Diabetes Reduces Aortic Endothelial Gap Junctions in ApoE-deficient Mice: Simvastatin Exacerbates the Reduction

Charles Jia-Yin Hou, Cheng-Ho Tsai, Cheng-Huang Su, Yih-Jer Wu, Su-Jen Chen, Jing-Jing Chiu, Ming-Shi Shiao, and Hung-I Yeh

Departments of Internal Medicine and Medical Research, Mackay Memorial Hospital, Mackay Medicine, Nursing and Management College, Taipei, Taiwan (CJ-YH, C-HT, C-HS, Y-JW, S-JC, H-IY); Taipei Medical University, Taipei, Taiwan (C-HT, H-IY); and Department of Life Science, Chang Gung University, Tao-Yuan, Taiwan (J-JC, M-SS)

SUMMARY We examined the endothelial gap junctions in diabetic hyperlipidemic mice. Male apolipoprotein E (apoE)-deficient mice were made diabetic by streptozotocin. Three weeks later, the animals were treated with simvastatin for 2 weeks. The expression of aortic gap junctions in the non-diabetic (n=10), untreated diabetic (n=10), and simvastatin-treated diabetic animals (n=6) was analyzed. There was a >4-fold increase in serum cholesterol level and >50% increase in plaque areas in the diabetic mice, regardless of simvastatin treatment. Western blotting of aortae showed reduced expression of connexin37 (Cx37) and Cx40 in the diabetic mice, which were further decreased in the simvastatin-treated diabetic mice. Immunoconfocal microscopy showed that endothelial gap junctions made of Cx37 and Cx40 were both reduced in the untreated diabetic mice compared with the non-diabetic mice (decrease: Cx37, 41%; Cx40, 42%; both p<0.01). The reduction was greater in the simvastatin-treated mice (decrease in treated diabetic vs non-diabetic: Cx37, 61%; Cx40, 79%; both p<0.01; decrease in treated diabetic vs untreated diabetic: Cx37, 34%; Cx40, 63%; both p<0.01). Cx37 and Cx40 were decreased in the endothelium of plaque surface. Cx43 appeared in the medial layer and inner layer of the intima. All three connexins were rarely expressed in monocytes/macrophages inside the plaques. In conclusion, in apoE-deficient mice, streptozotocin-induced diabetes is associated with downregulation of endothelial Cx37 and Cx40 gap junctions. Short-term treatment with simvastatin exacerbates the downregulation.

KEY WORDS

gap junction
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CELL–CELL INTERACTION is an essential element in atherogenesis. Previous studies have shown that direct intercellular communication through cell membrane protein channels made of connexins, called gap junctions, is involved in several steps of atherogenesis (Severs et al. 2001; Haefliger et al. 2004; Chadjichristos et al. 2006). In animal studies, endothelial gap junctions have been reported to be downregulated in the presence of risk factors of atherosclerotic disease, such as aging (Yeh et al. 2000a), hypertension (Yeh et al. 2006), and hyperlipidemia (Yeh et al. 2003b), in which endothelial dysfunction exists (Esper et al. 2006). In addition, the downregulation of endothelial gap junctions in hypertension and hyperlipidemia can be, respectively, attenuated by antihypertensive and lipid-lowering drugs (Yeh et al. 2003b, 2006), which are known to improve impaired endothelial function (Esper et al. 2006). The evidence has suggested that downregulation of gap junctions in the endothelium reflects endothelial dysfunction. Because clinical studies have shown that endothelial dysfunction is more pronounced in patients with multiple risk factors (Vita et al. 1990; Esper et al. 2006), this raises the possibility that addition of another risk factor to the existing factors mentioned above may result in further downregulation of endothelial gap junctions. Apart from hypertension and hyperlipidemia, diabetes is a major modifiable risk factor of atherosclerotic cardiovascular disease. Although in vitro studies have shown that high glucose reduces the expression of endothelial gap junctions and gap-junctional com-
munication function (Sato et al. 2002), to date, the in vivo effect of diabetes on endothelial gap junctions remains unclear. On the other hand, a recent study reported that connexin37 (Cx37), a component of endothelial gap junctions, was involved in the proatherogenic activity of monocytes/macrophages (Wong et al. 2006). However, the connexin expression profile of monocytes/macrophages in the vascular wall is not detailed.

To examine these issues, we studied the aortic endothelial gap junctions in apolipoprotein E (apoE)-deficient mice made diabetic by streptozotocin. Induction of diabetes by streptozotocin in apoE-deficient mice was reported to result in a rapid increase of aortic atherosclerotic lesions (Park et al. 1998; Candido et al. 2004), which facilitated the observation of connexins during the development of plaques. We also examined the effect of simvastatin on the connexins. In our previous study, we showed that short-term treatment with simvastatin attenuates the depressed connexin expression induced by hyperlipidemia and that the aortic endothelial gap junctions in apoE-deficient mice are made of Cx37 and Cx40, whereas Cx43 exists in the medial smooth muscle (Yeh et al. 2003b).

Materials and Methods

Animals, Diets, and Tissue Processing

Twenty-six 29- to 30-week-old male homozygous apoE-deficient mice of C57BL/6 background, supplied by the National Cheng Kung University Animal Center, were divided into three groups. Group 1 (n=10) was used as a control and groups 2 (n=10) and 3 (n=6) received streptozotocin (166.7 mg/kg/day for consecutive 3 days, intraperitoneally). Three weeks later, group 3 received oral feeding of simvastatin (10 mg/kg/day; kindly donated by Merck Sharp & Dohme, Taiwan) for 14 days. All animals were fed normal chow (rodent chow 5010; Purina, St. Louis, MO) throughout the experiment. The serum glucose and cholesterol levels were determined at the start, 3 weeks later, and weekly until the end of the experiment.

At the end of 5 weeks, all animals were anesthetized with ether inhalation and were perfusion-fixed through direct intracardiac injection, initially with heparinized PBS (10 units/ml), followed by phosphate-buffered 2% paraformaldehyde (pH 7.4) for 7 min. In all animals, the thoracic aortae were dissected and cut into transverse rings for rapid freezing in isopentane at −160°C. The samples were stored in liquid nitrogen before immunolabeling. Samples of the aortic arch were stained with Sudan IV for evaluating the burden of atheroma. This work was conducted in accordance with the government (Republic of China) Animal Protection Law (Scientific Application of Animals), 1998.

Immunodetection of Connexins and Macrophage Foam Cells

Anti-connexin and Anti-macrophage Foam Cell Antibodies. Three antibodies were used for the immunofluorescence detection of Cx37, Cx40, and Cx43. The polyclonal antisera against Cx37, Cx40, or Cx43 were produced in rabbits [designated Cx37(R382), Cx40(R2), and Cx43(R530), respectively] against the synthetic peptides corresponding to residues 266–281 (for Cx37), 256–270 (for Cx40), or 314–322 (for Cx43) of the cytoplasmic C-terminal tail of rat connexins. In addition, guinea pigs were used to generate anti-Cx40 antisera [designated Cx40(GP8)]. These polyclonal sera were affinity purified and have previously been confirmed to be isotype specific (Yeh et al. 2000a,2003a). For detection of macrophage foam cells, rat monoclonal anti-mouse Mac-2 antibody (Cedarlane Laboratories; Hornby, Ontario, Canada) was used.

Western Blotting. Aortic samples, stripped of the outer fatty layer, were put in SB20 buffer (containing 20% SDS, 0.1 M Tris, pH 6.8, and 10 mM EDTA) and ground

Figure 1 Serum glucose (top histogram) and cholesterol (bottom histogram) levels in animals of different groups. See text for details. *p<0.001 for top histogram and p<0.05 for bottom histogram compared with each of the other bars of the same time points. CT, control; STZ, streptozotocin; STZ + SI, streptozotocin plus simvastatin.
using a bullet blender (Next Advance; Averill Park, NY), followed by addition of 2.5% 2-mercaptoethanol. SDS-PAGE was performed using 10% gels. Twenty μg of sample was loaded in each lane, subjected to electrophoresis, and transferred onto nitrocellulose membranes. The blots were blocked with 10% BSA for 1 hr and detected with the primary antibodies specific for Cx37 [Cx37 (R382); 1:100] and Cx40 [Cx40 (GP8); 1:100] for 1 hr at room temperature. Thereafter, alkaline phosphatase–conjugated donkey anti-rabbit or rabbit anti-guinea pig IgG (both 1:5000 in TBST plus 10% BSA), purchased from Chemicon (Temecula, CA), was added. Immunoreactivity was visualized using the CDP-star system (Roche; Mannheim, Germany) according to the manufacturer’s instructions. Finally, the blots were stripped with stripping buffer (69 mM SDS, 100 mM 2-mercaptoethanol, 93.75 mm Tris-HCl, pH 6.8) at 56°C and incubated with anti-β-actin antibody (1:10,000; Abcam, Cambridge, UK) as an internal control.

Secondary Antibody/Detection Systems. Donkey anti-rabbit, anti-guinea pig, and anti-rat immunoglobulin conjugated to either CY3 or CY5 (Chemicon) were used to visualize immunolabeled connexins. For single labeling of individual connexins, CY3-conjugated antibodies were used. For double labeling of two connexins or one connexin and macrophage foam cells, one CY3-conjugated antibody and one CY5-conjugated antibody were used in combination.

Immunolabeling of Connexins and Macrophage Foam Cells. The perfusion-fixed aortic rings or cryosections of the rings were rinsed in PBS for 5 min, blocked in 0.5% BSA (15 min), and incubated with anti-Cx37 (1:100), anti-Cx40 (1:100 for either Cx40 (R2) or Cx40 (GP8)), anti-Cx43 (1:500), or anti-Mac-2 (1:100) at 37°C for 2 hr. The samples were treated with CY3-conjugated secondary antibody (1:500, room temperature, 1 hr). In double labeling experiments, incubation was with a mixture of anti-Cx37 (1:200) plus anti-Mac-2 (1:100), anti-Cx40 (Cx40 (GP8), 1:100) plus anti-Mac-2 (1:100), or anti-Cx43 (1:500) plus anti-Mac-2 (1:100), followed by incubation with a mixture of the two corresponding species-specific secondary antibodies (CY3 and CY5; 1:500).

Confocal Laser Scanning Microscopy

Immunostained samples were examined by confocal laser scanning microscopy using a Leica TCS SP equipped with argon/krypton and UV laser (Heidelberg, Germany). Single connexin-labeled samples were used for semiquantification of gap junctions. After the signal on the top of the sample was observed, the images were collected using the ×40 objective lens and zoom 1.0 computer setting so that each pixel represented 0.24 μm. Each image recorded consisted of 1024 × 1024 pixels, and projection views of consecutive optical sections taken at 0.4-μm intervals through the full thickness of endothelial connexin signal were recorded.

Figure 2 Comparison of the burden of atheromatous plaque (in red) between groups 1 (A), 2 (B), and 3 (C) by Sudan IV staining. Note that to better show the anatomical distribution of atherosclerotic plaques, the images were taken before the arches were cut open. See text for details. *p < 0.05 compared with each of the other bars. Abbreviations are the same as in Figure 1. Bar = 1 mm.
for analysis. The mean thickness of the vascular wall studied was 6 μm. For double labeling, the images were taken using simultaneous dual-channel scanning.

Image Analysis and Statistics

Analysis of connexin labeling from the confocal images, using QWIN image analysis software (Leica), was conducted as previously described (Yeh et al. 2000a,b). In single connexin-labeled samples, for each animal, four randomly selected fields were analyzed. Mean values (±SD) of the total area of immunolabeled gap junctions, expressed as percentage of the luminal surface area, were obtained.

Serum glucose and lipid levels were determined using routine enzymatic methods. Data were compared statistically by one-way ANOVA and t-test.

Results

Serum glucose level was elevated to >300 mg/dl in mice 3 weeks after administration of streptozotocin and persisted to the end of the experiment (at the end of experiment: group 1, 77 ± 44 mg/dl; group 2, 404 ± 60 mg/dl; group 3, 365 ± 54 mg/dl; group 1 vs either group 2 or 3, both p<0.001; Figure 1). Similarly, serum cholesterol level was elevated to >4-fold of the basal level in mice made diabetic (at the end of experiment: group 1, 481 ± 100 mg/dl; group 2, 2266 ± 644 mg/dl; group 3, 2306 ± 365 mg/dl; group 1 vs either group 2 or 3, both p<0.001; Figure 1). Treatment with simvastatin for 2 weeks did not lower the cholesterol level.

Sudan IV staining of the aortic arch showed the presence of atheromatous plaques in all three groups (Figure 2). Analysis of the percentage of aortic luminal surface area occupied by the plaques showed that the burden of plaques was markedly increased in mice made diabetic (group 1, 22.5 ± 1.7%; group 2, 34.8 ± 8.7%; group 3, 34.7 ± 9.1%; group 1 vs either group 2 or 3, both p<0.05; Figure 2). Treatment with simvastatin did not affect the burden of atheroma.

Gap Junction Distribution and Connexin Expression

Western blotting of the stripped aortae showed that, for Cx37 and Cx40, the content differed between the groups. For both Cx37 and Cx40, animals without diabetes possessed more than those made diabetic; for diabetic animals, those without simvastatin treatment possessed more than those with the treatment (Figure 3).

En face confocal views of the luminal surface of the animals after single labeling clearly displayed punctate signal for Cx37 and Cx40, typical of gap junctions. For the majority of the luminal surface outside the plaque areas, signals of both connexins more or less evenly delineated the borders of endothelial cells (Figure 4). Compared with the control animals (group 1), the animals made diabetic (group 2) had less signals of both connexins (group 2 vs group 1, reduction in total gap junction area: Cx37, 41%; Cx40, 42%, both p<0.01). In animals made diabetic and treated with simvastatin (group 3), the signals were even less (decrease, group 3 vs group 1: Cx37, 61%; Cx40, 79%, both p<0.01; group 3 vs group 2: Cx37, 34%; Cx40, 63%, both p<0.01; Figure 3). Double-labeling experiments to detect both Cx37 and Cx40 showed that the majority of the two connexins were colocalized (data not shown).

In all groups, the signals of both connexins were less and unevenly distributed at the luminal surface of the plaque areas (Figure 5). Cx37 has been reported to exist in macrophage foam cells. To further clarify the relationship between Cx37 and macrophage foam cells, a strategy of double labeling for simultaneous detection of macrophage foam cells and Cx37 was applied. As shown in Figure 5, the labels of Cx37 in all three groups were less or even rare in the areas containing Mac-2-positive macrophage foam cells and the surrounding zone. However, in some areas, the labels of Cx37 were overlapped with those of the macrophage foam cells. Therefore, cryosections of the
aortic rings were double labeled to further clarify the spatial relationship between Cx37 and macrophage foam cells. As shown in Figure 6, in a cross-sectional view of the aortic wall, the labels of Cx37 were located at the luminal surface, and most of the labels of Cx37 appeared away from the Mac-2-positive area, regardless of the size of the plaques. Such a distribution pattern is also seen for Cx40 (Figure 6). In contrast, labels...
of Cx43 were mainly located at the medial layer, whereas less occurred inside the plaque and rarely at the luminal surface (Figure 6).

Discussion
This study showed that, in aortic endothelium of apoE-deficient mice, the development of diabetes not only increases the burden of atheroma but also changes the endothelial gap junctions. Specifically, in the diabetic animals, outside the plaque areas, endothelial gap junctions and their component connexins, Cx37 and Cx40, were downregulated. Also, the downregulation of the junctions was further exacerbated by a 2-week treatment with simvastatin, an HMG-CoA reductase inhibitor. In addition, gap junctions made of Cx37 or Cx40 were few at the luminal surface of the plaque areas and rare inside the plaques, regardless of the presence of diabetes or simvastatin treatment. In contrast, Cx43 gap junctions were mainly located in the medial layer, few in the intima, and extremely few at the luminal surface. These findings provide novel information regarding the impact of diabetes and simvastatin on endothelial gap junctions in the hyperlipidemic state.

The findings from Western blotting and immunoconfocal microscopy are complementary to each other. Because of the difficulty in isolating endothelial cells from the mouse aorta, the samples for Western blotting were aortae stripped of the adventitial layer. Although one may question that the findings from Western blotting of the stripped aortae would not necessarily represent the Cx37 and Cx40 of endothelial cells, immunoconfocal microscopy showed that the signals of Cx37 and Cx40 were by far predominately located in the endothelium and therefore supported that the signals of both connexins in Western blots were mainly contributed by the endothelial cells. However, the different burdens of atheroma in the aortic wall make it difficult to compare the amounts of endothelial Cx37 and Cx40 among the three groups by Western blotting. In this regard, data from en face immunoconfocal microscopy are more suitable for such comparisons. We therefore did not conduct densitometric analysis of the Western blots; instead, we analyzed the en face immunoconfocal images to compare the endothelial expression of Cx37 and Cx40 gap junctions.

Our previous study showed that endothelial gap junctions are downregulated in the hyperlipidemic animals (Yeh et al. 2003b). An in vitro study also showed that endothelial gap junctions were reduced in the milieu of high concentrations of glucose (Sato et al. 2002). However, whether the in vitro effect of glucose
can be seen in animals with hyperlipidemia remained unknown. In this study, by using streptozotocin to induce diabetes in apoE-deficient mice, we unequivocally showed that the effect of high glucose on cultured cells does exist in hyperlipidemic animals. On the other hand, our previous study showed that the downregulation of endothelial gap junctions in the hyperlipidemic animals can be attenuated by short-term treatment with simvastatin, which also lowered the serum cholesterol level. In contrast, in this study, treatment with simvastatin did not affect the serum cholesterol level but further downregulated endothelial gap junctions. Statins have been known to have a biphasic effect on endothelial cells (Weis et al. 2002). In vitro studies showed that, whereas statins at low concentrations enhanced proliferation of endothelial cells, the drugs at high concentrations not only inhibited the proliferation but also induced apoptosis (Weis et al. 2002). Opposite effects of different doses of statins in angiogenesis were also reported in apoE-deficient mice (Weis et al. 2002). In this study, compared with those without diabetes, apoE-deficient mice made diabetic were found to have more severe fatty liver (animals given streptozotocin all had evenly whitish liver, whereas those without streptozotocin only have whitish patches in the liver). This raised the possibility that, in this study, the plasma level of simvastatin in the diabetic animals was higher, because of the slower clearance of the drug by the liver, and therefore the effect of simvastatin shown in this study is different from our previous report (Yeh et al. 2003b). In contrast, altered glucose metabolism in the streptozotocin-treated apoE-deficient mice may affect lipid metabolism, and thus simvastatin did not lower the cholesterol level or increase the endothelial connexins in the diabetic animals. Clinical studies have shown that statins are able to improve endothelial function (Dilaveris

Figure 6  Section view of the aortic wall double labeled for Mac-2 and Cx37 (A,E,I), Cx40 (C,G), or Cx43 (K). In all color images, Mac-2 is in red, whereas connexins are in green. For each color image, the corresponding phase contrast image (B,D,F,H,J,L) is displayed at the right to show that the whole layer of plaque, if present, is not only made of cells positive for Mac-2. Note that Cx37 and Cx40 are distributed at the luminal surface of the plaques (E,G,I), whereas Cx43 is at the inner layer of intima or in the medial layer (K). Images are oriented with the luminal side up. Bar = 30 μm.
et al. 2007) and slow the progression of or even cause regression of atherosclerosis (Balk et al. 2004). The findings from this study suggest that, in diabetic patients with very high serum cholesterol levels, such effects of statins do not exist.

Recently, Cx37 hemichannels formed in monocytes were reported to be antiatherogenic (Wong et al. 2006). In that report, an increase of atheroma burden seen in Cx37 knockout animals was attributed to an enhancement of monocyte adhesion because of the lack of Cx37. One interesting finding in this study is that, in the cross-section of the aorta, Cx37 was located at the luminal surface and rarely, if any, found inside the plaques, regardless of the size of the plaques. In addition, double-labeling experiments confirmed that Cx37 was rarely colocalized with MAC-2–positive macrophage foam cells. These results indicated that, once monocytes entered the vascular wall and became macrophages, they soon lost Cx37 expression. Furthermore, as shown in this study, the macrophage foam cells also lacked Cx40 and Cx43 gap junctions, suggesting that these cells are insulated from the gap junctional communication network of the vascular wall, in which the component endothelial and smooth muscle cells express Cx37, Cx40, and Cx43.

In conclusion, diabetes induced by streptozotocin is associated with downregulation of endothelial Cx37 and Cx40 gap junctions in apoE-deficient mice. Short-term treatment with simvastatin is associated with an even more severe downregulation of the gap junctions. In addition, Cx37 is rarely expressed in the macrophage foam cells inside the plaques. Clarification of the mechanisms modulating endothelial and monocytes/macrophages gap junction expression in the vascular wall in diabetic hyperlipidemic environment and those underlying the effect of simvastatin requires further experiments.

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Literature Cited


