主論文の要約

Molecular hydrogen suppresses activated Wnt/β-catenin signaling

（分子状水素は活性化された Wnt/β-catenin シグナル伝達系路を抑制する）

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Background

Molecular hydrogen (H_2) has been proved to be effective for many rodent models and human diseases. However, molecular bases of H_2 have not been fully elucidated. Cumulative evidence indicates that H_2 functions as a signaling modulator. We examined whether H_2 affects Wnt/β-catenin signaling.

Methods

The activation of Wnt/β-catenin signaling was measured by Topflash luciferase reporter assay. Total protein levels of β-catenin and phosphorylated β-catenin were analyzed by Western blotting. The levels of gene expressions measured by qRT-PCR. To check the effect of H_2 on osteoarthritis (OA), we performed surgical destabilization of the medial meniscus (DMM) to induce OA on rat knees. Rats drank degassed water or supersaturated H_2 water for 8 weeks after DMM surgery. Safranin O staining and immunofluorescent staining were performed on the sections of rat knees.

Results

We conducted Topflash luciferase reporter assay to quantify activation of Wnt/β-catenin signaling and found that H_2 suppresses activated Wnt/β-catenin signaling induced by Wnt3a, LiCl or BIO (Fig. 1a). Consistently, H_2 reduced accumulation of β-catenin, the transcriptional co-activator, induced by Wnt3a, LiCl, or BIO in L cells (Fig. 1b - d). H_2 has been reported to inhibit JNK signaling. A previous report shows that inhibition of JNK decreases β-catenin accumulation. We found that inhibition of JNK activity by SP600125 did not block the effect of H_2 on decreasing β-catenin levels, indicating that H_2 suppressed Wnt/β-catenin signaling independent of JNK signaling (Fig. 1e).

H_2 has no effect on mRNA levels of β-catenin in L cells (Fig. 2a), indicating that H_2 acts on β-catenin protein synthesis/degradation rather than its gene expression. Then, we used cycloheximide (CHX) to block protein biosynthesis of β-catenin and conducted the CHX chase assay. Because endogenous β-catenin is hard to be detected in L cells, we expressed myc-β-catenin in L cells and found that H_2 accelerated degradation of myc-β-catenin (Fig. 2b). Intracellular β-catenin is degraded by the ubiquitin-proteasome system. Consequently, inhibition of β-catenin degradation by MG132, which is a proteasome inhibitor, minimized the suppressive effect of H_2 on BIO-induced β-catenin accumulation (Fig. 2c). We also observed that H_2 facilitated ubiquitination of β-catenin (Fig. 2d). These results suggest that H_2 enhances proteasome-mediated β-catenin degradation.

In canonical Wnt/β-catenin signaling, β-catenin is sequentially phosphorylated at Ser45 by casein kinase 1 (CK1) and at Ser33/Ser37/Thr41 by glycogen synthase kinase 3

(GSK3) in a complex with Axin1 and adenomatous polyposis coli (APC). We then tested whether the effect of H_2 was on either phosphorylation or degradation of β-catenin. Since the level of phospho-β-catenin is dependent on the total amount of β-catenin, we added MG132 to block β-catenin degradation in the proteasome pathway. H_2 increased phosphorylation of β-catenin at Ser45 and at Ser33/Ser37/Thr41 with the treatment of MG132 (Fig. 3a). We also found that H_2 was able to decrease β-catenin levels induced by low BIO concentrations (1 μM and 2 μM) but not by high BIO concentrations (4 μM and 6 μM; Fig. 3b). These results indicates that the suppressive effect of H_2 on activated Wnt/β-catenin signaling requires GSK3 activity. Then, we examined the effect of H_2 in two cell lines, HCT-116 colon cancer cells and HepG2 liver carcinoma cells, which carry mutations in *CTNNB1* encoding β-catenin at its phosphorylation sites. HCT-116 cells are heterozygous for a deletion mutation at Ser45, the CK1 regulatory site, in the β-catenin gene. HepG2 cells are heterozygous for an inframe-deletion lacking a potential GSK3-phosphorylation site in the β-catenin gene. We found that the inhibitory effect of H_2 on Wnt3a- or BIO-induced β-catenin accumulation in HCT-116 cells (Fig. 3c) was less than those observed in L cells (Fig. 1b, d). The reduction of the H_2 effect in HCT-116 cells was likely due to the presence of a deletion at Ser45. In HepG2 cells, Wnt3a or BIO failed to increase the truncated β-catenin and H_2 showed no suppressive effect on the truncated β-catenin (Fig. 3d). In contrast, wild-type β-catenin in HepG2 cells was increased by Wnt3a or BIO, and was suppressed by H_2 (Fig. 3d). Next, we found that H_2 did not affect GSK3-mediated phosphorylation of glycogen synthase in L cells (Fig. 3e). Thus, H_2 may not affect GSK3 activity. In addition to kinase activities, phosphorylation status of β-catenin also depends on phosphatase activities. We observed that okadaic acid, which is an inhibitor for protein phosphatase 2A (PP2A), could not abrogate the suppressive effect of H_2 on β-catenin accumulation in BIO-treated cells (Fig. 3f), indicating that H_2 promoted β-catenin degradation independent of PP2A.

In canonical Wnt/β-catenin signaling, two scaffold proteins, APC and Axin1, bind to β-catenin to form a degradation complex, and facilitate phosphorylation and ubiquitination of β-catenin. We then investigated whether APC, Axin1, or both are involved in H_2-medicated β-catenin degradation. H_2 has no effect on mRNA levels of APC and Axin1 (Fig. 4a, b). Knock-down of APC by siRNA in L cells abrogated the suppressive effect of H_2 on β-catenin accumulation induced by BIO (Fig. 4c, e). Similarly, knock-down of Axin1 also attenuated the effect of H_2 (Fig. 4d, f). Accordingly, the effect of H_2 requires CK1/GSK3-phosphorylation sites of β-catenin, as well as the β-catenin degradation complex comprised of CK1/GSK3, APC, and Axin1.

Abnormal activation of Wnt/β-catenin signaling has been reported to be involved in the development and aggravation of osteoarthritis (OA). We confirmed that, in human osteoarthritis chondrocyte (OAC) cells stimulated by Wnt3a or BIO, H_2 decreased

β-catenin accumulation (Fig. 5a) and suppressed mRNA levels of *Axin2* (Fig. 5b), the target gene of Wnt/β-catenin signaling. Additionally, H_2 partly rescued Wnt3a- or BIO-induced downregulation of Sox9, which plays an essential role in chondrogenic differentiation (Fig. 5c). We also conducted Alcian blue staining in differentiated ATDC5 mouse chondrogenic cells, and found that both Wnt3a- and BIO-induced loss of proteoglycans were marginally reversed by H_2 (Fig. 5d). Next, we observed a tendency that 8-week administration of H_2 water after surgery partially improved Safranin O-staining on the articular surface and minimally preserved the structure of articular cartilage (Fig. 6a). We also found that H_2 decreased the accumulation of β-catenin (Fig. 6b) and increased the expression of Sox9 (Fig. 6c) in cartilage chondrocytes in the DMM group without affecting the expression of β-catenin and Sox9 in sham groups (Fig. 6b, c). These data indicate that H_2 suppresses Wnt/β-catenin signaling in articular chondrocytes and partially ameliorates cartilage degradation and OA progression in a rat OA model.

Discussion and Conclusion

Although the effects of H_2 have been reported in 166 disease models and human diseases, molecular target(s) of H_2 are still unknown. H_2 was initially reported as a scavenger of hydroxyl radical (•OH). However, as the reaction rate constant of H_2 and •OH is low and the dwell time of H_2 in our body is short, H_2 is unlikely to efficiently remove •OH. Thus, yet unidentified mechanisms should underlie the therapeutic effect of H_2. Previous studies show that H_2 acts as a gaseous signal modulator.

We observed that H_2 inhibited activated Wnt/β-catenin signaling in L and HeLa cells. H_2 promoted phosphorylation, ubiquitination, and subsequent degradation of β-catenin without affecting its mRNA level. The effect of H_2 required CK1/GSK3-phosphorylation sites on β-catenin, the CK1/GSK3 activities, as well as APC and Axin1 activities. We confirmed the suppressive effect of H_2 on Wnt/β-catenin signaling in chondrocytes. Oral intake of H_2 water tended to ameliorate cartilage degradation in a DMM-induced rat OA model through attenuating β-catenin accumulation. We demonstrate that H_2 suppresses abnormally activated Wnt/β-catenin signaling, which accounts for the protective roles of H_2 in a fraction of diseases.