Protective effects of histamine on $G_\text{q}$-mediated relaxation in regenerated endothelium.

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Running head: protection of bradykinin relaxation

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Abstract

In the porcine coronary artery, regenerated endothelium is dysfunctional as regards the responses to endothelium-dependent agonists. The current study aimed to determine the possible involvement of histamine in such dysfunction. Pigs were treated chronically with pyrilamine (H₁ receptor inhibitor, 2mg/kg/day) with part of their coronary endothelium and allowed to regenerate for 28 days after balloon denudation. The results showed a reduction in relaxation to bradykinin (Gq protein dependent) only in the pyrilamine treated group [area under the curve (AUC) 269.7 ± 13.4 vs 142.0 ± 31.0, native endothelium vs regenerated endothelium] but not in the control group (253.0 ± 22.1 vs 231.9 ± 29.5, native endothelium vs regenerated endothelium). Whereas the differences in the relaxation to serotonin (Gi protein dependent) between native and regenerated endothelium were not affected by the pyrilamine treatment (control group 106.3 ± 17.0 vs 55.61 ± 12.7 and pyrilamine group 106.0 ± 8.20 vs 49.30 ± 6.31, native endothelium vs regenerated endothelium). These findings indicate that during regeneration of the endothelium the activation of H₁ receptors by endogenous histamine may be required in order to maintain the endothelium-dependent Gq protein-mediated relaxation to bradykinin, suggesting a beneficial role of the monoamine in the process of endothelial regeneration.
Introduction

The turnover of endothelial cells is slow, but can be accelerated when exposed to excessive stress, such as hypoxia, oxidative stress or detrimental shear stress. The apoptotic, dying endothelial cells will detach from the internal elastic membrane and be washed away by the flowing blood, which liberates adjacent endothelial cells from “contact inhibition”, thus permitting mitosis and regeneration (28, 35, 36). Although the regenerated endothelial cells form a new monolayer lining, and can produce nitric oxide (NO), they are dysfunctional and constitute a weaker spot in the intima which favors the occurrence of the inflammatory response that ultimately will lead to the atherosclerotic plaque (13, 26, 36).

Primary cultures derived from regenerated endothelial cells in the porcine coronary artery exhibit a significantly different genomic profile compared to those derived from native endothelium (13). The genes responsible for anti-oxidative mechanisms [including superoxide dismutase (Mn type) and glutathione peroxidase 3] and expression of tissue factor pathway inhibitor (TFPI) were down-regulated (13). These changes can lead to augmented oxidative stress, and causes the cells to become pro-thrombotic, which possibly increase the production of tissue factor and can subsequently lead to fibrin deposition (5, 21, 29).

Histamine is synthesized from histidine by histidine decarboxylase and rapidly degraded by histamine N-methyltransferase or diamine oxidase (16) unless stored in mast cells and basophils (33). Upon stimulation, histamine is released to carry out a diverse range of functions as pro-inflammatory chemokine (16). In particular, histamine
contributes to the regulation of vascular tone. When it binds to H₁ receptors on vascular smooth muscle cells vasoconstriction results (17, 31). Acute activation of H₁ receptors on the endothelium leads to increased production of nitric oxide (NO) and endothelium-dependent vasodilatation (12, 31).

Mast cells are a major source of histamine and are associated with atherosclerotic lesions (1, 8). Genetically engineered animals with histamine deficiency exhibited reduced atherosclerosis (38). Human studies also showed the presence of activated mast cells is associated with atherosclerotic plaque instability (11). During the atherosclerotic process, tissue factor is released and exacerbates intimal thickening (29). In regenerated endothelial cells, the expression of tissue factor pathway inhibitor (TFPI) is down-regulated (13), implying that unchecked activity of tissue factor may contribute to the occurrence of endothelial dysfunction upon regeneration. Histamine may also contribute to the progression of the process since it induces the release of tissue factor, but not that of its physiological antagonist TFPI (29). Therefore, the present experiments were designed to test the hypothesis that chronic in vivo treatment with pyrilamine (H₁ receptor antagonist) prevents the dysfunction, as well as the resulting thickening of the intima-medial layer, associated with endothelial regeneration in the porcine coronary artery. The experiments were performed in parallel with another study (6) of which the untreated control group was used for statistical comparison with the present results obtained in pyrilamine-treated pigs.
**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 fed with laboratory chow (Labdiet, St Louis, MO, USA) and water *ad libitum*. They were kept at 21 ± 1°C in a 12 hour light and 12 hour dark cycle.

Under anesthesia (isoflurane, 2 %) mixed with breathing gases, the endothelium of approximately 2 cm of the left anterior descending artery (immediately distal to the first branch) was removed by balloon angioplasty, as described (6, 25) An angiogram was taken to ensure that no immediate vasospasm occurred in the denuded region. The animals were then given antibiotics and analgesics and observed until they recovered from the anesthesia. After recovery, they were randomly assigned to be treated once a day with pyrilamine maleate [2 mg/kg/day, based on the recommended daily dose for humans and the 1:1 conversion ratio for human to pig according to the FDA (http://www.fda.gov/downloads/Drugs/Guidances/UCM078932.pdf)] which was mixed with chow, n = 5) or control (n = 6) and for twenty eight days; earlier work has demonstrated that the regenerated endothelial lining is complete after four weeks, endothelial dysfunction is fully established and the atherosclerotic process has commenced (25, 26). The pigs were anesthetized and euthanized with an intramuscular injection of zoletil (20mg/kg) and xylazine (1.5mg/kg). The hearts were harvested and immersed in ice-cold Krebs-Ringer bicarbonate buffer of the following composition: 120
mM NaCl, 25 mM NaHCO₃, 5.5 mM glucose, 4.76 mM KCl, 1.18 mM MgSO₄·7H₂O, 1.18 mM NaH₂PO₄·2H₂O, and 2.5 mM CaCl₂·2H₂O (control solution). The previously denuded left anterior descending artery (identified using the angiogram obtained during the denudation procedure) and the left circumflex artery were isolated and the fat and connective tissue of the adventitia was removed. The arteries were cut into rings (approximately 3mm in length) for functional studies in organ chambers and histological examination.

Isometric Tension Recording

Rings, with either native or regenerated endothelium of the same hearts, were equilibrated for one hour 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. 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 minutes. They were then exposed to 60 mM potassium chloride twice before the actual experiment to obtain a reference contraction.

The direct pharmacological effect of pyrilamine on vascular responsiveness was assessed in acute in vitro studies, whereby coronary arterial rings (with native or regenerated endothelium) of six untreated pigs were incubated for 40 minutes in
control solution with or without pyrilamine [30 minutes, $10^{-6}$ M; H$_1$-histaminergic antagonist (7)]; the rings were contracted with the TP receptor agonists prostaglandin $F_2\alpha$ ($3 \times 10^{-6}$ M, for the response to serotonin) or U46619 ($3 \times 10^{-8}$ M, for the responses to bradykinin, detaNONOate and isoproterenol) until a plateau contraction was obtained. Then 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 preparations of the chronic pigs (control or treatment with pyrilamine), relaxations to 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) were obtained, following an identical protocol as for the acute studies except that there was no addition of pyrilamine to the organ chambers at any time.

**Intima-medial cross-sectional area**

Frozen sections (5 µM thick) of the region of the LAD with endothelial denudation and of the left circumflex coronary artery at approximately the same distance from the aorta were stained with modified Curtis's Ponceau solution and counter stained with Mayer's hematoxylin solution and compared. 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 x2 using a microscope (Olympus, Hong Kong) and analyzed with a computer package (Olympus) (6).
**Calculation and Data Analysis**

The relaxations are expressed as percentage decreases in tension from the maximal contraction level to U46619 or PGF$_{2\alpha}$. 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 one-way or two-way ANOVA with Bonferroni’s *post hoc* test. The results were calculated, analyzed and graphed by the computer packages, Excel 2007 (Microsoft, USA) or Prism version 5 (GraphPad Software, La Jolla, California, USA). The area under the concentration response curves (AUC) were calculated using Prism version 5 and the averages were analyzed and compared with Student’s t-test for unpaired observations with Prism version 5. When P was less than 0.05, the differences were considered to be statistically significantly different.

**Drugs and Chemicals**

U46619 [(Z)-7-([(1S,4R,5R,6S)-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid] was purchased from Biomol International (Plymouth, Pennsylvania, USA). All other chemicals were purchased from Sigma Chemicals (Shanghai, China).
Results

1. Lack of acute effect of pyrilamine on vascular responsiveness:

Acute *in vitro* exposure of porcine coronary arteries with either native or regenerated endothelium to pyrilamine (10^-6 M), had no significant effect on the relaxations to endothelium-dependent (bradykinin and serotonin) and endothelium-independent (DetaNONOate and isoproterenol), as demonstrated by the lack of significant differences when compared to control (Table 1a). However the preparations with regenerated endothelium showed significantly smaller 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 (34)] in rings with regenerated endothelium than in those containing native endothelial cells (Table 1a). By contrast, relaxations to another endothelium-dependent vasodilator [bradykinin (10^-10 to 10^-6 M), and endothelium-independent vasodilators [DetaNONOate (10^-8 to 10^-4 M) and isoproterenol (10^-9 to 10^-5 M)] were comparable in the two types of preparations (Table 1a). The reference contractions to high potassium were comparable in the different experimental groups (Table 1c).

2. Chronic effect of pyrilamine on vascular responsiveness:

After chronic *in vivo* treatment with pyrilamine (2mg/kg/day, 28 days), the coronary arterial preparations showed no significant difference in relaxations to detaNONOate and isoproterenol (Table 1b) compared with controls; those responses which were comparable to those obtained in the acute experiments (Table 1a). The reduced
relaxation to serotonin observed in the controls with regenerated endothelium was also present in previously denuded coronary rings of the pigs with chronic treatment with pyrilamine (Table 1b). However, unlike in the control preparations the relaxation to bradykinin was significantly reduced in the arteries with regenerated endothelium of the chronically pyrilamine-treated group (Figure 1), with approximately 50% of reduction in the area under the concentration-relaxation curves (table 1b).

3. Lack of effect of pyrilamine on intimal-medial thickening:

The analysis of the cross-sectional area showed that intima-medial thickening occurred in the arteries with regenerated endothelium (Figure 2) and was not significantly affected by the chronic treatment with pyrilamine.
Discussion

Bradykinin is a peptide that is part of the kallikrein-kinin system (4). It preferentially activates constitutive B₂-kinin receptors (15, 23). When endothelial B₂ receptors are activated, G₉ 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 A₂ and the downstream release of vasodilator prostaglandins as well as it initiates endothelium-dependent hyperpolarizations (9, 18). In hypertensive patient taking angiotensin converting enzyme (ACE) inhibitors, the bradykinin level is shown to be increased due to the reduced hydrolysis of the peptide itself, studies also demonstrated potentiating effects independent of the hydrolysis aspects (32). This further enhances the importance of this pathway in the hypertensive subjects.

Coronary arteries with regenerated endothelium maintain a normal responsiveness to bradykinin, implying that G₉ protein coupling is unaltered by the regeneration process (6, 25, 26). The current results demonstrate a major reduction in the relaxations to this peptide in rings with regenerated but not native endothelium of animals treated chronically with the H₁ antagonist, pyrilamine. Such depression was not observed upon in vitro administration of the compound, ruling out a persistent pharmacological effect from the treatment. The lack of effects of the treatment with pyrilamine on the relaxation to the endothelium-independent NO donor, detaNONOate, demonstrates that the effect of pyrilamine is limited to the endothelium and not on the
vascular smooth muscle cells. Hence, the present findings strongly suggest a beneficial role for endogenous histamine in the maintenance of a normal response to bradykinin in regenerated endothelium.

In endothelial cells histamine activates H₁ receptors with a diverse range of effects. Acutely, activation of H₁ receptors initiates endothelium-dependent relaxations of human internal mammary arteries (39) and can release prostacyclin (which causes vasodilatation and prevents platelet aggregation) from human umbilical vein (2) and human coronary artery (22) endothelial cells. Upon the activation of H₁ receptors, histamine up-regulates eNOS expression through the calcium/calmodulin-dependent protein kinase II pathway (14). In addition, histamine stimulates the expression of eNOS and cyclooxygenase-1 and releases vasoactive molecules such as prostaglandin E₂ (30). In rats, histamine attenuates lipopolysaccharide-induced liver injury (19). By contrast, chronic treatment with pyrilamine is correlated with an increased incidence of inflammation (10). The present findings do not permit to speculate further concerning the identity the specific beneficial endothelial effect(s) of histamine counteracted by the chronic exposure to pyrilamine in regenerated endothelium, explaining the blunting of the endothelium-dependent relaxation to bradykinin. For example, a chronic reduction in the production of protective endothelium-derived mediators (NO and prostacyclin) would be expected to blunt not only the relaxation to bradykinin but also to cause further deterioration of the response to serotonin, which was not the case.
By contrast to bradykinin, the response to serotonin was significantly reduced to the same extent in preparations with regenerated endothelium of the control group (6) and in those of animals treated chronically with pyrilamine, indicating that the G\textsubscript{i}-protein mediated pathway is not affected by H\textsubscript{1} receptor blockade. Previous findings concluded that the decrease in relaxation to serotonin is due mainly to reduced activity rather than a reduced protein level of G\textsubscript{i}-proteins to induce activation of the eNOS (3, 24). The lack of effect of pyrilamine on serotonin-induced relaxation is compatible with a selective modulation of G\textsubscript{q}-mediated responses by endogenous histamine.

Despite the fact that H\textsubscript{1} receptor activation augments the expression of early growth response factor-1 [a transcription factor responsible for proliferation of smooth muscle cell (37)], the present histological analysis showed no significant effects of the histamine receptor H\textsubscript{1} inhibitor on the intima-medial thickening caused by the denudation of endothelium. This then suggests a lack of involvement of endogenous histamine in the thickening process caused by endothelial regeneration. The lack of worsening by pyrilamine of the intima-medial thickening resulting from the denudation procedure also implies that the preservation of a normal response to bradykinin, unlike that to serotonin (6), does not protect the coronary wall against intimal-medial thickening in untreated animals, suggesting a minimal role for the peptide and G\textsubscript{q}-coupled activation of eNOS under normal conditions.
The present findings demonstrate that key relaxation pathways in vascular smooth muscle, due to the production of either cyclic-GMP or cyclic-AMP (20, 40), are preserved in preparations with regenerated endothelium. This is the case whether or not the smooth muscle cells are exposed (acutely or chronically) to pyrilamine, as indicated by the lack of differences in the response to detaNONOate and isoproterenol, respectively. The present results thus confirm that the function of the underlying vascular smooth muscle cells is not affected majorly by the endothelial regeneration process (25, 27) and demonstrate that the treatment of pyrilamine does not affect it either.

Conclusion

The present results suggest that the Gq-protein mediated endothelium-dependent relaxations, which remain unchanged in regenerated endothelium, are reduced by a chronic treatment with pyrilamine. Thus, endogenous histamine may exert a protective role in the vascular wall.

Acknowledgments

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Legends

Table 1: Area under the concentration-relaxation curves (AUC) to bradykinin (10^{-10} to 10^{-6} M), detaNONOate (10^{-8} to 10^{-4} M), serotonin (10^{-9} to 10^{-6} M) and isoproterenol (10^{-9} to 10^{-6} M) in rings of porcine coronary artery lined with native or regenerated endothelium with (a) acute (10^{-6} M, 30 minutes) and (b) chronic (2 mg/kg/day for 28 days) treatment with pyrilamine. The relaxations are expressed as AUC (arbitrary units). (c) The baseline contractile responses to KCl (60 mM). Results were shown as means ± SEM; n = 5-6; * P<0.05 Native vs. Regenerated.

Figure 1: Chronic effects of vehicle (Control; Upper; data from Chan, Zhao et al., 2013, by permission), pyrilamine (2 mg/kg/day; Lower) 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 pre-contraction to U46619 (3 x 10^{-6} M) and shown as means ± SEM; n = 5-6; * P<0.05 Native vs. Regenerated.

Figure 2: Cross-sectional area (cm²) of the intima-medial layer of porcine coronary arteries with native or regenerated endothelium after chronic treatment for 28 days with vehicle (Control²) and pyrilamine (2 mg/kg/day). Left: Representative pictures of the arteries stained with modified Curtis's Ponceau. Right: 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.

1 data from (6), by permission
References


(a) Acute

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<tr>
<td>Bradykinin</td>
<td>246.4 ± 20.6</td>
<td>264.2 ± 24.9</td>
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<tr>
<td>DetaNONOate</td>
<td>153.2 ± 14.0</td>
<td>158.1 ± 13.7</td>
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<td>Serotonin</td>
<td>90.95 ± 24.6*</td>
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<td>Isoproterenol</td>
<td>169.5 ± 28.6</td>
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(b) Chronic

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<tr>
<td>Bradykinin</td>
<td>253.0 ± 22.1</td>
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<td>DetaNONOate</td>
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<td>Isoproterenol</td>
<td>174.5 ± 23.9</td>
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(C) KCl contraction (grams)

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<td>Chronic</td>
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Control

Native
Regenerated

Relaxation (% U46619)

Bradykinin (log M)

Pyrilamine (2 mg/kg/day)

Native
Regenerated

Relaxation (% U46619)

Bradykinin (log M)
Control Pyrilamine

Native

Regenerated

Intimal-medial cross-section area (mm²)

* *

Native Regenerated

Control Pyrilamine

0 1 2 3

0 1 2 3