Role of epigenetics in pulmonary hypertension

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PULMONARY HYPERTENSION (PH) is a devastating, chronic, and severely debilitating disease, for which there is no cure. It affects up to 18–32% of Americans, according to statistics from the National Health and Nutrition Examination Survey (NHANES) (39). A mean pulmonary artery pressure (PAP), >25 mmHg at rest or >30 mmHg with exertion, is diagnostic for PH (28, 39). Symptoms of PH are often nonspecific and include progressive dyspnea and fatigue but can also rarely lead to syncope due to markedly decreased carbon dioxide and significantly elevated PAP (2, 31). Three pathologic vascular remodeling mechanisms have been found to contribute to PH, which include muscularization of peripheral arteries (PAs), medial hypertrophy and neointima formation in proximal muscular arteries, and plexiform lesion formation (25, 33, 38). PAs, which include arteries at the level of the alveolar wall and duct, are normally not muscular (6). However, in PH, these PAs undergo muscularization secondary to differentiation of precursor cells into smooth muscle cells (SMCs), which then proliferate (7, 15, 37). These precursor cells include stem cells, fibrocytes, or endothelial cells (ECs). Medial hypertrophy of proximal muscular arteries and neointima formation both occur secondary to differentiation of progenitor cells to SMCs, which subsequently migrate and replicate (19, 42, 43). Plexiform lesions form after the progression of the above processes with resultant aberrant replication of ECs and subsequent formation of irregular endothelial channels in the nearly obliterated lumen of the vessel. Apoptosis resistance may also play a role in the formation of these lesions (4, 18, 35). The analogous mechanisms and the causes behind them have been well studied in the pathogenesis of peripheral hypertension but have not been well studied in the context of PH (31).

The role of epigenetics in the development of PH is a rapidly growing field of research. Epigenetics is defined as changes in gene expression secondary to factors unrelated to change in DNA sequence (12). Similar to the vascular remodeling described above, epigenetic mechanisms have been well studied in other diseases but not in the context of PH (17). Recently, the main focus of epigenetics research in relation to PH has been on microRNAs (miRNAs). However, two other epigenetic mechanisms that have received less attention are DNA methylation and histone modification. DNA methylation is defined as the addition of a methyl group to cytosine residues in CpG islands in the promoter region of a gene. Methylation occurs via DNA methyltransferases (DNMTs) and usually silences genes. Histone acetylation and deacetylation are forms of post-translational modification that lead to increased and decreased transcription of genes, respectively. Acetylation occurs via histone acetyltransferases (HATs), whereas deacetylation is accomplished via histone deacetylases (HDACs) (17). HDAC inhibitors (HDIs) can be used to halt expansion of tumor cells by arresting the cell cycle and stimulating apopto-
sis. They accomplish this by hyperacetylating histones, which lead to both expression of certain genes and somewhat paradoxically, repression of other genes (29). This silencing of certain genes may possibly be indirect. For example, HDI hyperacetylation may activate genes that act similarly to tumor-suppressor genes, which then inactivate other genes. It is likely that dysfunction of these epigenetic mechanisms may lead to PH in similar ways as seen with peripheral hypertension (34).

There are several different molecular mechanisms that are believed to be consequences of maladaptive epigenetic modifications in PH, many of which are intimately related. For example, chronic hypoxia creates an inflammatory environment in pulmonary vasculature and leads to increased generation of reactive oxygen species (ROS). Both inflammation and ROS have been shown to increase pulmonary vascular remodeling. Inflammation leads to release of growth factors, cytokines, and chemokines that stimulate cells to proliferate and increase cell survival (10, 22). Persistent inflammation can also lead to DNA damage, which in turn, leads to apoptotic resistance (24). ROS are known to activate fibroblasts in the adventitia, which after being acted upon, growth factors, like TGF-β, can differentiate into myofibroblasts, which can then travel to the medial pulmonary vessel layer causing medial hypertrophy. They can also produce and lay down fibronectin, collagen, and elastin in the ECM. These ECM proteins then stimulate further pulmonary artery SMC (PASMC) proliferation, thus creating a vicious cycle that eventually leads to PH (10). All of these molecular mechanisms can be exacerbated or repressed by different forms of epigenetic regulation.

This review will discuss the major findings of epigenetics in relation to hypertension, which could be instrumental in understanding epigenetics in PH.

Possible Epigenetic Mechanisms in PH

As discussed earlier, the role of epigenetics in PH has gained much attention, as previous studies revealed exciting information about the potential effects of gene repression. DNA methylation and histone modification are several important principles that could be beneficial in determining the epigenetic cause of PH and in turn, finding novel treatments that could suppress it (Fig. 1).

**DNA methylation.** It is believed that hypomethylation of promoter regions of genes promoting SMC differentiation and proliferation (30), as well as decreased expression of DNMTs, leads to the development of atherosclerotic plaques and neo-intima formation in PH (11).

Hiltunen et al. (11) set out to determine whether changes in DNMT expression and DNA methylation contributed to the development of atherosclerotic plaques. The study was conducted by measuring the level of 5-methylcytosine (5-mC) and the amount of transcriptional activity in normal human arteries vs. atherosclerotic arteries. They discovered that compared with the $3.2\% \pm 0.2$ 5-mC in normal human arteries, the level of 5-mC in atherosclerotic plaques was decreased at $2.9 \% \pm 0.1 (P < 0.05)$. This can be quantified as a decreased global DNA methylation of 9%. It was also discovered that certain genes, such as 15-lipoxygenase (15-LO), which is strongly linked to atherosclerotic plaque development, also had decreased methylation of CpG islands. This finding corresponded to overexpression of 15-LO in the plaques, which suggests that hypomethylation of 15-LO contributes to vascular remodeling. Results also showed that in the intimal SMCs of human vessels with atherosclerotic plaques, the overall mRNA level was elevated compared with SMCs in the intima and media of vessels without plaques. This is likely secondary to global hypomethylation, leading to decreased gene silencing with subsequent increased gene transcription and SMC hyperproliferation. Interestingly, levels of DNMTs were increased in the SMCs of the plaques, possibly indicating a compensatory response to globally decreased methylation. Despite this increase in DNMT expression, the hypomethylation was not reversed (11). Similar studies should be carried out to determine if DNA hypomethylation is indeed associated with the formation of plexiform lesions, peripheral abnormally muscularized arteries, and proximal medially hypertrophied arteries with neo-intima formation.
Other studies examining DNA methylation and atherosclerosis development have shown similar results. However, some of these studies demonstrated surprising results regarding the genes being affected. For example, Laukkanen et al. (20) examined the methylation status of extracellular SOD (EC-SOD), which normally is protective against the atherogenic effects of free radicals. It is expected that in atherosclerotic vessel SMCs, EC-SOD would be hypermethylated and thus silenced. However, Laukkanen et al. (20) found that in rabbits with aortic atherosclerotic plaques, there was generalized hypermethylation of EC-SOD genes. It was hypothesized that hypomethylation may lead to secondary alterations of the EC-SOD gene structure, rendering it nonfunctional (20). These results conflict with research examining a similar gene, SOD2, in the development of PH. Kim et al. (17) demonstrated that hypermethylation and subsequent downregulation of SOD2 were increased in PASMCs in rats with PH. However, both studies appear to reflect the fact that abnormalities in methylation, whether hypo- or hypermethylation, can lead to vascular lesions (17, 20). Furthermore, studies examining hypo- and hypermethylation of genes in the development of PH should be conducted to determine their role in vascular remodeling.

Histone modification. Aberrant histone acetylation and deacetylation have been implicated in the development of hypertension via many different mechanisms (13). Increased acetylation of histone H3 has been linked to the development of essential hypertension (26). Irmak and Sizlan (14) demonstrated that excess melatonin stimulates neurons in the area postrema that stimulate vasomotor catecholamine-producing neurons in the rostral ventrolateral medulla (RVLM). This results in increased sympathetic output from the RVLM, which has been shown in other studies to contribute to the development of essential hypertension (26). It was also found that melatonin leads to increased acetylation of H3 and expression of HATs. Irmak and Sizlan (14) postulated that this may contribute to essential hypertension by increasing transcription of genes involved in neurogenesis in the area postrema and subsequent aberrant RVLM stimulation. It can be deduced that if this pathway is involved in essential hypertension, then it may contribute to PH as well. Whereas not directly related to the vascular remodeling seen with PH, increased sympathetic output may lead to PA constriction, increased flow rate through the pulmonary vasculature, and subsequent shear-stress damage to the vessel walls. This may contribute to vascular remodeling in PH. Hyperacetylation of certain genes has been implicated in the overproliferation of SMCs in PH. Regarding PH, research has been carried out examining the effect of hyperacetylation in endothelial nitric oxide synthase (eNOS), a gene that has been connected with the development of persistent PH of the newborn (PPHN) (17). Xu et al. (40) induced PPHN in newborn rats via in utero induction of hypoxia through administration of indomethacin to induce premature closure of the ductus arteriosus. They found that eNOS was increased markedly in the pulmonary arterial ECs of rats and surmised that this was due to increased eNOS promoter acetylation (40). Xu et al. (40) did not investigate reversal of this upregulation of eNOS; therefore, the exact mechanism behind this hyperacetylation was not determined (17). Further research should be conducted to determine the cause of this hyperacetylation and how it can be prevented in the treatment of PH.

One possible future therapy for PH is HDIs, which have been shown to downregulate SMC proliferation (29). Findeisen et al. (9) used the HDI, Scriptaid, in vascular SMCs and found that it inhibited cyclin D1 expression and concomitantly increased expression of the cyclin-dependent kinase (CDK) inhibitor CDKN1B. These effects both lead to G1-phase cell-cycle arrest and subsequent decreased SMC proliferation and neointima formation (9). Okamoto et al. (27) produced similar results using the HDI trichostatin A (TSA), and the main difference was that TSA leads to upregulation of CDKN1A vs. CDKN1B with Scriptaid. Mathew et al. (23) found increased expression of both CDKN1A and CDKN2B, as well as decreased expression of CDKs Cdk2, Cdk4, and Cdk6 with the HDI butyrate, which was shown, like Scriptaid and TSA, to inhibit SMC proliferation and neointima formation. However, an unexpected finding was that butyrate caused upregulation of cyclin D1 and D3, the opposite of what was seen with Scriptaid and TSA. The increase in cyclin D1 and D3 did not lead to SMC proliferation, because their effects were canceled out by the ability of butyrate to block phosphorylation of retinoblastoma, the G1/S-phase checkpoint gene (23). Collectively, these studies appear to support the claim that HDIs, as a class, lead to inhibition of SMC proliferation (9).

Research has also been conducted to investigate the effectiveness of HDIs as future treatment modalities for patients with PH. Li et al. (21) discovered that increased class I HDAC activity leads to activation of fibroblasts in the pulmonary vascular adventitia of hypoxic calves. These activated fibroblasts went on to produce a large amount of chemokines and cytokines, creating an excessively inflammatory environment. This inflammatory environment initiated the subsequent activation of macrophages, which in turn, secreted even more inflammatory cytokines and chemokines. This propelled the exacerbation of overall inflammatory damage and eventually led to PH. Li et al. (21) investigated further and found that class I HDIs significantly inhibited the activated fibroblast’s secretion of inflammatory cytokines and chemokines and subsequently decreased activation of macrophages. Thus this further supports that HDIs may help decrease inflammatory processes that lead to PH in humans.

Cavasin et al. (3) used two HDIs, MGCD0103 and MS-275, which selectively inhibit class I HDACs in hypoxic rats. They found that PAP was reduced in rats treated with these HDIs and in fact, show a greater decrease in PAP compared with rats treated with tadalafil, the current therapy for PH in humans. The arteries of the rats treated with selective HDIs showed decreased medial hypertrophy of the proximal PAs, believed to be secondary to decreased SMC proliferation through the mechanisms described previously. The selective HDIs can be used as targeted therapies and may prove to be more effective than nonselective HDIs, such as Scriptaid, TSA, and butyrate (3). Although the effect of HDIs was not examined in the previously mentioned study by Xu et al. (40), research should be conducted to determine the effect of HDIs on eNOS expression. Since HDIs lead to hyperacetylation of genes, it is known that nonselective HDIs may actually increase eNOS expression further and thus worsen PH (17, 40). However, it may be possible to use selective HDIs to upregulate genes that suppress eNOS to reverse vascular remodeling in PH.

Yang et al. (41) recently examined HDAC inhibition in the development of pulmonary arterial hypertension in newborns.
sheep. The harvested sheep PASMCs were exposed to HDIs apicidin (a class I HDI), HDI (HDACi) VIII (a class II HDI), and Tenovin-1 (a class III HDI). They examined the resulting expression of cell-cycle genes, such as p53, p21, cyclin D1, CDK4, and CDK6. In addition, Yang et al. (41) quantified the expression of anti- and pro-oxidative enzymes, as well as the effects of inhibiting class I HDACs on DNA methylation. Their results revealed that both apicidin and HDACi VIII suppressed PASMC proliferation in a dose-dependent manner. Tenovin-1, on the other hand, did not demonstrate a statistically significant effect on PASMC proliferation. These results suggest that class I and II HDACs play a role in the development of PH. The results were strengthened further by the fact that PASMCs exposed to apicidin and HDACi VIII underwent cell-cycle arrest in G1, with apicidin more so than HDACi VIII. These cells also underwent corresponding decreases in cell-cycle regulatory proteins, such as cyclin D1, p21, CDK4, and CDK6. PASMCs exposed to Tenovin-1 did not experience the same results. Surprisingly, p53 expression was not altered by any of the HDIs examined in this experiment. Apicidin also significantly decreased levels of the pro-oxidant enzymes dual oxidase 1 and 2 and simultaneously increased levels of the anti-oxidant enzymes SOD2 and SOD3. It can be extrapolated from these results that HDIs may shield PASMCs from oxidative insults. Yang et al. (41) also discovered that apicidin led to decreased global DNA methylation in PASMCs. Further examination of the role of apicidin and other class I HDIs in the development of or the protection against PH is needed (41).

Histones can also be modified through methylation by histone methyltransferases (HMTs), which like methylation of promoter regions of genes by DNMTs, leads to decreased translation of genes (5). Aljubran et al. (1) examined the role of the HMT, enhancer of zeste homolog 2 (EZH2), in PH. They found that increased levels of EZH2 in mice led to apoptotic resistance, as well as increased proliferation and migration of PASMCs. This was possibly secondary to silencing of proapoptotic and antiproliferative genes. Human studies examining the effect of EZH2 in the development of PH, as well as examining the therapeutic benefit of EZH2 inhibition, should be conducted (1).

Conclusion

Although epigenetic mechanisms, such as DNA methylation and histone modification, have been well studied in the arena of hypertension, their contribution in the development of PH is still poorly understood. A significant amount of research examining the role of miRNAs in the development of PH has been conducted; however, more attention to histone modification and DNA methylation as important players in the pathogenesis of this devastating disease is necessary. This may also allow further understanding as to how histone modification and DNA methylation affect and are affected by miRNA regulation. It is important to identify and comprehend these mechanistic processes in relation to PH, because it may help develop novel therapeutic treatments for this disease. Hence, further research is needed to investigate the epigenetics of PH in conjunction with the experimental models mentioned in this review.

There are numerous research methods in the future direction of epigenetic PH. One promising research technique is the use of methylation mapping analysis by paired-end sequencing (Methyl-MAPS). This technique can determine the methylation state in all classes of sequencing, such as interspersed repeats, promoters, and genomic DNA. Genomes are fractionated into methylated and unmethylated components via fine methylation-sensitive restriction enzymes that only cuts methylated CpG sites and a methylation-dependent endonuclease that only cuts methylated CpG sites, called McrBC. Then, the sequencing of the paired end-tags from each compartment is performed, which is then mapped to the genome. CpG sites are then assigned methylation states. Methyl-MAPS offers several advantages over other techniques, because it can quantify both unmethylated and methylated DNA. Also, Methyl-MAPS is far more cost effective compared with the current standard, whole-genome bisulfite (8, 32, 36). Other promising techniques for assessing methylation status that are currently being developed include formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-Seq), bisulfite sequencing (MethylC-Seq) chromatin immunoprecipitation sequencing (ChIP-Seq), and the innovative nucleosome occupancy and methylyme sequencing (NOMe-Seq) (16). Future PH studies would also greatly benefit from combing both genetic and epigenetic analysis techniques. This would be greatly applicable to patients with hereditary PH secondary to the bone morphogenetic protein receptor type 2 mutation.

In conclusion, DNA methylation and histone modification show much promise for novel treatments in PH. Among the HDIs, apicidin appears to be promising in histone modification, as it may play a role in global DNA methylation in PASMCs (41). Future development of targeted DNMTs to silence proproliferative or antiapoptotic genes may also show great potential and should be investigated further. Compared with histone modification, there appears to be a paucity of research examining the role of DNA methylation regarding PH and hypertension in general. Further resources need to be dedicated to examine the effect of DNA methylation in the development of PH. In all, epigenetics provides multiple options in future research for improved treatment modalities against PH.

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