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A-FABP and Oxidative Stress Underlie the Impairment of Endothelium-Dependent Relaxations to Serotonin and the Intima-Medial Thickening in the Porcine Coronary Artery with Regenerated Endothelium

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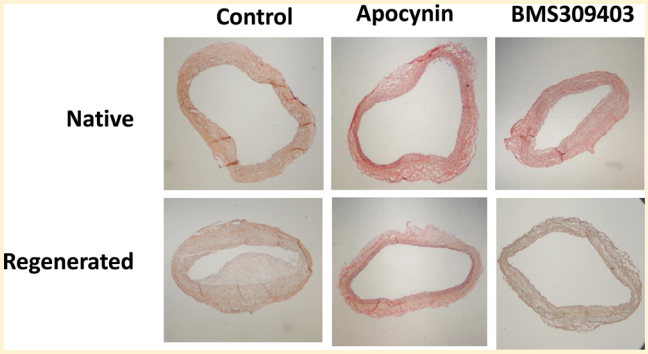
ABSTRACT: Experiments were designed to determine the cause of the selective dysfunction of G_i proteins, characterized by a reduced endothelium-dependent relaxation to serotonin (5-hydroxytryptamine), in coronary arteries lined with regenerated endothelial cells. Part of the endothelium of the left anterior descending coronary artery of female pigs was removed in vivo to induce regeneration. The animals were treated chronically with vehicle (control), apocynin (anti-oxidant), or BMS309403 (A-FABP inhibitor) for 28 days before functional examination and histological analysis of segments of coronary arteries with native or regenerated endothelium of the same hearts. Isometric tension was recorded in organ chambers and cumulative concentration-relaxation curves obtained in response to endothelium-dependent [serotonin (G_i protein mediated activation of eNOS) and bradykinin (G_q protein mediated activation of eNOS)] and independent [detaNONOate (cGMP-mediated), isoproterenol (cAMP-mediated)] vasodilators. The two inhibitors tested did not acutely affect relaxations of preparations with either native or regenerated endothelium. In the chronically treated groups, however, both apocynin and BMS309403 abolished the reduction in relaxation to serotonin in segments covered with regenerated endothelium and prevented the intima-medial thickening caused by endothelial regeneration, without affecting responses to bradykinin or endothelium-independent agonists (detaNONOate and isoproterenol). Thus, inhibition of either oxidative stress or A-FABP likely prevents both the selective dysfunction of G_i protein mediated relaxation to serotonin and the neointimal thickening resulting from endothelial regeneration.



KEYWORDS: *Apocynin, BMS309403, bradykinin, endothelial cells, G_i proteins*

Serotonin (5-hydroxytryptamine) is a monoamine produced in the gastrointestinal wall and in the central nervous system. In the blood, it is stored by the platelets, and released when the aggregation process begins. Its effect on the platelets is to further amplify aggregation by potentiating the response to other inducers of platelet aggregation.[1,2] In most blood vessels, if the smooth muscle cells are exposed to serotonin, vasoconstriction occurs following activation of 5-HT2A and 5-HT1B/1D receptors.[3] However, in some arteries, the monoamine possesses vasodilator effects because it activates endothelial 5-HT_{1D} receptors on the endothelial cells.[2,4−7] In native endothelial cells, activation of G_i protein coupled cell membrane receptors responding to serotonin or to catecholamines leads to increased activity of endothelial NO synthase (eNOS) with a resulting larger release of nitric oxide (NO) to cause relaxation of the underlying vascular smooth muscle.[8−11] Regenerated endothelial cells are dysfunctional because of loss of function of these G_i proteins (Figure 1).[1,9,12] Therefore serotonin, released by activated platelets, in regions lined with such dysfunctional endothelial cells will cause contraction of the underlying vascular smooth muscle, rather than activating a protective response, hence promoting vasospasm, platelet aggregation, coagulation and local inflammation.[1,9,12−14]

Primary cultures derived from regenerated porcine coronary arterial endothelial cells exhibit a different genomic profile compared to those derived from native endothelium of the same hearts.[15] In regenerated endothelial cells the down-regulation of cyclooxygenase-1 (source of prostacyclin) and eNOS (source of NO) will diminish the production of these two vasodilator and antiplatelet molecules. Genes responsible for encoding proteins [superoxide dismutase (Mn type), glutathione peroxidase 3 and thioredoxin reductase] assuming

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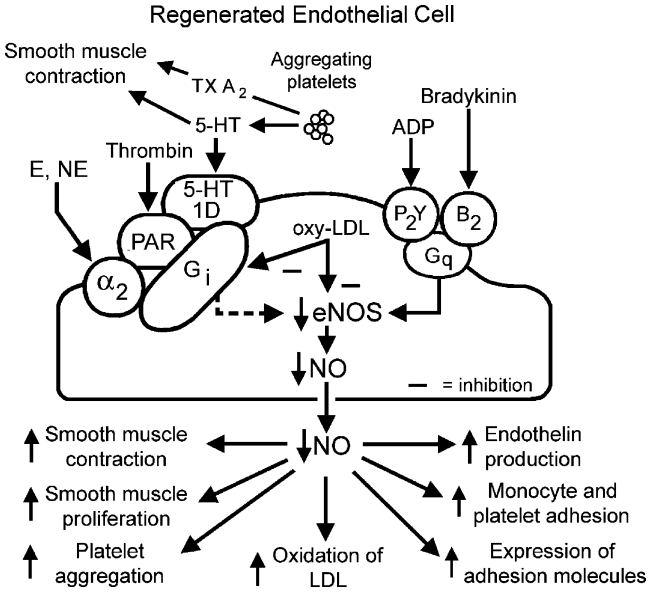

Figure 1. Effects of oxidized low-density lipoproteins (oxy-LDL) in a regenerated endothelial cell, resulting in the selective dysfunction of G_i proteins and reduced release of nitric oxide (NO). Abbreviations: 5-HT, serotonin receptor; B, bradykinin receptor; P, purinoceptor; G, coupling proteins (From Vanhoutte et al 2009. By permission).[9]

the defense against oxidative stress are down-regulated in regenerated endothelial cells.[15,16] The latter exhibit an exaggerated production of reactive oxygen species (ROS).[17−19] The resulting augmented oxidative stress can damage the cells directly through oxidation of several cellular proteins. In particular, the produced superoxide anions bind to NO produced by eNOS to form peroxynitrite,[20,21] which further promotes endothelial dysfunction.

The most striking genomic change in cultures derived from regenerated endothelium is the marked mRNA overexpression of adipocyte fatty acid binding protein (A-FABP).[15] A-FABP facilitates the binding and endocytosis of free fatty acids in macrophages.[22,23] At the early stages of atherosclerosis, macrophages are recruited by the endothelial cells and migrate through the endothelial barrier toward the smooth muscle. The increased expression/presence of A-FABP would allow excessive uptake of LDL-cholesterol by the macrophages to form foam cells, the hallmark of atherogenesis. The macrophages also release ROS, but regenerated endothelial cells lack the protective mechanisms against oxidative stress, resulting in more damage and more recruitment of macrophages and allowing the propagation of the fatty streak formation with subsequent intimal thickening and formation of the atherosclerotic plaque.[12,24,25]

The present study was designed to test the hypothesis that chronic in vivo treatment with either an antioxidant agent (apocynin) or an inhibitor of A-FABP (BMS309403) prevents the dysfunction (as well as the resulting thickening of the intima-medial layer) associated with endothelial regeneration.

RESULTS AND DISCUSSION

Functional Studies. *Acute Effects.* First, the acute effect, if any, of in vitro exposure to the inhibitors used for the chronic treatment was determined on endothelium-dependent (bradykinin and serotonin) and endothelium-independent (detaNONOate and isoproterenol) relaxations of porcine coronary artery rings with native or regenerated endothelium. The relaxations to increasing concentrations (10^{-9} to 10^{-6} M) of serotonin [in the presence of ketanserin (10^{-6} M) to prevent activation of vascular smooth muscle[7]] were significantly smaller, without a change in EC_{50}, in rings with regenerated endothelium than in those containing native endothelial cells

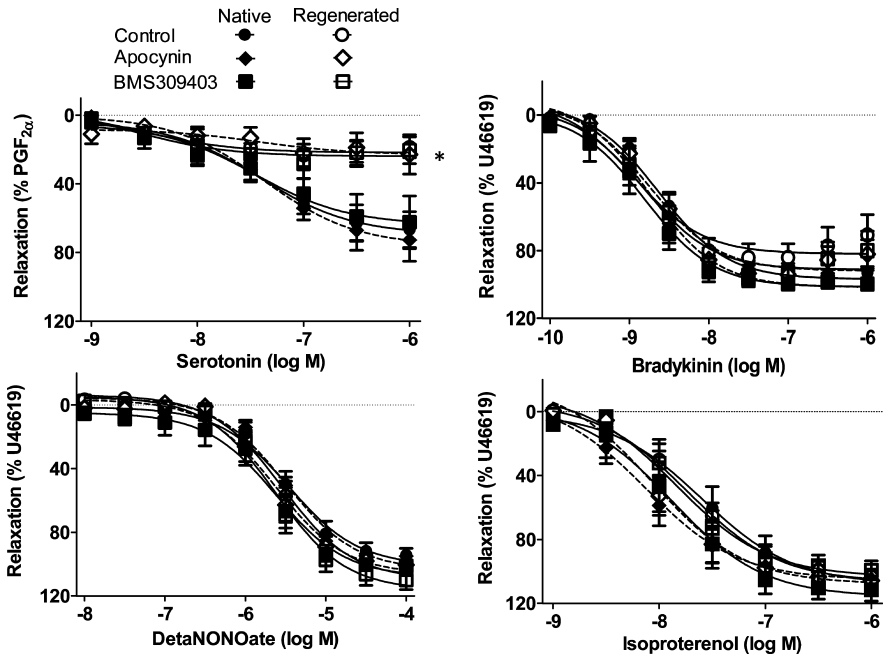

Figure 2. Lack of acute effects of apocynin (10^{-4} M) and BMS309403 (3×10^{-6} M) on the relaxation to serotonin (10^{-9} to 10^{-6} M; upper left), bradykinin (10^{-10} to 10^{-6} M; upper right), detaNONOate (10^{-8} to 10^{-4} M; lower left), and isoproterenol (10^{-9} to 10^{-6} M; lower right) in rings of porcine coronary artery lined with native or regenerated endothelium. The rings with endothelium were incubated with or without the drugs for 40 min. The relaxations are expressed as percentage of the precontraction and shown as means ± SEM; $n = 5–6$; *$P < 0.05$ Native vs Regenerated.

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Table 1. EC_{50} and Maximal Response to Serotonin (10^{-9} to 10^{-6} M), Bradykinin (10^{-10} to 10^{-6} M), detaNONOate (10^{-8} to 10^{-4} M), and Isoproterenol (10^{-9} to 10^{-6} M) in Porcine Coronary Arteries with Native or Regenerated Endothelium, in Control Solution or after Acute Incubation (30 minutes) with Apocynin (10^{-4} M) or BMS309403 (3×10^{-6} M)[a]

		EC_{50} (M)					
		control		apocynin		bms309403	
		native	regenerated	native	regenerated	native	regenerated
serotonin	mean	4.85×10^{-8}	6.63×10^{-8}	4.42×10^{-8}	2.27×10^{-8}	$1.88 \times 10{-}7$	5.9×10^{-8}
	SEM	1.4×10^{-8}	3.96×10^{-8}	1.87×10^{-8}	6.44×10^{-9}	6.42×10^{-8}	4.33×10^{-8}
bradykinin	mean	2.76×10^{-9}	1.98×10^{-9}	2.38×10^{-9}	2.46×10^{-9}	2.27×10^{-9}	2.35×10^{-9}
	SEM	5.54×10^{-10}	3.94×10^{-10}	4.22×10^{-10}	7.27×10^{-10}	$5.69 \times 10{-}10$	$8.84 \times 10{-}10$
DetaNONOate	mean	3.72×10^{-6}	3.19×10^{-6}	3.00×10^{-6}	2.87×10^{-6}	$2.46 \times 10{-}6$	$2.72 \times 10{-}6$
	SEM	8.17×10^{-7}	7.85×10^{-7}	3.71×10^{-7}	9.74×10^{-7}	$8.11 \times 10{-}7$	$8.47 \times 10{-}7$
isoproterenol	mean	4.14×10^{-8}	2.75×10^{-8}	2.12×10^{-8}	1.73×10^{-8}	2.11×10^{-8}	2.2×10^{-8}
	SEM	2.17×10^{-8}	7.54×10^{-9}	1.32×10^{-8}	8.31×10^{-9}	7.71×10^{-9}	6.92×10^{-9}

		maximal relaxation (%)					
		control		apocynin		BMS309403	
		native	regenerated	native	regenerated	native	regenerated
serotonin	mean	73.1	33.1*	86.1	31.4*	82.7	26.3*
	SEM	16.5	19.8	14.2	8.4	16.5	5.6
bradykinin	mean	94.4	89.4	97.1	89.5	95.8	91.7
	SEM	3.5	17.0	1.1	5.4	2.5	7.3
DetaNONOate	mean	91.1	106.3	96.7	100.0	108.7	112.4
	SEM	4.2	4.7	2.0	3.0	14.5	6.5
isoproterenol	mean	108.2	101.7	100.6	108.1	105.3	98.5
	SEM	7.4	5.6	2.6	4.0	5.1	6.2

[a]The data are shown in mol L^{-1} (EC_{50}) or as a percentage of the reference contraction to 60 mM potassium chloride. $n = 5{-}7$; *$P < 0.05$ Native vs. Regenerated.

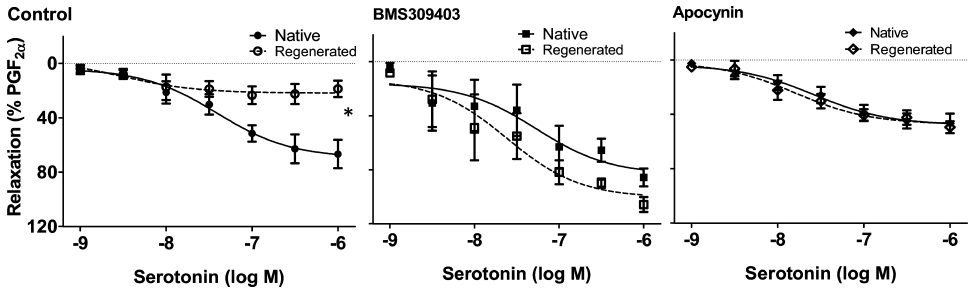

Figure 3. Chronic effects of vehicle (Control; left), BMS309403 (1.5 mg/kg/day; middle) and apocynin (4 mg/kg/day; right) on the relaxation to serotonin (10^{-9} to 10^{-6} M) in porcine coronary arterial rings with native or regenerated endothelium. The concentration-relaxation curves are expressed as percentage of the precontraction and shown as means ± SEM; $n = 5{-}6$; *$P < 0.05$ Native vs Regenerated.

(Figure 2; Table 1). By contrast, relaxations to another endothelium-dependent vasodilator [bradykinin (10^{-10} to 10^{-6} M)] were comparable in the two types of preparations (Figure 2; Table 1). Bradykinin is a peptide which is part of the kallikrein-kinin system.[26] It activates endothelial B_2-kinin receptors.[27] When endothelial B_2 receptors are activated, G_q protein coupling to the phospholipase C pathway increases the intracellular calcium concentration, the phosphorylation of eNOS and thus the production of NO. In addition, the increase in calcium also stimulates phospholipase A_2 and the downstream release of vasodilator prostaglandins as well as it initiates endothelium-dependent hyperpolarizations.[28,29] The present findings thus confirm the previous demonstration[30,31] that endothelium-dependent relaxations of coronary arteries with regenerated endothelium that involve the G_i protein mediated pathway, in particular those to serotonin, are blunted selectively, whereas the G_q protein mediated endothelium-dependent relaxations, such as those in response to bradykinin, remain unchanged (Figure 1).[7,31,32] Previous findings concluded that the decrease in relaxation to serotonin is due mainly to reduced activity rather than absence or reduced levels of G_i proteins to induce activation of eNOS.[32] By contrast, the relaxation pathways in the vascular smooth muscle cells are preserved in preparations with regenerated endothelium, as indicated by the lack of differences in the response to detaNONOate (10^{-8} to 10^{-4} M) and isoproterenol (10^{-9} to 10^{-5} M) (Figure 2). DetaNONOate stimulates soluble guanylyl cyclase increasing the production of cyclic guanosine monophosphate (cGMP) which causes relaxation of vascular smooth muscle by opening of cyclic nucleotide gated potassium channels, hyperpolarization and activation of protein kinase G; these actions concur to reduce the intracellular calcium concentration and inhibit the contractile process. The β-adrenergic agonist isoproterenol activates adenylyl cyclase producing cyclic adenosine monophosphate (cAMP) which in turn phosphorylates protein kinase A, also leading to a reduction in intracellular calcium and inhibition of the contractile proteins.[33] In certain arteries, isoproterenol causes

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Table 2. EC_{50} and Maximal Response to Serotonin (10^{-9} to 10^{-6} M), Bradykinin (10^{-10} to 10^{-6} M), detaNONOate (10^{-8} to 10^{-4} M), and Isoproterenol (10^{-9} to 10^{-6} M) in Control Porcine Coronary Arteries with Native or Regenerated Endothelium or after Chronic Treatment with Apocynin (4 mg/kg/day) and BMS309403 (1.5 mg/kg/day)[a]

		EC_{50} (M)					
		control		apocynin		BMS309403	
		native	regenerated	native	regenerated	native	regenerated
serotonin	mean	4.24×10^{-8}	5.58×10^{-8}	3.75×10^{-8}	2.49×10^{-8}	4.68×10^{-7}	1.03×10^{-7}
	SEM	1.33×10^{-8}	3.4×10^{-8}	1.02×10^{-8}	9.55×10^{-9}	4.44×10^{-7}	1.7×10^{-8}
bradykinin	mean	2.62×10^{-9}	1.89×10^{-9}	3.47×10^{-9}	2.86×10^{-9}	5.56×10^{-9}	1.49×10^{-8}
	SEM	7.45×10^{-10}	5.91×10^{-10}	1.56×10^{-9}	1.1×10^{-9}	1.53×10^{-9}	4.83×10^{-9}
DetaNONOate	mean	3.72×10^{-6}	3.19×10^{-6}	2.77×10^{-6}	2.18×10^{-6}	3.91×10^{-6}	3.98×10^{-6}
	SEM	8.17×10^{-7}	7.85×10^{-7}	6.93×10^{-7}	5.26×10^{-7}	9.72×10^{-7}	7.15×10^{-7}
isoproterenol	mean	4.97×10^{-8}	2.75×10^{-8}	2.43×10^{-8}	2.65×10^{-8}	3.75×10^{-8}	3.13×10^{-8}
	SEM	2.46×10^{-8}	7.54×10^{-9}	8.24×10^{-9}	5.14×10^{-9}	6.66×10^{-9}	6.01×10^{-9}

		maximal relaxation (%)					
		control		apocynin		BMS309403	
		native	regenerated	native	regenerated	native	regenerated
serotonin	mean	73.1	33.1*	47.1	48.7	73.9	77.7
	SEM	16.5	19.8	13.1	6.8	6.9	25.0
bradykinin	mean	94.4	89.4	81.9	74.8	97.1	82.1
	SEM	3.5	17.0	9.2	2.8	3.0	13.0
DetaNONOate	mean	91.1	106.3	99.1	103.3	96.9	112.2
	SEM	4.2	4.7	1.6	3.6	2.4	8.0
isoproterenol	mean	108.2	101.7	106.2	106.3	105.6	96.1
	SEM	7.4	5.6	5.5	3.3	4.1	3.9

[a]The data are shown in mol L^{-1} (EC_{50}) or as a percentage of the reference contraction to 60 mM potassium chloride. $n = 5–7$; *$P < 0.05$ Native vs. Regenerated.

relaxations/dilatations that are partly endothelium-dependent (e.g., ref 34). This can be attributed to background basal [or in vivo shear stress induced] release of NO that amplifies the relaxation due to the production of cyclic AMP in the vascular smooth muscle cells. However, under the current experimental conditions, this is not the case for the porcine coronary artery and only the vascular smooth muscle cells are the target of the β-adrenergic agonist. The present results thus confirm that the function of the vascular smooth muscle cells is not significantly affected by the endothelial regeneration process.[30,31]

Incubation for 30 min with apocynin [10^{-4} M; antioxidant and inhibitor of NADPH oxidase[35,36]] or BMS309403 [3×10^{-6} M; A-FABP inhibitor[23,37]] had no significant effect on the responses, in terms of EC_{50} or maximal response, of porcine coronary arteries with either native or regenerated endothelium (Figure 2; Table 1) to serotonin, bradykinin, detaNONOate, and isoproterenol. Therefore, any effect (shown below) observed with these inhibitors after chronic treatment cannot be attributed to acute pharmacological actions per se of these agents.

Chronic Effects. Serotonin: G_i Protein Mediated Relaxation. The major finding of the present study is that chronic treatment with either apocynin or BMS309403 prevented the difference in relaxation to serotonin between porcine coronary artery rings with native and regenerated endothelium (Figure 3; Table 2), indicating that their targets play a key role in the selective loss of G_i protein mediated responses in the latter.[31,38] A summary of the EC_{50} and maximal relaxation values are listed in Table 2.

Apocynin, originally viewed as a selective NADPH (NOX4) inhibitor, is a potent antioxidant.[35,36] Relaxations to increasing concentrations of serotonin of coronary artery rings with native endothelium were not significantly different in the control group and after chronic treatment with apocynin (4 mg/kd/day; Figure 3; Table 2), indicating that in the native endothelial cells the production of ROS is not sufficient to interfere majorly with NO bioavailability.[39−41] However, the chronic treatment with the antioxidant abolished the difference in response to serotonin between preparations with native and regenerated endothelium (Figure 3; Table 2). The chronic effect of apocynin contrasts with the observation that the antioxidant does not acutely affect the responses to serotonin in arteries with regenerated endothelium (Figure 2; Table 1). This then suggests that apocynin exerts its effects at the genomic level. Earlier studies reveal that, in the regenerated endothelium, the protein presences of eNOS and Akt (an activator of eNOS) are reduced, while genes favoring oxidative stress are up-regulated.[15] The present findings, in conjunction with these earlier observations, strengthen the conclusion that the increased production of ROS plays a key role in the genomic changes leading to the dysfunction of G_i proteins in regenerated endothelium.[15,17] ROS are key players in the pathogenesis of atherosclerosis.[42] The recruitment of macrophages and the endocytosis of low density lipoprotein generate oxidized low density lipoprotein (oxy-LDL) and ROS, which contribute to the oxidative stress.[43] This not only causes oxidative damage to the eNOS pathway,[44] but by interacting with transcription factors (nuclear factor-κb and activator protein-1) promotes the generation of pro-inflammatory adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1) and cytokines (interleukin-6, tissue necrosis factor-α).[42] Oxy-LDL reduces the activity of G_i proteins and the subsequent release of NO.[45,46] Along with previous findings demonstrating the loss of G_i protein functions in regenerated endothelium,[32] the present results demonstrate that in vivo prevention of oxidative stress maintains the

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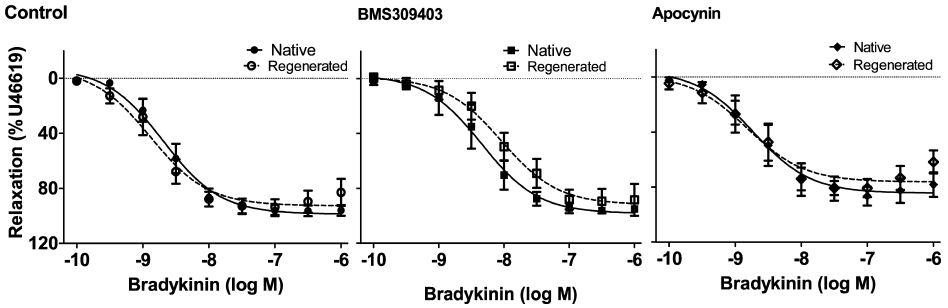

Figure 4. Chronic effects of vehicle (Control; left), BMS309403 (1.5 mg/kg/day; middle), and apocynin (4 mg/kg/day; right) on the relaxation to bradykinin (10^{-10} to 10^{-6} M) in porcine coronary arterial rings with native or regenerated endothelium. The concentration-relaxation curves are expressed as percentage of the precontraction and shown as means ± SEM; n = 5–6.

function of the G_i proteins during the endothelial regeneration process in the porcine coronary artery.

The present experiments demonstrate that chronic treatment with 1.5 mg/kg/day of the A-FABP inhibitor BMS309403[23,37] prevents the blunting of the relaxation to serotonin in regenerated endothelial cells compared to the response observed in rings with native endothelium (Figure 3); this is also observed in the maximal responses in Table 2. This finding strongly suggests that the adipokine plays an essential role in the selective loss of G_i protein activity in regenerated endothelial cells. This conclusion is consistent with the findings that in vivo treatment of $ApoE^{-/-}$ mice with BMS309403 prevents the vascular dysfunction that parallels the progressive overexpression of A-FABP in endothelial cells and that in vitro incubation with the inhibitor restores eNOS phosphorylation in human cell lines overexpressing the gene encoding for the adipokine.[11] It is tempting to speculate that the augmented intracellular lipid levels resulting from the overexpression of A-FABP stresses the endoplasmic reticulum with consequent activation of pro-inflammatory pathways, increased oxidative stress and hence augmented oxidation of lipids.[23] The higher intracellular levels of oxy-LDL in turn inactivate G_i proteins and their ability to enhance the release of NO.[45,46] This interpretation implies that the augmented presence of A-FABP precedes the increase in oxidative stress causing the selective dysfunction in regenerated endothelial cells. The observations that BMS309403 potentiates, while apocynin only protects the response to the monoamine in preparations with regenerated endothelium favors the primary importance of A-FABP in causing the selective dysfunction of the G_i proteins.

Bradykinin: G_q Protein Mediated Relaxation. The relaxations to increasing concentrations of bradykinin were not significantly different between preparations with native or regenerated endothelium of the control, apocynin-treated or BMS309403-treated pigs (Figure 4), which suggests that under the present experimental conditions oxidative stress or A-FABP overexpression do not majorly affect G_q protein mediated endothelium-dependent responses[31] during the regeneration process.

Endothelium-Independent Relaxations. The relaxations to detaNONOate and isoproterenol were not significantly different in preparations with native or regenerated endothelium of the different treatment groups (Figure 5). These observations suggest that changes in smooth muscle responsiveness cannot explain the enhanced response to serotonin observed in rings with regenerated endothelium of the BMS309403-treated group.

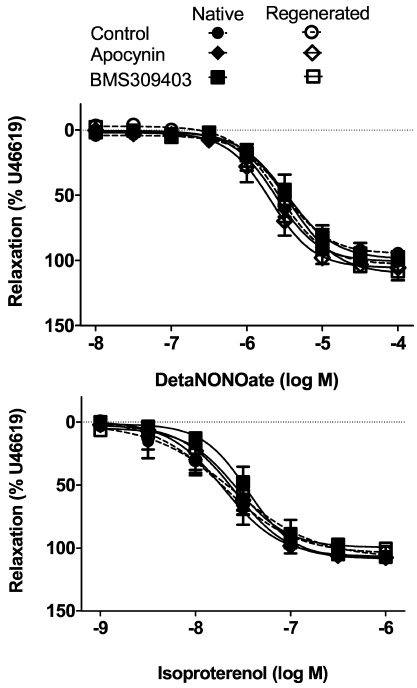

Figure 5. Lack of chronic effects of vehicle (Control), apocynin (4 mg/kg/day), and BMS309403 (1.5 mg/kg/day) on the relaxation to detaNONOate (10^{-8} to 10^{-4} M; upper) and isoproterenol (10^{-9} to 10^{-6} M; lower) in porcine coronary arterial rings with native or regenerated endothelium. The concentration–relaxation curves are expressed as percentage of the precontraction and shown as means ± SEM; n = 5–6.

Histological Analysis. The analysis of the cross-sectional area showed that intima-medial thickening had occurred in the arteries with regenerated endothelium (Figure 6), in confirmation of earlier observations.[31] The chronic treatment with either apocynin or BMS309403 prevented the intima-medial thickening observed in rings with regenerated endothelium of the control animals (Figure 6). The lack of intimal thickening observed with the antioxidant and the inhibitor of A-FABP is in line with the preserved endothelium-dependent responsiveness to serotonin and illustrates the causal role of endothelial dysfunction due to the selective loss of G_i protein mediated responses in the initiation of the atherosclerotic process.[12]

■ CONCLUSION

The present results demonstrate that adipocyte fatty acid binding protein (A-FABP) and oxidative stress play a key and sequential role in causing both the loss of G_i protein mediated

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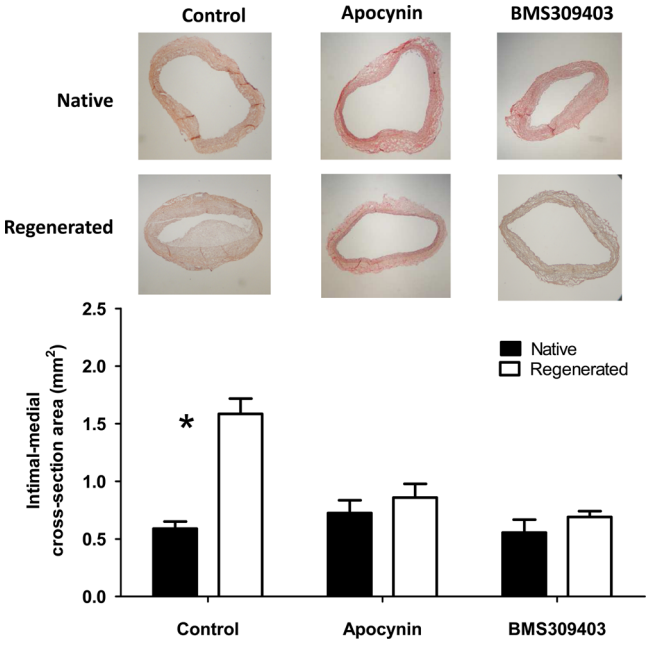

Figure 6. Cross-sectional area of the intima-medial layer of porcine coronary arteries with native (black bars) or regenerated (white bars) endothelium after chronic treatment for 28 days with vehicle (Control), apocynin (4 mg/kg/day) and BMS309403 (1.5 mg/kg/day). Upper: Representative pictures of the arteries stained with modified Curtis's Ponceau. Lower: Quantification of the cross-sectional area of the intima-medial layer in the different treatment groups. The data are shown as means ± SEM; n = 5–6; *P < 0.05 Native vs Regenerated.

relaxations and the thickening of the intima-medial layer following endothelial regeneration. Inhibiting A-FABP and reducing endothelial oxidative stress may represent privileged therapeutics targets to preserve endothelial function and prevent atherosclerosis.

METHODS

The present studies were approved by the Institutional Animal Care Committee of the University of Hong Kong.

Source and Holding Conditions. Female pigs (3–4 months old, weighing between 25 and 35 kg) were purchased from local farms in Hong Kong and housed in the Laboratory Animal Unit of the University of Hong Kong. The animals were kept for a week to familiarize them with the holding facility before angioplasty was performed. During their stay, the animals were fed with laboratory chow (Labdiet, St Louis, MO) and water ad libitum and were kept at 21 ± 1 °C in a 12 h light/dark cycle.

Percutaneous Transluminal Coronary Angioplasty. Once the pigs were acclimatized, they were anesthetized with an intramuscular injection of Zoletil (2 0 mg/kg) plus xylazine (1.5 mg/kg); anesthesia was maintained with isoflurane (2%) mixed with breathing gases. A coronary guiding catheter (Cordis Webster, Baldwin Park, CA) was inserted into the carotid or femoral artery with the help of an introducer, which was then advanced to the opening of the coronary artery in the aorta. An intravenous injection of heparin sodium (30 μg/kg) was made. Then the balloon catheter was inserted into the guiding catheter along with the guide wire to the left anterior descending artery (LAD). Endothelial denudation was achieved by inflating the balloon (diameter of 2.52 mm, balloon to artery ratio of about 1.2:1, pressure 8 bar). Approximately 2 cm of the LAD distal to the first branch were denuded. After the procedure, an angiogram was taken to ensure that no immediate vasospasm had occurred in the denuded region. The animals were then given antibiotics and analgesics and observed until they recovered from the anesthesia.

Drug Treatment. For the acute studies, all pigs were fed with control diet without drug treatment. In the chronic study, the pigs were assigned randomly to the control group or a drug treatment group. In the drug treatment groups apocynin (4 mg/kg/day) or BMS309403 (1.5 mg/kg/pig) were mixed with chow and fed to the pigs once a day for 28 days. In the control group, the pigs were fed with the chow only.

Tissue Collection and Isolation. Twenty eight days after angioplasty, the pigs were anesthetized and euthanized with an intramuscular injection of Zoletil plus xylazine. The heart was harvested and immediately immersed in ice-cold modified Krebs-Ringer bicarbonate buffer of the following composition: 120 mM NaCl, 25 mM $NaHCO_3$, 5.5 mM glucose, 4.76 mM KCl, 1.18 mM $MgSO_4 \cdot 7H_2O$, 1.18 mM $NaH_2PO_4 \cdot 2H_2O$, and 2.5 mM $CaCl_2 \cdot 2H_2O$ (control solution). The region of the LAD that underwent angioplasty (lined with regenerated endothelium) and the left circumflex coronary artery (LCX; lined with native endothelium) of the same hearts were cleared of fat and adventitial connective tissue and cut into rings (approximately 3 mm in length) for functional studies in organ chambers or for histological analysis.

Isometric Tension Recording. Rings, with either native or regenerated endothelium of the same hearts, were equilibrated for 1 h in organ chambers containing control solution aerated with 95% oxygen and 5% carbon dioxide (Hong Kong Oxygen & Acetylene Co., Ltd., Hong Kong) and maintained at 37 °C by means of a water jacket. They were suspended in the solution between two metal hooks, of which the top one was connected to a force transducer (AD Instruments, Sydney, Australia) for isometric tension recording (PowerLab, AD Instruments). The rings were allowed to equilibrate at optimal basal tension (2 g) for 60 min. They were then exposed to 60 mM potassium chloride twice before the actual experiment. The resulting increase in tension was used as reference contraction.

For the acute studies, coronary arterial rings (with native or regenerated endothelium) were incubated for 40 min in control solution with or without apocynin (10^{-4} M) or BMS309403 (3×10^{-6} M). To study relaxations to serotonin (Shimokawa, Flavahan et al. 1989),[30] the rings were contracted with the individual concentration of prostaglandin $F_{2\alpha}$ (10^{-7} M to 3×10^{-6} M) which caused approximately 50% of the reference contraction to KCl; in the control group, the increase in tension in responses to prostaglandin $F_{2\alpha}$ averaged 2.19 ± 0.2 and 2.3 ± 0.2 g in preparations with native and regenerated endothelium, respectively. To study relaxations to the other vasodilators, the preparations were exposed to the TP receptor agonist U46619 (3×10^{-8} M);[47−49] the contractions to U46619 averaged 4.13 ± 0.3 and 4.4 ± 0.3 g in control preparations with native and regenerated endothelium, respectively. The contractions to either prostaglandin $F_{2\alpha}$ or U46619 were not significantly different in preparations of the treatment groups compared to control (data not shown). Once the contractions had reached a plateau, cumulative concentration-relaxation curves were obtained for serotonin [10^{-9} to 10^{-6} M; in the presence of ketanserin (10^{-6} M)], bradykinin (10^{-9} to 10^{-6} M), detaNONOate (10^{-10} to 10^{-6} M), or isoproterenol (10^{-9} to 10^{-6} M). For the study of coronary arteries from pigs treated chronically with drugs or control diet only, the experimental procedure was the same for the acute study, except for the incubation with apocynin or BMS309403.

Cross Section Area of the Intima-Medial Layer. Sections of the region of the LAD with endothelial denudation and of the LCX at approximately the same distance from the aorta were compared. The rings were frozen in liquid nitrogen and stored at −80 °C before being embedded in optimal cutting temperature compound (Sakura, Torrance, CA) and then sliced into sections (5 μM thick). The sections were stained with modified Curtis's Ponceau solution and counter stained with Mayer's hematoxylin solution to identify the collagen in the basal membranes. The intima-medial layer was identified as the region between the lumen and the basal membrane outside of the medial layer. The image was captured at ×2 using a microscope (Olympus, Hong Kong) and analyzed with a computer package (Olympus).

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Calculation and Data Analysis. All contractions are expressed as a percentage of the second reference contraction to 60 mM KCl obtained at the beginning of the experiment. The relaxations are expressed as percentage decreases in tension from the maximal contraction level to U46619 or prostaglandin $F_{2\alpha}$. The results are presented as means ± standard error of mean (SEM); *n* refers to the number of experimental animals tested. The results were analyzed statistically by two-way ANOVA and Bonferroni's post hoc test. The results were calculated, analyzed and graphed by the computer packages, Excel 2007 (Microsoft, Redmond, WA) or Prism version 5 (GraphPad Software, La Jolla, CA). When *P* was less than 0.05, the differences were considered to be statistically significantly different.

Drugs and Chemicals. BMS309403 was synthesized as described previously.[11] U46619 [(*Z*)-7-[(1*S*,4*R*,5*R*,6*S*)-5-[(*E*,3*S*)-3- hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid] was purchased from Biomol International (Plymouth, PA). Apocynin was purchased from Calbiochem Biochemicals (San Diego, CA). All other chemicals were purchased from Sigma Chemicals (Shanghai, China).

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Author Contributions

C.K.C. performed most of the experiments, analyzed the data, and drafted the manuscript. Y.Z. performed some of the experiments. S.Y.L. and Y.L.Z. performed the angioplasty. M.Y.K.L., A.X., and H.F.T. provided advice for the experimental design (A.X. also provided BMS309403 for the chronic treatment). P.M.V. was responsible for the experimental design and the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

A-FABP, adipocyte fatty acid binding protein; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; ROS, reactive oxygen species

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Due to a production error, this paper was published on the Web on October 2, 2012, with the second author's last name misspelled. The corrected version was reposted on October 5, 2012.