Diabetes Mellitus Downregulates Expression of Connexin43 in Rat Aortic Medial Smooth Muscle Cells and Can Be Reversed by Simvastatin and Losartan Therapy

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Background: Diabetes mellitus (DM) plays a crucial role in the pathogenesis of initiation and propagation of atherosclerosis. Although previous studies have suggested that interactions between cells form the framework for understanding the pathogenesis of atherosclerosis, little is known about how DM impacts intercellular communication within arteries, which occurs via connexin43 (Cx43) gap junctions (GJs). This study tested the hypothesis that DM suppresses expression of Cx43 GJs, and that this suppression can be abrogated via simvastatin or losartan treatment.

Methods: An experimental model of DM (induced by streptozocin 60 mg/kg body weight) in adult male rats (n = 24) was utilized to investigate Cx43 expression in the aorta. These rats were divided into group I (insulin therapy only), group II (insulin plus simvastatin 20 mg/kg/day) and group III (insulin plus losartan 20 mg/kg/day). Twenty-four diabetic rats and 8 healthy rats (group IV) were sacrificed 3 weeks after DM induction for Western blot and immunofluorescence analysis.

Results: By day 21, the blood sugar level was significantly higher than the respective baseline values in groups I, II and III (all values of $p < 0.0001$). Additionally, the final blood sugar levels of groups I-III were significantly higher than that of group IV ($p < 0.0001$). The final body weight in group IV was significantly higher than that in groups I-III (all values of $p < 0.0001$). Experimental results demonstrated that Cx43 expression in the aortic wall did not differ among groups II-IV ($p > 0.1$). However, compared with groups II-IV, Cx43 expression in the aortic wall was significantly mitigated in group I (all values of $p < 0.05$). Western blot results showed that relative density of Cx43 to β-actin was significantly higher in groups II-IV than in group I ($p < 0.01$).

Conclusions: DM markedly suppressed expression of Cx43 in rat aortic walls. Both simvastatin and losartan treatment significantly reversed the effects of DM on integrity of Cx43 expression.

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Key words: diabetes mellitus, connexin43, pharmacomodulation

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Atherosclerosis is a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation,[1-3] which are initiated by various pathogenetic mechanisms that converge on an essential common target of altering behavior in arterial smooth cells.[1-6] These cells migrate to the intima, proliferate and become highly active synthetically.[1-3,7] In addition to these complex pathogenetic mechanisms in the formation of atherosclerosis, cell-to-cell communication mediated by gap junctions has also been implicated.[6,8,9] In deed, gap junctions, which are composed of connexin (Cx) subunits, play a key role in direct intercellular exchanges of ions, secondary messengers and small signaling molecules, and have a crucial role in tissue homeostasis and the regulation of growth, differentiation and developments.[9-11] It is well recognized that the principal Cx isoform expressed by vascular smooth muscle is Cx43.[9] Experimental data suggest that enhanced expression of gap junctions between smooth muscle cells likely plays an essential role in maintaining phenotypic transformation of these muscle cells in response to vascular injury during early growth of atherosclerosis.[6,9] Nevertheless, exactly how diabetes mellitus (DM) impacts the expression of Cx43 gap junctions in vascular smooth muscle cells is currently unclear. Although the pleiotropic effects of statins and angiotensin type I receptor blockades have recently been recognized,[12-14] little is known about the impact of these pharmacological agents on improving expression of Cx43 gap junctions in a DM setting. Therefore, this study tests the hypothesis that DM decreases expression of Cx43 in the aortic walls of adult male Sprague Dawley (SD) rats, and that simvastatin or losartan therapy reverses the effects of DM on CX43 expression.

METHODS

All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at Chang Gung Memorial Hospital (CGMH), and were performed in accordance with the 'Guide for the Care and Use of Laboratory Animals' (NIH publication No. 85-23, National Academy Press, Washington, DC, U.S.A., revised 1996).

Diabetic animal model, protocol and procedure

Thirty-two adult male SD rats, including 8 healthy rats that served as the control group (group IV), weighing 325-350 grams (Charles River Technology, BioLASCO Taiwan Co. Ltd., Taiwan) were utilized in this study. Experimental diabetes was induced in the rats using subcutaneous streptozocin (Sigma, U.S.A.) injection (60 mg/kg body weight) over the abdominal area for 3 consecutive days.

These 24 rats were randomly assigned to group I (insulin therapy only, n = 8), group II (insulin plus simvastatin 20 mg/kg/day, n = 8) and group III (insulin plus losartan 20 mg/kg/day, n = 8). The blood glucose level of each rat was examined daily between 8:00-9:00 a.m. using a blood glucose monitor (ACCU-CHEK-Active; Roche).

The protocol of subcutaneous insulin administration (monotard® HM, Novo Nordisk) was as follows: (1) 4 units of insulin were given to the rat when blood glucose was 200-300 gm/dL; (2) 6 units of insulin were given to the rat when blood glucose was > 300 and ≤ 400 gm/dL; (3) 8 units of insulin were given to the rat when blood glucose was > 400 and ≤ 500 gm/dL; and (4) 10 units of insulin were given to the rat when blood glucose > 500 gm/dL.

Preparation of samples

All animals were sacrificed on day 21 following DM induction. Ascending and thoracic aortic tissue samples were obtained, frozen rapidly in liquid nitrogen and stored at –80°C for immunolabeling examination of Cx43.

Immunolabeling of Cx43

Three serial sections of aortic tissues (4 μm thick) were prepared by Cryostat (Leica CM3050S) for Cx43 immunolabeling. Samples were fixed in acetone for 15 min at –20°C and blocked in Beat Blocker Kit (Zymed Company, #50-300) solution A and B for 30 min and 10 min at room temperature, respectively. To co-localize troponin I and Cx43 in the same sample, the tissue sections were first incubated with a mixture of the polyclonal anti-Cx43 (1:200) (Zymed) plus anti-Troponin I (1:200) (Abcam) for 24 h at 4°C, followed by incubation with anti-mouse fluorescein isothiocyanate (FITC) (1:200) (Molecular Probe) and anti-rabbit Rhodamine (1:200) (Molecular Probe) for 30 min at

room temperature, respectively. All experiments with aortic sections served as positive controls, whereas those without primary antibodies were used as negative controls.

For each rat, three sections were chosen for Cx43 immunolabeling study. For each slide, the aggregation of Cx43-labeled spots in the aortic muscle layer was quantified under wide field fluorescent microscopy in three randomly chosen high-power fields (HPF) (×400). The average CX43-positively stained spots for each animal per high-power field was then obtained by summing these spots and dividing by 9. Calculation of the integrated area (μm^2) of Cx43 spots in the same slide was achieved using Image Tool 3 (IT3) image analysis software (The University of Texas Health Science Center in San Antonio, UTHSCSA, Image Tool for Windows, version 3.0, U.S.A.). The quantification procedure for three slides chosen for each rat was as follows. Three randomly selected HPFs were analyzed in each slide. The number of pixels in each Cx43 spot per HPF was first determined, followed by summation of the pixel numbers obtained from the 3 HPFs in each slide. The procedure was repeated for two other slides for each animal. The mean pixel number per HPF for each animal was then determined by summation of all pixel numbers divided by 9. The mean area of Cx43 per HPF was obtained by adopting a conversion factor of 19.24 (1 μm^2 represented 19.24 pixels).

Western blots

Equal amounts (30 μg) of protein extracts were loaded and separated by SDS-PAGE using 10% acrylamide gradients. After electrophoresis, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, U.S.A.). Nonspecific sites were blocked by incubation of the membrane in blocking buffer [5% nonfat dry milk in T-TBS (TBS containing 0.05% Tween 20)] overnight. The membranes were incubated with the indicated primary antibodies (Cx43 1:1000, Zymed), for 1 h at room temperature. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:2000, Amersham Biosciences) was used as a second antibody for 1 h at room temperature. The washing procedure was repeated 8 times within 1 h, and immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposure to Biomax L film (Kodak). For quantitation, ECL signals were digitized using Labwork software (UVP). The same membrane was re-probed with anti-actin antibody after complete removal of antibodies.

Statistical analysis

Data are expressed as means (± standard error of mean, SEM). The significance of differences between groups was evaluated with a Student's *t*-test or repeated measurements of ANOVA, followed by Scheffe's multiple comparison test. Logarithmic transformation of the integrated area (μm^2) of clustered Cx43 spots was used to improve normality for statistical analysis. Statistical analysis used SAS statistical software for Windows, version 8.2 (SAS Institute, Cary, NC, U.S.A.). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics of study and control rats

Table 1 shows the baseline characteristics of study and control rats. No differences existed for initial body weight and initial blood sugar among the four groups. DM was induced on the third day in all rats. Final daily mean blood sugar levels after DM induction (from days 3-21) also did not differ among groups I, II and III. Final blood sugar levels were significantly higher than the respective baseline values in groups I, II and III (all values of $p < 0.0001$). Final blood sugar levels of groups I-III were significantly higher than that of group IV.

Final body weight in group IV was significantly higher than that in groups I-III (all values of $p < 0.0001$). However, final body weight did not differ among groups I-III (all values of $p > 0.5$) or when compared with initial body weight for each group (all values of $p > 0.5$).

Quantitative immunofluorescence image study

Fig. 1 shows the results of quantification of the integrated area (μm^2) of clustered Cx43 spots in each group of rat aortas. The clustered Cx43 spots did not differ among the control group (IV), losartan treatment group (group III) and simvastatin treatment group (group II) (all values of $p > 0.1$). However, the

Table 1. Baseline Characteristics of Study and Control Rats

Variables	Group I (n = 8)	Group II (n = 8)	Group III (n = 8)	Group IV (n = 8)	*p* value
Initial body weight (kg)	355.4 ± 6.0	353.8 ± 7.2	355.0 ± 6.5	354.2 ± 7.0	0.671
Final body weight (kg)*	352.3 ± 7.2[a]	348.0 ± 7.9[a]	352.0 ± 8.2[a]	423.2 ± 7.6[b]	< 0.0001
Initial blood glucose (gm/dL)	114.3 ± 6.6	119.4 ± 4.9	112.2 ± 5.9	116.2 ± 5.3	0.462
Final blood glucose (gm/dL)*	365.7 ± 26.1[a]	372.4 ± 24.4[a]	358.5 ± 28.7[a]	124.2 ± 6.7[b]	< 0.0001

Data are expressed as mean value ± standard error of mean (SEM).

*: Means with different letters (a, b) indicate significant difference (at level $p = 0.05$) by Scheffe's multiple comparison procedure.

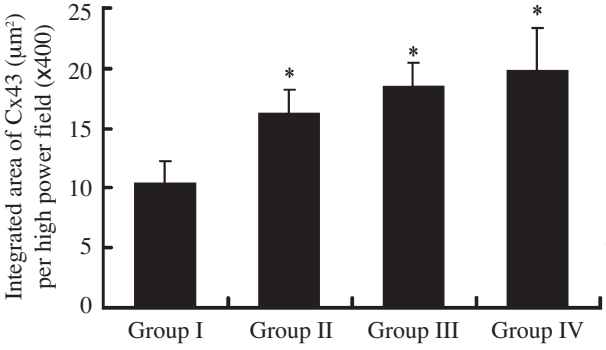

Fig. 1 Quantification of the integrated area (μm²) of clustered connexin43 (Cx43) spots in each group of rat aortas per high-power field (x400). Group IV (control) versus group II (insulin plus simvastatin) or group III (insulin plus losartan), $p > 0.1$. *versus group I (insulin only), $p < 0.05$.

integrated area of Cx43 spots was substantially larger in groups II-IV than in group I (all values of $p < 0.05$).

Image analysis of immunolabeling Cx43

Fig. 2 (A through D) presents the results of semi-qualitative analysis of immunolabeling Cx43. Immunolabeled sections were examined by immunofluorescence microscopy. Fig. 2A shows positive staining and the homogeneous distribution of Cx43 spots in healthy rats (group IV). Large cluster-sized Cx43-positive spots were observed in groups II, III and IV (Figs. 2A, 2C and 2D). Semi-quantitative immunolabeled images at higher magnification (x 400) in group I (Fig. 2B) diabetic rats identified a relatively decreased number of Cx43-positive spots existed within muscle layers of the aorta compared to the normal controls (group IV). The appearance of the Cx43 gap junctions was inconspicuous in group I (Fig. 2B). Sporadic distribution and small sized Cx43-positive spots were observed in group I.

Western blot findings of Cx43

Fig. 3 shows the density of Cx43 protein relative to β-actin (Fig. 3A) and Western blot finding for Cx43 (Fig. 3B). The experimental results demonstrated that relative density of Cx43 protein did not differ among groups II-IV (all values of $p > 0.1$). However, the relative density of Cx43 protein was significantly higher in groups II-IV than in group I (all values $p < 0.01$).

DISCUSSION

This study, which investigated the impact of DM on Cx43 expression, yielded two striking implications. First, compared with normal control rats, expression of Cx43 in diabetic rats was markedly suppressed. This finding suggests that direct intercellular communication via the gap junction was affected in smooth muscle cells of the vascular wall in a diabetic setting. Second, both simvastatin and losartan therapies significantly reversed the adverse effect of DM on the expression of CX43.

Cardiovascular disease is responsible for 80% of deaths among diabetic patients, the majority of cases being attributed to coronary artery disease.[15-18] In patients undergoing angiography, DM is associated with increased severity and diffuse coronary atherosclerosis, high plaque burden, small vessel reference diameter, frequent association with left main stem disease, multiple vessels and distal coronary tree involvement, as well as poor collateral circulation.[19-22] Previous studies have demonstrated that activation of multiple signal transduction pathways is responsible for the proliferation and migration of smooth muscle cells. Additionally, synthesis of extracellular

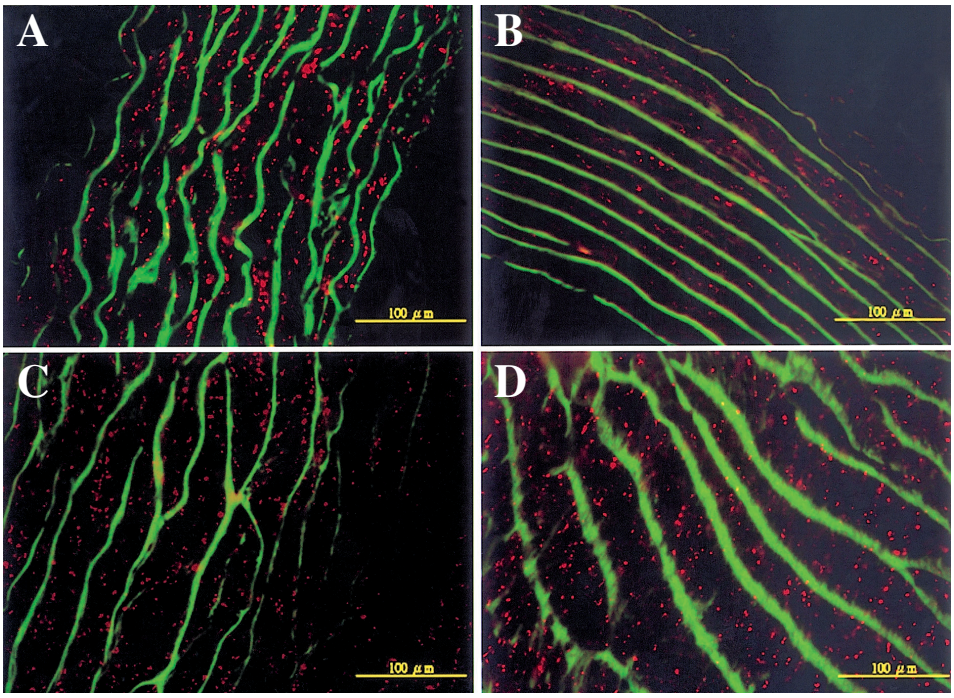

Fig. 2 A through D presents immunolabeling Cx43. Immunolabeled sections were examined by immunofluorescence microscopy (x400). The scale bars in the right lower corner represent 100 μm. Fig. 2A shows positive staining and the homogeneous distribution of Cx43 spots in group IV (normal control). Large cluster-sized Cx43-positive spots were observed in groups II, III and IV (Figs. 2A, 2C and 2D). However, immunolabeled images in group I (Fig. 2B) diabetic rats identified a relatively decreased number of Cx43-positive spots existed within muscle layers of the aorta compared to groups II-IV.

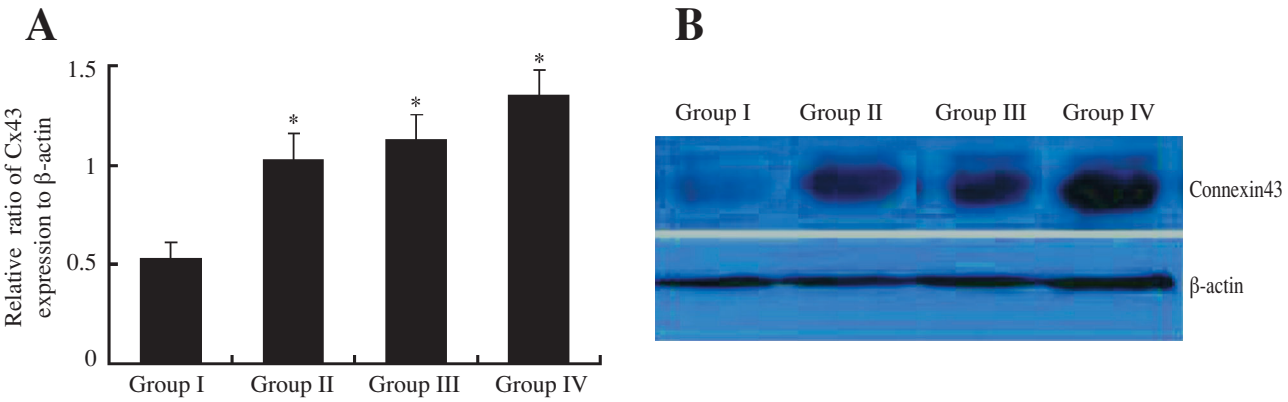

Fig. 3 Density of Cx43 protein relative to β-actin (Fig. 3A) and Western blot finding for Cx43 (Fig. 3B). (3A) Group IV versus group II or group III, $p > 0.1$; *versus group I, $p < 0.01$. (3B) Group I indicates insulin therapy only, group II indicates insulin plus simvastatin therapy, group III indicates insulin plus losartan therapy and group IV indicates healthy control.

matrix by these cells is essential for underlying atherosclerotic disease and for any healing response to vascular injury following catheter-based coronary interventions.[23-26] Two previous studies by Yeh and Blackburn et al.[6,9] further demonstrated that direct intercellular communication via Cx43 gap junctions may also play an important role in participating in and maintaining smooth muscle phenotypic transfor-

mation during early growth of the atherosclerotic plaque, and in response to balloon catheter injury of arterial walls. Surprisingly, in contrast to findings obtained by Yeh and Blackburn et al.,[6,9] Cx43 gap junctions were upregulated in the early stages of atherosclerosis, this study showed that, compared to normal healthy rats, Cx43 expression in the aortic wall was markedly suppressed by DM. Why such a discrepancy exists between this study and findings in the previous studies remains unclear.[6,9] However, we propose that two rationales likely explain this discrepancy. First, this difference is probably due to the different baseline conditions utilized in this study and those of Yeh and Blackburn et al.[6,9] Second, the different stages of disease probably affect intensity of Cx43 gap junction expression. Cx43 gap junction expression in the early stages of atherosclerosis was utilized for investigation in Yeh and Blackburn et al.[6,9] However, in this study, diabetic rats with mean blood glucose levels exceeding 250 mg/dl, an index of DM with relatively poor control and reflective of advanced disease stage, were utilized. Blackburn et al.[6] suggested that a decline in gap junction quantity appeared in advancing stages of the disease, which is in parallel with the accumulation of extracellular matrix material. Following this process, the number of gap junctions gradually decreases, and the size of gap junctions increases and becomes sparsely scattered. At this stage, the gap-junctional communication is probably of little further significance to lesion genesis or its fate. Accordingly, the experimental results in this study further support this suggestion[6] and indicate that different disease processes induce different expressions of Cx43 gap junctions within smooth muscle cells of arterial walls.

Interestingly, Blackburn et al. suggested that upregulation of Cx43 gap junctions plays a key role in the early stages of atherosclerosis.[6] This study also found that DM, which plays a crucial role in endothelial dysfunction and atherosclerosis, significantly downregulated Cx43 expression. Additionally, previous studies suggested that gap junctions, composed of Cx subunits are essential for tissue homeostasis and growth regulation, differentiation and development.[9-11] Therefore, we propose that an inappropriate expression of the Cx43 gap junction (either downregulated or upregulated) interferes with intercellular communication, which in turn activates synthetic phenotypes in smooth muscle cells. Accordingly, interactions between cells form the framework for understanding the pathogenesis of atherosclerosis.[6]

One novel finding in this experimental study was that simvastatin treatment significantly reversed DM-induced downregulation of Cx43 expression in rat aortas. Although the mechanisms underlying this finding are not easily explained, we speculate that this suppression may be due to, at least in part, the anti-inflammatory effect elicited by simvastatin. As shown previously, diabetic patients typically have high circulating levels of high-sensitivity C-reactive protein, an index for inflammation.[13] In addition to their anti-inflammatory effects, statins have been shown to have immunomodulatory and lipid-lowering properties.[27,28] Furthermore, statins have been shown to promote potent systemic antioxidant effects through specific anti-inflammatory pathways.[29] It is likely that the validity of utilizing the anti-inflammatory effect of simvastatin in treating endothelial dysfunction and cardiovascular diseases is, in part, based on its protective effects on the integrity of Cx43 gap junctions and its ability to appropriately upregulate Cx43 expression.

A second novel finding in this study was that losartan treatment also abrogated the effect of DM on suppressed Cx43 expression in rat aortic walls. Whether such protective effects are also due to an anti-inflammatory effect or other mechanism(s) remains uncertain. As shown previously, activation of the angiotensin II type 1 (AT1) receptor has a powerful pro-inflammatory effect, promoting expression of many pro-inflammatory mediators, such as cytokines, chemokines and adhesion molecules, via activation of signaling pathways.[30] An experimental study[12] previously demonstrated that, in addition to an anti-hypertensive effect, losartan also has anti-inflammatory and anti-aggregatory properties. Perhaps these findings[12,30] provide some information that supports the uncertain mechanism(s) of this study.

Study limitations

This study has several limitations. First, without investigating how time courses of DM impact Cx43 expression, this study did not provide the serial changes in Cx43 expression. Second, the exact mechanism(s) of losartan or simvastatin therapy in abrogating the effect of DM on Cx43 expression

remains uncertain. However, investigating these mechanisms is beyond the scope of this study.

In conclusion, this experimental study supports the proposition that direct intercellular communication via the gap junction is perturbed in smooth muscle cells of vascular walls by DM. Both simvastatin and losartan treatment reversed the adverse effect of DM on integrity of intercellular communication. These experimental results raise the need for further studies investigating the mechanisms underling these findings.

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糖尿病抑制老鼠大動脈連接素 (Connexin43) 的表現可藉由 Simvastatin 和 Losartan 的治療而改善

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背 景： 研究顯示糖尿病對於形成及加促血管動脈硬化 (atherosclerosis) 扮演重要的角色。雖然之前的研究認爲，可以利用細胞與細胞之間的聯繫所形成架構去了解粥狀動脈硬化病理機轉。可是有關糖尿病經由影響間隙接合 (gap junction) 中連接素 [connexin43 (Cx43)] 對於大動脈細胞間聯繫的影響，目前仍不是很了解。本研究假設糖尿病會抑制間隙接合 (gap junction) 中連接素 connexin43 (Cx43) 的表現，但可藉由 Simvastatin 和 Losartan 的治療可逆轉此現象。

方 法： 二十四隻成年大白鼠 (Sprague Dawley) 利用 streptozocin (STZ) 注射導致第一型糖尿病爲模型，之後進一步來研究連接素 43 (Cx43) 在大動脈的表現。實驗的老鼠分成三個組別：第一組（只有胰島素的治療），第二組 (胰島素治療加上 simvastatin 20 mg/kg/day)，第三組 (胰島素治療加上 losartan 20 mg/kg/day)。在第三週時，這二十四隻成年糖尿病大白鼠和另外八隻健康大白鼠被犧牲 (sacrificed) 後，再經由西方蛋白質轉漬法 (Western blot) 和免疫螢光染色法 (immunofluorescence) 來分析。

結 果： 研究結果顯示，第一、第二及第三組大白鼠在第二十一天最後的血糖值都比第一天的基準血糖值高 (所有 p 值 < 0.0001)。另外，第一到第三組大白鼠最後的血糖值比第四組大白鼠來得高 (p 值 < 0.0001)。此外，第四組大白鼠最後的體重比第一到第三組大白鼠最後的體重來得重 (p 值 < 0.0001)。實驗結果發現，第二組到第四組連接素 43 (Cx43) 在大白鼠大動脈的表現彼此間並無不同 (p 值 > 0.1)，然而第一組與其他組別間 Cx43 在大白鼠大動脈的表現有明顯的減少 (所有 p 值 < 0.05)。此外，第二組到第四組β肌蛋白 (β-actin) 和連接素 43 (Cx43) 的相對密度值明顯高過於第一組 (p 值< 0.01)。

結 論： 糖尿病很明顯抑制大白鼠大動脈壁連接素 43 (Cx43) 的表現。而 Simvastatin 和 Losartan 的治療明顯改善糖尿病對細胞間的聯繫的傷害。

(長庚醫誌 2008;31:136-44)

關鍵詞： 糖尿病，連接素 43 (connexin43)，藥理調節

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