Histamine induces activation of protein kinase D that mediates tissue factor expression and activity in human aortic smooth muscle cells

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Submitted 17 May 2011; accepted in final form 17 September 2012

Hao F, Wu DD, Xu X, Cui M. Histamine induces activation of protein kinase D that mediates tissue factor expression and activity in human aortic smooth muscle cells. Am J Physiol Heart Circ Physiol 303: H1344–H1352, 2012. First published September 21, 2012; doi:10.1152/ajpheart.00500.2011.—Histamine, an inflammatory mediator, has been shown to influence the pathogenesis of vascular wall cells. However, the molecular basis of its influence is not well understood. Our data reveal that histamine markedly induces protein kinase D (PKD) activation in human aortic smooth muscle cells. PKD belongs to a family of serine/threonine protein kinases, and its function in vascular disease is largely unknown. Our data show that histamine-induced PKD phosphorylation is dependent on the activation of histamine receptor 1 and protein kinase C (PKC). To determine the role of PKD in the histamine pathway, we employed a small-interfering RNA approach to downregulate PKD expression and found that PKD1 and PKD2 are key mediators for expression of tissue factor (TF), which is the key initiator of blood coagulation and is important for thrombosis. Our results show that PKD2 predominantly mediates histamine-induced TF expression via the p38 mitogen-activated protein kinase (MAPK) pathway, whereas PKD1 mediates histamine-induced TF expression through a p38 MAPK-independent pathway. We demonstrate that histamine induces TF expression via the PKC-dependent PKD activation. Our data provide the first evidence that PKD is a new component in histamine signaling in live cells and that PKD has a novel function in the histamine signaling pathway leading to gene expression, as evidenced by TF expression. Importantly, our data reveal a regulatory link from histamine to PKD and TF, providing new insights into the mechanisms of coagulation and the development of atherosclerosis.

Histamine receptor; protein kinase activation; phosphorylation; tissue factor; vascular wall cells

HISTAMINE IS A SMALL MOLECULE amine that is mainly produced by histidine decarboxylase (HDC) from mast cells and monocyte-derived macrophages (18, 32). HDC is found in macrophage-derived foam cells (10), and HDC knockout mice show reduced neointimal thickening (22), suggesting a role of histamine in the development of vascular disease.

As a potent inflammatory molecule, histamine increases blood vessel permeability and endothelial dysfunction (21, 26) and induces relaxation (15, 36) and constriction (13) of blood vessels. Through the cross talk between calcium flow and mitogen-activated protein kinase (MAPK) induction, histamine may be a risk factor for hypertension (5). Histamine exerts its function through its four receptors (32). We reported previously that histamine induces expression of the master transcription factor Egr-1 in human aortic endothelial cells (8). Histamine also induces tissue factor (TF) expression in smooth muscle cells (SMCs) related to acute coronary syndrome (27). In addition, histamine increases the expression of cytokines such as interleukin (IL)-6 and IL-8 (4, 11). The antagonists of histamine receptor 1 (H1) abolish the effect of histamine and reduce the formation of intimal hyperplasia (14). Recently, it has been reported that histamine H1 receptor promotes the development of atherosclerotic lesions by increasing vascular permeability for low-density lipoprotein (LDL) (19).

Protein kinase D (PKD), a family of serine and threonine protein kinases, consists of PKD1, PKD2, and PKD3. PKDs have been implicated in various cellular functions (7, 9, 25). However, the relationship between histamine and PKD in live cells and the functional role of PKD in the histamine pathway have not been revealed. Furthermore, although histamine activation of vascular SMCs is well documented, the activation cascade is still unclear. In the present study, we investigated the effect of histamine on activation of PKD in human aortic smooth muscle cells (HASMCs). PKD function in histamine-regulated cellular events, and its implication in vascular diseases. Our results provide the first evidence that histamine activates PKD in living cells as evidenced by using HASMCs. Furthermore, the current study documents the functional role of PKD in histamine-induced cellular events. Importantly, our results reveal that histamine-activated PKD controls the expression of the key coagulation initiator TF and implies a new role for PKD in coagulation, atherothrombosis, and possibly other inflammatory diseases.

MATERIALS AND METHODS

Reagents. Histamine, mepyramine, and cimetidine were obtained from Sigma; pertussis toxin (PTX), SB-203580, and Ro-31–8220 were from Biomol International (Plymouth Meeting, PA). Antibodies against PKD1, PKD3, phospho (p)-PKD-activation loop (Ser\textsuperscript{738/742} of human PKD1, Ser\textsuperscript{706/710} of human PKD2, and Ser\textsuperscript{734/735} of human PKD3), p-PKD1 COOH-termini (Ser\textsuperscript{910} of human PKD1), p-extra-cellular signal-regulated kinase (ERK), p-p38 MAPK, and p-c-Jun NH\textsubscript{2}-terminal kinase (JNK) were from Cell Signaling Technology (Beverly, MA). Antibody against p-PKD2 COOH-termini (Ser\textsuperscript{876} of human) was from Millipore (Billerica, MA). Antibodies against PKD2 were from Bethyl Lab (Montgomery, TX). Antibody against β-actin was from Sigma-Aldrich (St. Louis, MO). Nonsilencing control small-interfering RNA (siRNA), PKD1 siRNA, PKD2 siRNA, and PKD3 siRNA were from Qiagen (Valencia, CA). The siRNA transfection reagent RNAi Max was from Invitrogen. Antibodies against human TF antibody and α-actin as well as TF surface activity assay kit were from American Diagnostica (Stamford, CT). Goat anti-rabbit IgG Alexa Fluor 488 was from Invitrogen (Carlsbad, CA), and rhodamine red-X-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell culture. HASMCs supplied by Cascade Biologics were cultured in Medium 231 with special SMGS supplements (Cascade...
Histamine rapidly and markedly induces activation of PKD in HASMCs. The regulatory relationship between histamine and PKD is currently unknown. To determine whether histamine activates PKD in HASMCs, we examined PKD phosphorylation in HASMCs upon stimulation with histamine using a specific antibody that recognizes phosphorylated serine residues at the activation loop common to all three isoforms of PKD (for human PKD1, at Ser738 and Ser742; for human PKD2, at Ser706 and Ser107; and for human PKD3, at Ser731 and Ser735). Phosphorylation of these serine residues correlates to PKD catalytic activity (20). Cultured HASMCs were starved for 24 h and then treated with 10 μM histamine for various times. As shown in Fig. 1, B and C, we observed that PKD activation (phosphorylation at the PKD activation loop) was markedly and rapidly induced with a peak at around 45 s to 2 min. It has been shown that phosphorylation of the serine residues at the COOH-terminus of PKD1 (Ser910 for human and Ser916 for mouse) occurs by autophosphorylation and correlates with the activation status of PKD1 (12, 34); similarly, it was found that the corresponding COOH-terminal serine in PKD2 (Ser879) was also phosphorylated during activation of PKD2 and the degree of Ser876 phosphorylation correlated to PKD2 catalytic activity (28). However, PKD3 lacks a COOH-terminal phosphorylation site; therefore, its activation status cannot be examined at COOH-terminal phosphorylation. We found that histamine rapidly and strikingly induced the phosphorylation of PKD1 (Ser910) and PKD2 (Ser876) in HASMCs with a peak at around 2–5 min (Fig. 1, B and C). The histamine-induced phosphorylation of serine residues at COOH-termini of PKD1 and PKD2 as well as the PKD activation loop declined to baseline at around 30–60 min (Fig. 1, B and C). As shown in Fig. 1, D and E, histamine dose-dependently induced phosphorylation of PKD1, PKD2, and the common PKD activation loop. These data indicate that histamine markedly induces activation of PKD1 and PKD2 in HASMCs. In the following studies, the histamine concentration of 10 μM was chosen to stimulate HASMCs because it maximally induces PKD activation (Fig. 1C) and because this dose is in the range found in neointimal lesions (6).

Activated PKD is located in plasma membranes of HASMCs. To examine the location of histamine-induced phosphorylated PKD in HASMCs, we used an immunofluorescence technique with antibodies against phosphorylation sites of PKDs and found that histamine-induced phosphorylated PKD1 and PKD2 accumulated in the plasma membranes of HASMC as shown in Fig. 1, F and G.

Histamine-induced activation of PKD is mediated by the H1 receptor but not histamine receptor 2. Histamine exerts its proinflammatory function through four receptors. H1 and histamine receptor 2 (H2) are expressed in vascular smooth muscle cells (24, 30, 32). To determine which histamine receptor mediates histamine-induced PKD phosphorylation, we used the specific H1 receptor antagonist mepyramine and the H2 receptor antagonist cimetidine to pretreat HASMCs for 40 min; then the cells were treated with 10 μM histamine for 5 min, and the phosphorylation of PKD1 and PKD2 was examined by Western blotting. As shown in Fig. 2, A and B, pretreatment with the H1 receptor antagonist mepyramine dose-dependently blocked phosphorylation of PKD1 and PKD2; however, pretreatment with the H2 receptor antagonist cimetidine had no effect on the activation of PKD1 and PKD2 (Fig. 2, C and D).
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A

HASMC HEK293

PKD1

PKD2

PKD3

B

Histamine treatment (min)

0 0.75 2 5 10 15 30 60

p-PKD1 (Ser 910)

PKD1

p-PKD2 (Ser 876)

PKD2

p-PKD (activation loop)

C

Phosphorylation of PKDs (fold induction)

0.0 2.5 5.0 7.5 10.0

Time (min)

0 10 20 30 40 50 60 70

p-PKD1 (Ser 910)

p-PKD2 (Ser 876)

p-PKD (activation loop)

D

Histamine (µM)

0 0.1 1 10 100

p-PKD1 (Ser 910)

PKD1

p-PKD2 (Ser 876)

PKD2

p-PKD (activation loop)

E

Phosphorylation of PKDs (fold induction)

0 5 10 15 20 25 30 35 40

Histamine (µM)

0 0.1 1 10 100

p-PKD1 (Ser 910)

p-PKD2 (Ser 876)

p-PKD (activation loop)

F

Histamine (10 µM)

Nucleus

α-actin

p-PKD1 (ser 910)

Merged

0 min

5 min

G

Histamine (10 µM)

Nucleus

α-actin

p-PKD2 (ser 876)

Merged

0 min

5 min

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00500.2011 • www.ajpheart.org
These results indicate that histamine-induced activation of PKD is mediated by the H₁ receptor, but not the H₂ receptor, in HASMCs.

Histamine-induced PKD phosphorylation is independent of Gᵢ/o proteins. Various G proteins are coupled with histamine receptors to mediate histamine-induced intracellular signaling. To determine whether Gᵢ/o proteins mediate histamine-induced phosphorylation of PKD1 and PKD2, we pretreated HASMCs with PTX (100 ng/ml), a specific inhibitor of Gᵢ/o proteins. Next, cells were treated with histamine (10 μM) for 5 min followed by the detection of PKD phosphorylation. As shown in Fig. 3, A and B, treatment with PTX had no effect on the phosphorylation of PKD1 and PKD2 induced by histamine, suggesting that histamine-induced PKD activation in HASMCs is independent of Gᵢ/o protein mediation.

Protein kinase C mediates histamine-induced PKD phosphorylation. Both protein kinase C (PKC)-dependent and -independent activation of PKD in mammalian cells have been reported (1–3). To assess whether histamine-induced PKD activation is mediated by PKC, we pretreated HASMCs with various concentrations of the PKC inhibitor Ro-31–8220 for 40 min and then stimulated cells with 10 μM histamine for 5 min. As shown in Fig. 3, C and D, we found that the PKC inhibitor Ro-31–8220 dose-dependently blocked histamine-induced PKD phosphorylation. Ro-31–8220 at 2 μM nearly completely eliminated the phosphorylation of PKDs. These results indicated that histamine-induced PKD activation is mediated by PKC in HASMCs.

PKD2 mediates histamine-induced phosphorylation of p38 MAPK but does not affect phosphorylation of ERK and JNK MAPKs. The role of PKD in the histamine signaling pathway has not been revealed. To begin to probe the role of PKD, we evaluated PKD’s role on MAPK activation, since histamine has been reported to activate MAPKs (27). We examined whether knockdown of PKD expression, using specific siRNA
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Fig. 3. Effect of the inhibitors of Gi/o proteins and protein kinase C (PKC) on histamine-induced PKD activation. HASMCs were pretreated with the Gi/o protein inhibitor pertussis toxin (PTX) overnight or the PKC inhibitor Ro-31–8220 for 40 min; then cells were stimulated with histamine (10 μM) for 5 min. A: Gi/o proteins are dispensable in histamine-induced PKD activation. Western blot analysis of the PTX effect on PKD phosphorylation induced by histamine. B: results of Western blot analysis of PKD phosphorylation were quantified by densitometry. Data are means ± SE from three experiments. C: PKC mediates histamine-induced PKD phosphorylation. Western blot analysis of the effect of PKC inhibitor Ro-31–8220 on PKD phosphorylation induced by histamine. D: results of Western blot analysis were quantified by densitometry. Data are means ± SE from three experiments. * and #P < 0.05 and ** and ###P < 0.01 vs. histamine alone.

PKD controls histamine-induced TF expression and activity in HASMCs. The above results reveal a novel role of PKD in the histamine signaling pathway. We demonstrated that PKD2 is responsible for histamine-induced phosphorylation of p38 MAPK. To explore the biological function of PKD in the histamine-induced cellular response, we examined whether PKD affects the coagulation pathway, an important pathway contributing to blood coagulation and thrombosis. Previously, it was reported that histamine induces TF expression via a MAPK pathway (27). Interestingly, our results demonstrated that PKD2 mediates histamine-induced p38 MAPK activation (Fig. 4A). Therefore, we examined the role of PKD in histamine-induced TF expression and TF activity.

Interestingly, we observed that knockdown of the expression of PKD2 with PKD2 siRNA resulted in completely blocking histamine-induced TF protein expression (Fig. 5A), whereas knockdown of PKD1 expression also resulted in remarkable inhibition of TF expression. However, depletion of PKD3 expression with siRNA had no effect on TF expression. We also examined the effect of knockdown of the specific PKD
isoform on TF activity. We found that knockdown of the expression of PKD2 with PKD2 siRNA resulted in completely blocking histamine-induced TF surface activity (Fig. 5B), whereas knockdown of PKD1 expression resulted in about 50% inhibition of TF activity. However, knockdown of PKD3 with PKD3 siRNA had no effect on the surface activity of TF in HASMCs (Fig. 5B). Collectively, these data (Fig. 5) plus the results shown in Fig. 4A, where knockdown of PKD2 blocks p38 phosphorylation, support a conclusion that PKD2 predominantly mediates histamine-induced TF expression and activity via the p38 pathway, and, while the role of PKD1 is mild compared with PKD2, PKD1 also mediates TF expression and activity via a p38-independent pathway. In contrast, PKD3 has no effect on histamine-induced TF expression.

PKC, an upstream kinase of PKD, mediates p38 MAPK activity and TF expression. As shown in Fig. 3, C and D, phosphorylation of PKDs is mediated by PKC. Moreover, data shown in Fig. 4A indicate that PKD2 mediates phosphorylation of p38 MAPK. We next determined whether PKC mediates histamine-induced phosphorylation of p38 MAPK. As shown in Fig. 6A, the PKC-specific inhibitor Ro-31–8220 at 2 μM markedly inhibited histamine-induced p38 MAPK phosphorylation, indicating that PKC, via the PKD2 pathway, mediates p38 MAPK activation in vascular SMCs.

To determine the effect of PKC, the upstream mediator of PKD, on histamine-induced TF expression, we examined whether pretreatment of HASMCs with the PKC-specific inhibitor Ro-31–8220 affects histamine-induced TF protein expression compared with the effect of the p38 MAPK-specific inhibitor SB-203580, which was shown to inhibit histamine-induced TF expression (27). As shown in Fig. 6B, pretreatment with the PKC-specific inhibitor Ro-31–8220 largely inhibited histamine-induced TF expression. Pretreatment with SB-203580 (10 μM), as expected, also largely blocked histamine-induced TF expression. These results indicate that PKC-dependent, PKD2-mediated p38 MAPK activation is required for histamine-induced TF expression.

DISCUSSION

Recent evidence supports histamine’s role in vascular diseases. In particular, histamine has been reported to stimulate vascular wall permeability and endothelial dysfunction (21, 26). Histamine also induces expression and secretion of various cytokines and chemokines (4, 11). Furthermore, histamine increases the formation of intimal hyperplasia (14, 19), and histamine H1 receptor promotes atherosclerotic lesion formation. Experimental data also show that knockout of the histamine-producing enzyme HDC reduces neointimal thickening (22). These findings suggest that histamine directly and markedly promotes atherogenesis. Vascular SMCs are the major constituents of blood vessel media, and histamine activation of SMCs has been implicated in the development of atherosclerosis (23). Therefore, understanding the histamine-induced intracellular signaling cascade of vascular SMC activation and identifying the role of the new components in the histamine...
signaling pathway are important to understanding the development of various vascular complications. In the present study, our results demonstrate that histamine rapidly and markedly induces PKD activation in HASMCs. PKD activation is the early signaling event triggered by histamine, leading to histamine-initiated HASMC activation. Our data provide the first evidence, to the best of our knowledge, that inflammatory factor histamine activates the PKD pathway in live cells. PKDs have been reported to contribute to survival, proliferation, and migration of vascular SMCs and endothelial cells in response to growth factors (7, 9). PKDs are also implicated in cardiac remodeling and could be a therapeutic target in heart failure (16). Our previous study reveals that PKD mediates human blood monocyte migration in a lysophosphatidylcholine-triggered pathway (31). Therefore, the present finding that histamine activates PKD1 and PKD2 in HASMCs strongly suggests that PKD1 and PKD2 mediate the histamine signal, leading to vascular pathologies.

Our results demonstrated that the H1, but not H2, receptor mediates histamine-induced activation of PKD1 and PKD2 in HASMCs, indicating a crucial role of H1 receptor in histamine-induced cell events. In line with our findings, it has been

![Fig. 5](http://ajpheart.physiology.org/)

**Fig. 5.** Effect of PKD on tissue factor (TF) expression and TF surface activity induced by histamine in HASMCs. The specific PKD siRNAs were transfected into HASMCs for 48 h followed by serum starvation for 24 h; then cells were stimulated with histamine (10 μM) for 5 h. **A**: top, effect of knockdown of the expression of PKD isoforms with PKD siRNAs on histamine-induced TF protein expression. Expression of PKDs and TF proteins was examined by Western blot analysis. **Bottom**, results were quantified by densitometry. **B**: effect of knockdown of the expression of PKD isoforms with PKD siRNAs on histamine-induced TF surface activity. Data are means ± SE from three experiments. **P** < 0.01 vs. histamine alone.

![Fig. 6](http://ajpheart.physiology.org/)

**Fig. 6.** Effect of PKC-specific inhibitor on p38 MAPK activity and TF protein expression. **A**: effect of PKC-specific inhibitor Ro-31–8220 on p38 MAPK activation. **Top**, phosphorylation of PKD2 and p38 MAPK was detected by Western blot analysis. HASMCs were pretreated with Ro-31–8220 for 40 min, followed by histamine (10 μM) stimulation for 5 min. **Bottom**, results were quantified by densitometry. **B**: effects of PKC-specific inhibitor Ro-31–8220 and p38 MAPK inhibitor SB-203580 on histamine-induced TF expression. **Top**, TF protein expression was examined by Western blot analysis. HASMCs were pretreated with inhibitors for 40 min and then stimulated with histamine (10 μM) for 5 h. **Bottom**, results were quantified by densitometry. Data are means ± SE from three experiments. **P** < 0.01 vs. histamine alone.
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reported that histamine increases TF expression (27), induces Egr-1 expression (8), and promotes inducible nitric oxide synthase expression (33) through the activation of the H1 receptor. Furthermore, the H1 receptor promotes the development of atherosclerotic lesions by increasing vascular permeability for LDL (19), and histamine H1 receptor antagonists abolish the effect of histamine and reduce the formation of intimal hyperplasia (14).

PKD activation is mediated by various G proteins, which couple to a variety of stimuli receptors. For instance, lysophosphatidic acid-induced PKD activation is mediated by G1 proteins in Swiss 3T3 and Rat-1 cells (17) and by cooperation of G1, Gq, and G12/13 in a murine embryonic cell line (35). The present study demonstrates that histamine-induced PKD activation is independent of G1 protein mediation in HASMCs, in agreement with our previous report that histamine activation of endothelial cells is mediated by non-G1 proteins (8). It has been reported that both PKC-dependent and -independent activation of PKDs exist (1–3). The present results show that PKC is required for histamine-induced PKD activation.

In searching for the downstream target of PKD in the histamine signaling pathway, we identified the specific effect of PKD isoforms on MAPK activation in HASMCs. Our data reveal the previously unknown regulatory relationship between PKD and MAPK (ERK, JNK, and p38 MAPK) in SMCs: that is, PKD2, but not PKD1, is required for p38 MAPK activation. We also evaluated the possible feedback of MAPK in mediating PKD activation, since it was previously shown that MAPK regulates PKD activation (29). However, our data indicate that, in the histamine-triggered signaling pathway in SMCs, MAPK is downstream of PKD and does not have a significant feedback effect on PKD activation.

Importantly, the present study not only reveals the new signaling molecule PKD in the histamine-triggered signaling pathway and PKD-p38 MAPK regulatory relationship, but also identifies the functional role of PKD in the histamine pathway and in the coagulation pathway. Our results indicate that PKD is required for TF expression and activity. TF, the key initiator of blood coagulation, plays an important role in thrombosis. We identified two possible pathways of how PKD contributes to TF expression: 1) the main pathway, PKD2 via p38 MAPK, regulates histamine-induced TF expression and TF cell surface activation, and 2) the minor pathway, PKD1 via a p38 MAPK-independent pathway, mediates TF expression and activation.

In summary, the present study reveals that PKD is a novel signaling component in the histamine pathway in live cells as evidenced by histamine activation of both PKD1 and PKD2 in HASMCs. Our data demonstrate that histamine-induced PKD activation is via the H1 receptor, independent of G1 proteins, and is mediated by PKC. More interestingly, the present study reveals a previously unknown role of PKD in the coagulation pathway: PKD is required for histamine-induced TF expression and TF cell surface activity. In line with growing evidence that histamine/histamine receptors are potent inflammatory factors, which worsen vascular lesions, our data suggest that PKD via its upregulation of TF may contribute to blood coagulation and atherothrombosis. Therefore, PKD may be a new and valuable therapeutic target for the treatment of coagulation and atherothrombosis.

REFERENCES


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