

主論文の要旨

**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 (H₂) has been proved to be effective for many rodent models and human diseases. However, molecular bases of H₂ have not been fully elucidated. Cumulative evidence indicates that H₂ functions as a signaling modulator. We examined whether H₂ 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 H₂ on osteoarthritis (OA), we performed surgical destabilization of the medial meniscus (DMM) to induce OA on rat knees. Rats drank degassed water or supersaturated H₂ 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 H₂ suppresses activated Wnt/β-catenin signaling induced by Wnt3a, LiCl or BIO (Fig. 1a). Consistently, H₂ reduced accumulation of β-catenin, the transcriptional co-activator, induced by Wnt3a, LiCl, or BIO in L cells (Fig. 1b - d). H₂ 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 H₂ on decreasing β-catenin levels, indicating that H₂ suppressed Wnt/β-catenin signaling independent of JNK signaling (Fig. 1e).

H₂ has no effect on mRNA levels of β-catenin in L cells (Fig. 2a), indicating that H₂ 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 H₂ 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 H₂ on BIO-induced β-catenin accumulation (Fig. 2c). We also observed that H₂ facilitated ubiquitination of β-catenin (Fig. 2d). These results suggest that H₂ 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 H₂ 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. H₂ increased phosphorylation of β -catenin at Ser45 and at Ser33/Ser37/Thr41 with the treatment of MG132 (Fig. 3a). We also found that H₂ was able to decrease β -catenin levels induced by low BIO concentrations (1 μ M and 2 μ M) but not by high BIO concentrations (4 μ M and 6 μ M; Fig. 3b). These results indicate that the suppressive effect of H₂ on activated Wnt/ β -catenin signaling requires GSK3 activity. Then, we examined the effect of H₂ 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 H₂ 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 H₂ 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 H₂ 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 H₂ (Fig. 3d). Next, we found that H₂ did not affect GSK3-mediated phosphorylation of glycogen synthase in L cells (Fig. 3e). Thus, H₂ 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 H₂ on β -catenin accumulation in BIO-treated cells (Fig. 3f), indicating that H₂ 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 H₂-mediated β -catenin degradation. H₂ 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 H₂ on β -catenin accumulation induced by BIO (Fig. 4c, e). Similarly, knock-down of Axin1 also attenuated the effect of H₂ (Fig. 4d, f). Accordingly, the effect of H₂ 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, H₂ decreased

β -catenin accumulation (Fig. 5a) and suppressed mRNA levels of *Axin2* (Fig. 5b), the target gene of Wnt/ β -catenin signaling. Additionally, H₂ 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 H₂ (Fig. 5d). Next, we observed a tendency that 8-week administration of H₂ 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 H₂ 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 H₂ 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 H₂ have been reported in 166 disease models and human diseases, molecular target(s) of H₂ are still unknown. H₂ was initially reported as a scavenger of hydroxyl radical (\bullet OH). However, as the reaction rate constant of H₂ and \bullet OH is low and the dwell time of H₂ in our body is short, H₂ is unlikely to efficiently remove \bullet OH. Thus, yet unidentified mechanisms should underlie the therapeutic effect of H₂. Previous studies show that H₂ acts as a gaseous signal modulator.

We observed that H₂ inhibited activated Wnt/ β -catenin signaling in L and HeLa cells. H₂ promoted phosphorylation, ubiquitination, and subsequent degradation of β -catenin without affecting its mRNA level. The effect of H₂ 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 H₂ on Wnt/ β -catenin signaling in chondrocytes. Oral intake of H₂ water tended to ameliorate cartilage degradation in a DMM-induced rat OA model through attenuating β -catenin accumulation. We demonstrate that H₂ suppresses abnormally activated Wnt/ β -catenin signaling, which accounts for the protective roles of H₂ in a fraction of diseases.