DP2-BSW Olympus Software and BX-41 microscope (Olympus Corp. Japan).

ER stress markers protein expression by western blot

Frozen heart tissues were weighed and homogenized as a protein lysate according to the protocol described previously (11). In brief, 40 µg of protein from each sample was applied and then detached by gel SDS-PAGE. After protein separation, protein was transferred electrically to the membrane of nitrocellulose. Primary antibodies applied were anti-GRP78 (goat polyclonal), anti-CHOP (mouse polyclonal), and anti-GAPDH (goat polyclonal) (Santa Cruz Biotechnology Inc. USA) and anti-caspase-12 rabbit polyclonal (BioVision Inc. USA). All primary and secondary antibodies were diluted. The average band expression from negative control was applied as one arbitrary unit (AU). Band densities were analysed quantitatively by Scion image software for densitometric analysis.

Statistical analysis

Data was processed digitally by Graph Pad software. In this quantitative experimental study, we used blood glucose level, apoptotic cells and densitometric analysis result of ER stress markers as parameters to detect the efficacy of olmesartan treatment. Thus, to detect differences of means in all parameters of all three groups, we analyse using Analysis of Variance (ANOVA). Significant differences were defined as probability values fewer than 0.05 ($p<0.05$). Numerical data were shown as means, percentages or standard of deviation (SD).

RESULTS

Diabetes animal model

BG levels of all mice before the protocol were normal, below 200 mg/dL. Five days after the STZ injection, BG level increased significantly above 300 mg/dL and was considered as diabetes (Table I).

Diabetic mice in the D group suffered from persistent significant hyperglycaemia ($p<0.01$) with BG changes reaching 148.99% ($p<0.05$) matched to 101.66% from week 1 to week 6 in the normal (N) group. Diabetic mice receiving olmesartan treatment in the O group also suffered significant hyperglycaemia ($p<0.01$) with BG changes reaching 135.20% ($p<0.05$) compared to 101.66% from week 1 to week 6 in the N group. Nevertheless, BG differences in the diabetic (D) and olmesartan (O) group (BG changes 148.99% vs.

Table I: Blood glucose (BG) changes during the protocol among groups

Group	BG before protocol	BG during the protocol (in g)			BG changes (%)
		Week 1	Week 3	Week 6	
Normal (N)	150.75±45.78	153.75±27.77	142.25±22.43	148.00±12.68	101.66±23.47
Diabetes (D)	129.17±17.35	411.67±58.57**	602.50±4.18**	601.67±2.88**	148.99±24.02*
Olmesartan (O)	137.25±9.43	415.75±54.01**	536.75±74.12**	569.00±62.00**	135.20±4.12*

BG changes were obtained from the BG at week 6 compared to BG at week 1 in percentage.
*p<0.05 and **p<0.01 compared to the normal (N) group.

Table II: BW changes and HW/BW ratio during the protocol among groups

Group	BW before protocol	BW during the protocol (in g)			BW changes (%)	HW/BW ratio
		Week 1	Week 3	Week 6		
Normal (N)	24.83±1.50	26.40±1.42	27.30±1.33	29.35±1.35##	111.21±1.08	4.45±0.34
Diabetes (D)	22.28±3.11	20.82±2.75*	21.17±2.70**	21.53±2.17**	103.91±6.26*	3.98±0,22
Olmesartan (O)	25.63±0.28	23.60±1.93*	22.48±2.33**	22.48±2.92**#	95.18±2.72*	3.68±0.17

BW changes were obtained from the BW in week 6 compared to BW in week 1 in percentage.
*p<0.05 and **p<0.01 compared to the normal (N) group.
#p<0.05 and ##p<0.01 compared to the BW before the protocol

135.20%) were not significant for the 6 weeks protocol. Therefore, in the diabetic condition, olmesartan treatment did not directly alter BG level. Additionally, BW loss was significantly observed in the diabetic mice group receiving olmesartan (95.18%) compared to the N (111.21%) and the D (103.91%) group (Table II).

Increment of BW was observed in the D group; however, the increment was not as high as the N group (103.91% vs. 111.21%). Furthermore, HW/BW ratios of diabetic mice receiving either olmesartan or vehicle were smaller than those in the N group. No significant differences were detected compared to the N group. Conclusively, the animal model of diabetic mice was confirmed through the significant increase of BG level and significant reduction of BW or delayed BW increment. Olmesartan did not prevent BW loss in the diabetic condition.

Cardiac apoptosis

Hyperglycaemia, dysfunctions of endothelial and mitochondrial tissue and cell death were known as the hallmark of diabetic cardiomyopathy (6). As shown from the TUNEL assay of cardiac histopathologic slides in Figure 1A and 1B, significant increment of cardiac apoptotic cells (brown stained cells) was observed in the D group matched to the N group with the significance of p<0.01. Further quantitative analysis had shown that apoptotic cardiac cells in the D group were five times higher than in the N group. Significant increment of cardiac apoptotic cells was detected also around twofold in the O group when compared to the N group (Figure 1C). Significant difference of cardiac apoptotic cells was observed between the D and the O group (Figure 1D). These results have conclusively shown that daily decoction of olmesartan for 6 weeks may play a beneficial role in reducing cardiac apoptotic cells in diabetes.

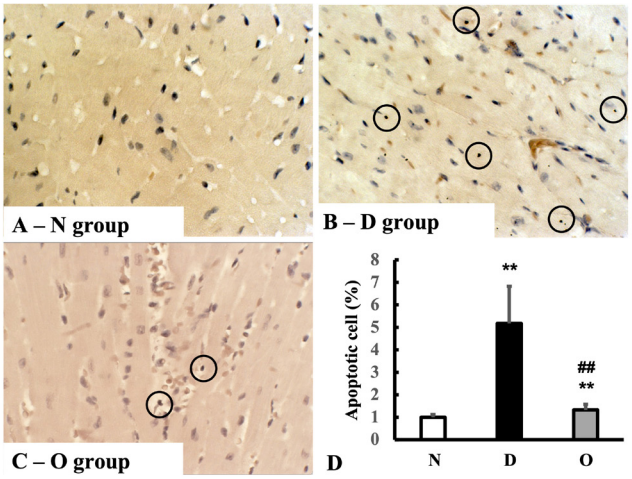

Figure 1: Apoptotic cardiac cells stained in brown (encircled in round shape) by the TUNEL assay. (A) Slides from the N group; (B) Slides from the D group showing 5 apoptotic cells; (C) Slides from the O group showing 2 apoptotic cells; (D) TUNEL assay analysis. All photos were taken in 200x magnification. N = Normal group; D = Diabetes group with vehicle treatment; O = Diabetes group with olmesartan treatment. **p<0.01 matched to the N group; ##p<0.01 matched to the D group

ER stress-associated protein expression

Persistent chronic hyperglycaemia is reported to be the culprit to activate both extrinsic and intrinsic pathways of apoptosis in diabetic cardiomyopathy (4,6,7). The intrinsic pathway of apoptosis was dominated by the mitochondrial axis or ER-related apoptosis. As depicted in Figure 2A, GRP78 protein bands were clearer in the diabetic rats compared to the normal ones. Quantitative analysis further confirmed the significant protein appearance of GRP78 almost twice higher in the D group than in the N group (p<0.01). These results indicate that ER stress chaperones were activated in the diabetic heart. We further investigated the initiating factor of ER stress-related apoptosis, CHOP. In the D (D1

and D2) group, CHOP bands predominantly appeared compared to the N (N1) group. The quantitative protein analysis showed that CHOP protein expression significantly increased compared to the N group (Figure 2B). These results enhanced the previous result that activation of ER stress chaperone would initiate further the apoptotic pathway in diabetes mellitus. To confirm the activation of ER stress-related apoptotic pathways, we investigate caspase12, a pro-death caspase which is specifically related to ER stress or disturbances of calcium homeostasis. Significant upregulation of cleaved-caspase12 were detected in the D (D3) group matched to the N group (Figure 2C). Furthermore, myocardial GRP78, cleaved-caspase12 and CHOP were less observed in the olmesartan group than in their counterparts' group with significance reaching $p<0.01$. Therefore, daily decoction of olmesartan 10 mg/kg BW for 6 weeks significantly alleviates ER stress from the upstream chaperone to the downstream key initiating factor in diabetic cardiomyopathy.

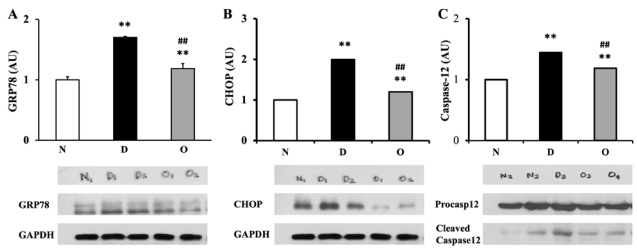

Figure 2: Myocardial expression of ER stress chaperone. (A) Protein expression of ER stress marker GRP78 compared to GAPDH; (B) Protein expression of CHOP compared to GAPDH; (C) Protein expression of cleaved caspase-12 compared to procaspase-12. All densitometric analysis was done with Scion Image. N=N1=N2=normal group; D=D1=D2=D3=Diabetes group with vehicle treatment; O=O1=O2=O3=O4=Diabetes group with olmesartan treatment. **p<0.01 vs. the N group and ##p<0.01 vs. the D group.

DISCUSSION

Significant findings of this study are: (1) Significant increase of myocardial apoptosis as well as significant expression of ER stress chaperone molecule were found in accordance with prolonged hyperglycaemia in diabetic mice. These results support the evidence that apoptosis related to ER stress were activated in the diabetic cardiomyopathy; (2) Daily decoction of olmesartan 150 mg/kg BW for 6 weeks did not alter directly blood glucose level nor body weight reduction in the diabetic mice (3) Daily decoction of olmesartan 150 mg/kg BW for 6 weeks significantly decreased not only the cardiac apoptosis but also the expression of ER stress chaperone from the upstream to the downstream apoptotic markers. Our results have demonstrated that olmesartan may prevent from diabetic cardiomyopathy at least in part by direct elimination of ER stress-related cardiac apoptosis but not by the glycemic control.

Prolonged hyperglycaemia has been reported to be associated with several detrimental effects on the cells including increased inflammation, increased oxidative cell and increased cell death in the diabetes mellitus and its complications (3,4). Recently, chronic hyperglycaemia has been reported also to hinder calcium entry capacity and to disturb the protein folding process which elicit the unfolded protein response (UPR). Accumulated unfolded protein will further activate the ER stress sensor molecules and trigger survival response at early events of diabetic cardiomyopathy (8,9). Thus, increased ER stress will activate several survival responses through the GRP-78 and Ire1α pathway, however, if the responses are failed, the process will continue to the initiation of ER stress apoptosis initiating factors, CHOP and caspase12 (4,6). We have demonstrated that prolonged hyperglycaemia in our 6 weeks diabetic animal elicited ER stress by expressing up-stream molecules of GRP-78. However, the survival process may have failed since cardiac apoptotic cells were found to be increased significantly along with the significant expression of downstream key initiating factor of CHOP and caspase12. CHOP and caspase12 were reported to be the specific molecule related to ER stress-associated apoptotic pathway. Conclusively, prolonged hyperglycemia in our 6 weeks diabetic model elicited ER stress, however the survival responses were failed so that cell death was activated through the GRP-78/CHOP/caspase12 pathway. Thus, inhibiting the GRP-78/CHOP/caspase12 pathway can be considered as the main strategy of preventing diabetic cardiomyopathy. To validate this hypothesis, we tried to inhibit ER stress by using an agent that may have properties as an ER stress inhibitor. Previously, we have reported the beneficial effect of olmesartan, an ARB, in the renal apoptosis by inhibiting CHOP-JNK-Caspase-12 pathway so that we used olmesartan in this study. Additionally, Ang-II has been reported to modulate the ER stress chaperone and activate the pro-apoptotic pathway in mice (13). Our study has validated that ER stress up-stream molecules GRP78, and key initiating factor of CHOP were significantly reduced along with reduced cardiac cell death in the diabetic mice receiving olmesartan treatment. Recent evidence has reported that long-acting ARB drug, valsartan, owned a property as an ER stress inhibitor and eliminated ER stress-related cell death in the diabetic hearts through the CHOP/PUMA pathway (14). Recently, telmisartan has been reported to possess the ER stress inhibitor properties by protecting high glucose-induced apoptosis (15) and by suppression of AMPK pathway in insulin resistance related to obesity (16). Finally, candesartan cilexetil is also reported to protect against ER stress-induced cardiotoxicity in the myosin-induced rat (17). Thus, ARB drugs in general may play a beneficial role in diabetes mellitus at least with their additional property as an ER stress inhibitor. In brief, olmesartan has been proven to protect against diabetic cardiomyopathy, partly, by inhibiting the pro-apoptotic pathway of GRP78/CHOP/Caspase12.

CONCLUSION

Prolonged hyperglycaemia elicited diabetic cardiomyopathy by activating several detrimental processes including ER stress. In our study, prolonged hyperglycaemia activates the up-stream molecule and apoptotic key initiating factor of ER stress which subsequently stimulate massive cardiac apoptosis. Olmesartan treatment for 6 weeks alleviates GRP78, CHOP and caspase12 along with cardiac apoptosis, partly, through the inhibition of pro-apoptotic pathway GRP78/CHOP/Caspase12. Thus, olmesartan, despite its ARB main activity, possesses a property as an ER stress inhibitor that may play essential support in preventing further diabetic complications.

REFERENCES

1. Athithan L, Gulsin GS, McCann GP, Levelt E. Diabetic cardiomyopathy: Pathophysiology, theories and evidence to date. World J Diabetes. 2019;10(10):490-510.
2. Dandamudi S, Slusser J, Mahoney DW, Redfield MM, Rodeheffer RJ, Chen HH. The prevalence of diabetic cardiomyopathy: a population-based study in Olmsted County, Minnesota. J Card Fail. 2014;20(5):304-9.
3. Bodiga VL, Eda SR, Bodiga S. Advanced glycation end products: role in pathology of diabetic cardiomyopathy. Heart Fail Rev. 2014;19(1):49-63.
4. Joubert M, Manrique A, Cariou B, Prieur X. Diabetes-related cardiomyopathy: The sweet story of glucose overload from epidemiology to cellular pathways. Diabetes Metab. 2019;45(3):238-47.
5. Heydari AH, Fathi M, Heydari S, Heidari ME. Advanced glycation end product blocker drugs have a great potential to prevent diabetic cardiomyopathy in an animal model of diabetes mellitus type-2. Cardiovasc Ther. 2022;2022:7014680.
6. Chen Y, Hua Y, Li X, Arslan IM, Zhang W, Meng G. Distinct types of cell death and the implication in diabetic cardiomyopathy. Front Pharmacol. 2020;11:42.
7. Sun S, Yang S, Dai M, Jia X, Wang Q, Zhang Z, et al. The effect of Astragalus polysaccharides on attenuation of diabetic cardiomyopathy through inhibiting the extrinsic and intrinsic apoptotic pathways in high glucose -stimulated H9C2 cells. BMC Complement Altern Med. 2017;17(1):310.
8. Yang L, Zhao D, Ren J, Yang J. Endoplasmic reticulum stress and protein quality control in diabetic cardiomyopathy. Biochim Biophys Acta. 2015;1852(2):209-18.
9. Lakshmanan AP, Harima M, Suzuki K, Soetikno V, Nagata M, Nakamura T, et al. The hyperglycemia stimulated myocardial endoplasmic reticulum (ER) stress contributes to diabetic cardiomyopathy in the transgenic non-obese type 2 diabetic rats: a differential role of unfolded protein response (UPR) signaling proteins. Int J Biochem Cell Biol. 2013;45(2):438-47.
10. Lakshmanan AP, Thandavarayan RA, Palaniyandi SS, Sari FR, Meilei H, Giridharan VV, et al. Modulation of AT-1R/CHOP-JNK-Caspase12 pathway by olmesartan treatment attenuates ER stress-induced renal apoptosis in streptozotocin-induced diabetic mice. Eur J Pharm Sci. 2011;44(5):627-34.
11. Sari FR, Watanabe K, Thandavarayan RA, Harima M, Zhang S, Muslin AJ, et al. 14-3-3 protein protects against cardiac endoplasmic reticulum stress (ERS) and ERS-initiated apoptosis in experimental diabetes. J Pharmacol Sci. 2010;113(4):325-34.
12. Sari FR, Hendarto H, Adhiyanto C, Ananditya F, Rifa A, Syabrina F, et al. Syzygium polyanthum protects against diabetic cardiac apoptosis in the chronic diabetes mellitus. International Journal of Human and Health Sciences (IJHHS). 2020;5(1), 16-21.
13. Xu J, Wang G, Wang Y, Liu Q, Xu W, Tan Y, et al. Diabetes- and angiotensin II-induced cardiac endoplasmic reticulum stress and cell death: metallothionein protection. J Cell Mol Med. 2009;13(8A):1499-512.
14. Wu T, Dong Z, Geng J, Sun Y, Liu G, Kang W, et al. Valsartan protects against ER stress-induced myocardial apoptosis via CHOP/Puma signaling pathway in streptozotocin-induced diabetic rats. Eur J Pharm Sci. 2011;42(5):496-502.
15. Wang Y, Xue J, Li Y, Zhou X, Qiao S, Han D. Telmisartan protects against high glucose/high lipid-induced apoptosis and insulin secretion by reducing the oxidative and ER stress. Cell Biochem Funct. 2019;37(3):161-8.
16. Huang Y, Li Y, Liu Q, Zhang J, Zhang Z, Wu T, et al. Telmisartan attenuates obesity-induced insulin resistance via suppression of AMPK mediated ER stress. Biochem Biophys Res Commun. 2020;523(3):787-94.
17. Arumugam S, Thandavarayan RA, Palaniyandi SS, Giridharan VV, Arozal W, Sari FR, et al. Candesartan cilexetil protects from cardiac myosin induced cardiotoxicity via reduction of endoplasmic reticulum stress and apoptosis in rats: involvement of ACE2-Ang (1-7)-mas axis. Toxicology. 2012;291(1-3):139-45.