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Simultaneous oral and inhalational intake of molecular hydrogen additively suppresses signaling pathways in rodents

Sayaka Sobue · Kazuaki Yamai · Mikako Ito · Kinji Ohno · Masafumi Ito · Takashi Iwamoto · Shanlou Qiao · Tetsuo Ohkuwa · Masatoshi Ichihara

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Abstract Molecular hydrogen (H_2) is an agent with potential applications in oxidative stress-related and/or inflammatory disorders. H_2 is usually administered by inhaling H_2-containing air (HCA) or by oral intake of H_2-rich water (HRW). Despite mounting evidence, the molecular mechanism underlying the therapeutic effects and the optimal method of H_2 administration remain unclear. Here, we investigated whether H_2 affects signaling pathways and gene expression in a dosage- or dose regimen-dependent manner. We first examined the H_2 concentrations in blood and organs after its administration and found that oral intake of HRW rapidly but transiently increased H_2 concentrations in the liver and atrial blood, while H_2 concentrations in arterial blood and the kidney were one-tenth of those in the liver and atrial blood. In contrast, inhalation of HCA increased H_2 equally in both atrial and arterial blood. We next examined whether H_2 alters gene expression in normal mouse livers using DNA microarray analysis after administration of HCA and HRW. Ingenuity Pathway Analysis revealed that H_2 suppressed the expression of nuclear factor-kappa B (NF-κB)-regulated genes. Western blot analysis showed that H_2 attenuated ERK, p38 MAPK, and NF-κB signaling in mouse livers. Finally, we evaluated whether the changes in gene expression were influenced by the route of H_2 administration and found that the combination of both HRW and HCA had the most potent effects on signaling pathways and gene expression in systemic organs, suggesting that H_2 may act not only through a dose-dependent mechanism but also through a complex molecular network.

Keywords Molecular hydrogen · Gene expression · NF-κB · p38 · ERK

Sayaka Sobue and Kazuaki Yamai contributed equally to this work.

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S. Sobue · K. Yamai · T. Iwamoto · S. Qiao · M. Ichihara (✉)
Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, Kasugai 487-8501, Japan
e-mail: ichihara@isc.chubu.ac.jp

K. Yamai · T. Ohkuwa
Department of Materials Science and Engineering, Nagoya Institute of Technology, Graduate School of Engineering, Nagoya 466-8555, Japan

M. Ito · K. Ohno
Division of Neurogenetics, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan

M. Ito
Research Team for Mechanism of Aging, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan

Introduction

Molecular hydrogen (H_2), together with NO, CO, and H_2S, is an antioxidant gas with medical applications [1]. Seven years after Ohsawa et al. reported that H_2 could be used as an antioxidant therapy [2], more than 200 articles have demonstrated that H_2 ameliorates pathological conditions in numerous diseases and disease models, mostly in rodents [3, 4].

H_2 is usually administered in vivo through inhalation of air with 2–4 % H_2, injection of H_2-rich saline into the peritoneal cavity, or oral intake of H_2-rich water (HRW); however, it is not clear which method of administration achieves maximal therapeutic effects for any particular

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disease model [3, 4]. Furthermore, several reports demonstrate that the antioxidant effects of H_2 are dose dependent [2, 5–7], although it remains unclear whether other effects of H_2, other than antioxidation, correlate with its in vivo concentration, because the precise pharmacological action of H_2 is not fully known. Inhalation of H_2-containing air (HCA) achieves persistent systemic H_2 concentrations, both in arterial and venous blood. In contrast, HRW contains only small amounts of H_2; for example, 1 L of H_2-saturated water contains at most 16 ml of H_2 gas. However, there have been no reports showing that HCA improves pathological conditions significantly more than oral administration; therefore, the effects of H_2 may not show dose dependency in target organs. This suggests that the biological mechanism of H_2's action is more complicated than expected.

When H_2 is administered in inflammatory conditions, it affects intracellular signals, including NF-κB [8, 9], ERK [9, 10], p38 [9, 10], JNK [8–10], and Nrf2 [11], and also the expression of a large number of genes, including inflammatory cytokines. However, the mechanism of these actions is unknown. It is also not known whether the effects of H_2 on signal transduction and gene expression are due to a secondary change accompanying remission of a disease condition or due to modifications of specific upstream molecules.

In this study, we first clarified the chronological change of blood H_2 concentration after the administration of HCA or HRW, as well as their organ concentrations. Next, we showed that H_2 altered gene expression and signal transduction in healthy liver tissue. Finally, we investigated whether the change in gene expression was influenced by the route of H_2 administration. We found that the combination of both HRW and HCA had the most potent (additive) effects on signaling pathways and gene expression in systemic organs.

Materials and methods

HRW

The HRW used in the experiments shown in Fig. 1 was provided by Blue Mercury (Tokyo, Japan). In other experiments, HRW was generated from distilled water with 0.44 mM Na_2SO_4 using Aquela Blue, a water-electrolyzing device, to produce electrolyzed H_2-saturated water at near-neutral pH. This device was provided by MiZ Co., Ltd. (Fujisawa, Japan). The H_2 concentration of HRW was approximately 0.6–0.8 mM for all experiments. As control water, we used dehydrogenized water that was made by leaving HRW uncovered for 24 h at 4 °C. We measured the H_2 concentrations in HRW, and control water with a hydrogen electrode (ABLE, Tokyo, Japan).

Animals and H_2 administration

All experimental procedures and protocols were approved by the Animal Care and Use Committee of Chubu University and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Eight-week-old male Wistar/ST rats, weighing 240–270 g, were purchased from Japan SLC (Hamamatsu, Japan). Each rat was food-deprived for 18 h before HRW administration and cannulation for blood sample collection. After food deprivation, each rat was anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg). The rats were either administered 4 ml of HRW into the stomach through a gastric tube or exposed to 2 % H_2 gas/98 % air (HCA) in a plastic cage. Meanwhile, 3-week continuous administration of HCA to mice was performed as described previously with slight modification [12]. Mice in standard plastic cages were placed in a 60-L air-tight acrylic chamber, which was continuously supplied with 10 L/min HCA. Control mice were also placed in the air-tight chamber, which was continuously supplied with 10 L/min air without H_2. HCA was generated by mixing compressed air from a scroll compressor SLP-15 EB (Anest Iwata, Yokohama, Japan) and 100 % H_2 (Taiyo Nippon Sanso, Tokyo, Japan) using a multi-flowmeter Model-1203 (Kofloc, Kyoto, Japan), and thereafter this gas mixture (HCA) was delivered into the chamber. Air samples from the chamber were periodically collected and H_2 concentrations monitored with an Optical Gas Monitor Model FI-21 (Riken Keiki, Tokyo, Japan).

Collection of blood and exhaled gas and measurement of H_2 in these samples

To obtain blood samples from the arteries and atria of rats, the subclavian vein and external carotid artery were cannulated with a silicone tube (Laboran Silicone Tube, As One, Osaka, Japan) while the rats were under anesthesia. The arterial catheter was placed in the common carotid artery, and the venous catheter was positioned in the right atrium. Each catheter was treated with heparinized saline. After administration of H_2, blood samples were sequentially collected through the catheters into a heparinized syringe. To obtain exhaled gas, the rat trachea was cannulated with an intravenous catheter (SurFlash 24G, Terumo, Tokyo, Japan) while the rats were under anesthesia. The H_2 concentrations of blood and exhaled air were measured as described previously using gas chromatography (EAG-analyzer, SensorTec, Ritto, Japan) [2].

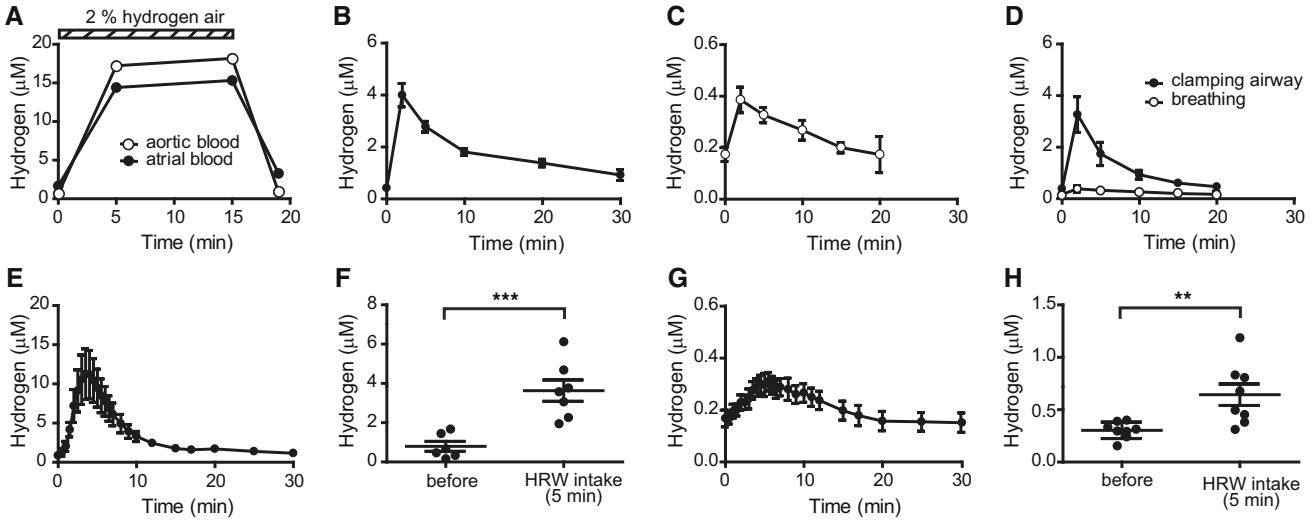


Fig. 1 Temporal profiles of the H_2 concentrations in blood and tissues after administration of H_2-rich water (HRW) or H_2-containing air (HCA). **A** Wister/ST rats were placed in HCA (2 % H_2) for 15 min, and the H_2 concentrations in aortic (*open circle*, $n = 3$) and atrial (*closed circle*, $n = 3$) blood were sequentially evaluated after HCA administration. **B** and **C** HRW (4 mL) was orally administered to Wister/ST rats, and the H_2 concentrations in atrial (**B**) and aortic (**C**) blood were sequentially evaluated ($n = 6$ or 7). **D** The H_2 concentration in aortic blood was compared with that with (*closed circle*) versus without (*open circle*) airway clamping after oral administration of HRW ($n = 6$). **E**, **F**, **G**, and **H** After oral administration of HRW, the H_2 concentrations in the liver (**E**) and kidney (**G**) were sequentially measured using a needle-type H_2 sensor ($n = 5$ for each organ). Five minutes after oral administration of HRW, the peak H_2 concentrations in the liver (**F**, $n = 6$ or 7) and kidney (**H**, $n = 8$) were confirmed using gas chromatography. The data are presented as the mean ± SEM. *Asterisks* indicate statistical significance as determined by Student's *t* test ($^{**}p < 0.01$, $^{***}p < 0.001$)

Measurement of H_2 in tissues

The H_2 concentrations in tissues were sequentially measured using a needle-type H_2 sensor (Unisense, Aarhus N, Denmark). We also measured the maximal H_2 concentrations in tissues using gas chromatography as described for measuring the blood H_2 concentration.

RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was prepared from 2 μg of RNA using a Transcriptor First-Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Real-time quantitative reverse transcription (RT)-qPCR was performed using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche, Mannheim, Germany) and the primer sets listed in Table S2. Reactions were performed and analyzed on a LightCycler DX400 (Roche, Mannheim, Germany).

Microarray experiments and data analysis

Ten-week-old male BALB/c mice were divided into two groups: (1) fed with control water in regular air ($n = 4$) and (2) fed with HRW in HCA ($n = 4$). HRW or control water was administered ad libitum to the mice using a 50-mL closed glass vessel equipped with an outlet line having a ball bearing. The H_2 concentration within the glass vessels remained greater than 0.2 mM after 24 h. After 3 weeks of administration, these mice were sacrificed, and their livers were extirpated for RNA extraction. After extraction of total RNA from each liver, an equal amount of RNA in each group was mixed and hybridized to a 3D-Gene Mouse Oligo chip 24 k (Toray Industries, Inc., Kanagawa, Japan). The microarray data were deposited with the Gene Expression Omnibus (GSE48826). Differentially expressed genes were described by functional annotation clustering using DAVID Bioinformatics Resources (david.abcc.ncifcrf.gov) and Ingenuity Pathway Analysis (IPA; Ingenuity System, www.ingenuity.com).

Western blot analysis

Nuclear and cytoplasmic proteins were extracted with nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). Western blot analysis was performed as described previously [13] with anti-p-p38 MAP Kinase, anti-p38 MAP Kinase, anti-p-SAPK/JNK, anti- SAPK/JNK, anti-p-ERK1/2, anti-ERK1/2, anti-p-Akt, anti-Akt, anti-NFAT C2, anti-NF-κB p65, anti-β-tubulin, and anti-Histone H3 antibodies (Cell Signaling Technology, Beverly, MA). ECL Prime Western Blotting Detection Reagent

(GE Healthcare, Buckinghamshire, UK) was used for detection. Band intensities were measured using Multi Gauge Ver3.0 software (Fujifilm, Tokyo, Japan). As a positive control for NF-κB activation in liver, we prepared liver nuclear and cytoplasmic proteins from mice 2 h after intraperitoneal injection with lipopolysaccharide (LPS) (from Escherichia coli 055:B5, Sigma-Aldrich, St. Louis, MO, USA) as described previously [14].

Statistical analyses

We performed statistical analyses using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Data are represented as the mean ± SEM.

Results

Temporal profiles of H_2 concentration in rat blood and tissues after administration of HCA or HRW

To investigate the blood kinetics of H_2 after administration of HCA or HRW, we first measured known concentrations of H_2 dissolved in water by gas chromatography and found that it was possible to measure blood H_2 over a wide range (Supplemental Fig. 1A).

We next measured H_2 concentrations in blood obtained from the atrium and carotid artery of rats under HCA. As shown in Fig. 1A, blood H_2 from both the atrium and the artery reached approximately 15 μM within 5 min and returned to basal levels within 5 min after discontinuing H_2 administration. The observed H_2 concentration in blood was equivalent to the value predicted by Henry's law for the solubility of a gas in a liquid.

We further evaluated the blood H_2 level after oral administration of HRW. After gastric administration of 4 mL of HRW, H_2 in atrial blood increased to its maximum value within 5 min and returned to the basal level within 30 min (Fig. 1B). The peak H_2 concentration in the atrium was 4.0 ± 0.45 μM ($n = 7$). H_2 in exhaled gas increased with a similar temporal profile (Supplemental Fig. 1B). The H_2 concentrations and the temporal profile in exhaled gas were also similar to those observed in humans [15].

Meanwhile, we found that the H_2 concentration in arterial blood was approximately one-tenth of that in atrial blood (Fig. 1C). To demonstrate that this decreased H_2 concentration was due to exhalation from the lungs, we clamped the airway during blood sampling and found that the value elevated to a level similar to that in atrial blood, suggesting that most of the H_2 in atrial blood was exhaled from the lung after oral intake of HRW (Fig. 1D).

To elucidate whether the H_2 concentration in arterial blood following oral HRW administration was consistent with that in organs, we evaluated H_2 levels in the liver and kidney. Measurement with H_2 electrodes indicated similar chronological changes in the liver (Fig. 1E) and kidney (Fig. 1G) compared with the atrial and arterial blood, respectively. Additionally, the results in Fig. 1E are similar to those observed by Kamimura et al. [16]. Using gas chromatography, we directly measured organ H_2 concentrations 5 min after the administration of HRW and found that H_2 increased in the liver and kidney to a similar extent to that measured with H_2 electrodes (Fig. 1F, H). These results show that the inhalation of HCA achieved sustained H_2 concentrations in the blood and organs, while drinking HRW led to a sudden change in the liver H_2 concentration and decreased H_2 in arterial blood.

H_2 alters gene expression profiles in healthy mouse liver

To investigate the molecular basis for the effects of H_2 in vivo, we evaluated gene expression in the liver upon the administration of H_2. We first extracted RNA from the livers of 8-week-old mice to which both HCA and HRW were administered for 3 weeks and compared the gene expression profile between control and H_2-treated mouse livers using microarray analysis. We excluded genes with low levels of expression because we observed insufficient reproducibility of these genes in our preliminary evaluation using qPCR. Finally, we found 31 up-regulated genes (≥2.1-fold change) and 109 down-regulated genes (≤0.48-fold change) (Table S1). We next evaluated the actual gene expression levels by qPCR using the primers listed in Table S2 and found that the differences in gene expression levels between control and H_2-treated mouse livers were reproducibly observed by qPCR in more than 80 % of the genes listed in Table S1 (data not shown). Supplemental Fig. 2 presents the relative mRNA levels of genes showing significant differential expression, as determined by qPCR. We added Bcl6 to Supplemental Fig. 2, because in a previous unpublished study we found Bcl6 to be differentially expressed after H_2 administration.

We characterized the down-regulated genes by Gene Ontology analysis using DAVID and found that H_2 treatment resulted in down-regulation of signal transduction-related genes (Table S3).

H_2 alters signaling pathways in the healthy mouse liver

To investigate which upstream pathways mediate the gene expression changes that were stimulated by H_2 treatment, we analyzed signaling pathways through IPA and found that NF-κB and nuclear factor of activated T-cells (NFAT)-related pathways were affected by both HRW and HCA administration (Fig. 2A). We then observed by Western

blot analysis that H_2 treatment attenuated the activation of p38, ERK, and NF-κB (Fig. 2B–F). On the other hand, we did not detect NFAT protein in the liver by Western blot analysis and did not demonstrate NFAT-related pathway activity through IPA using the results from qPCR (Supplemental Fig. 3A, B). This suggests that, from the microarray results, IPA might incorrectly implicate the NFAT pathway as an H_2-affected signaling pathway or that H_2 marginally affected NFAT pathway-related gene expression.

To date, numerous reports have demonstrated that H_2 suppresses a broad range of signaling pathways, including NF-κB, especially under pathological conditions [4]. Although we observed that H_2 treatment attenuated the activation of p38, ERK, and NF-κB in healthy mice, we wondered whether the mice used in our study might be exposed to stressful conditions in the air-tight acrylic chamber during the 3-week continuous administration of HCA. Therefore, we examined serum levels of the stress-hormone corticosterone after 3 weeks of housing without H_2 gas in a conventional clean rack or in the air-tight acryl chamber used for H_2 gas administration. We found no difference in serum corticosterone levels between the two groups, whereas mice under 20-h restraint stress showed a marked increase in the levels of corticosterone (Supplemental Fig. 4A). We further compared the activation of NF-κB under these two conditions and found no difference in activation (Supplemental Fig. 4B and C), suggesting that H_2 treatment does attenuate the activation of several signaling pathways in healthy mice under our experimental conditions.

Next, we investigated to what extend H2 treatment suppresses the activation of NF-κB in the liver compared with the full activation of NF-κB by intraperitoneal administration of LPS. We observed that the basal activation of NF-κB in the liver was as low as 22 % of its maximal activation by intraperitoneal LPS administration, while H_2 treatment decreased this basal activation from 22 to 8 % (Fig. 2G, H). These results indicate that, albeit with a modest response compared with that caused by severe LPS stimulation-induced inflammation, H_2 impacts a wide range of intracellular signaling pathways in healthy liver and may, thereby, alter gene expression.

HRW and HCA additively alter gene expression in liver and other systemic organs

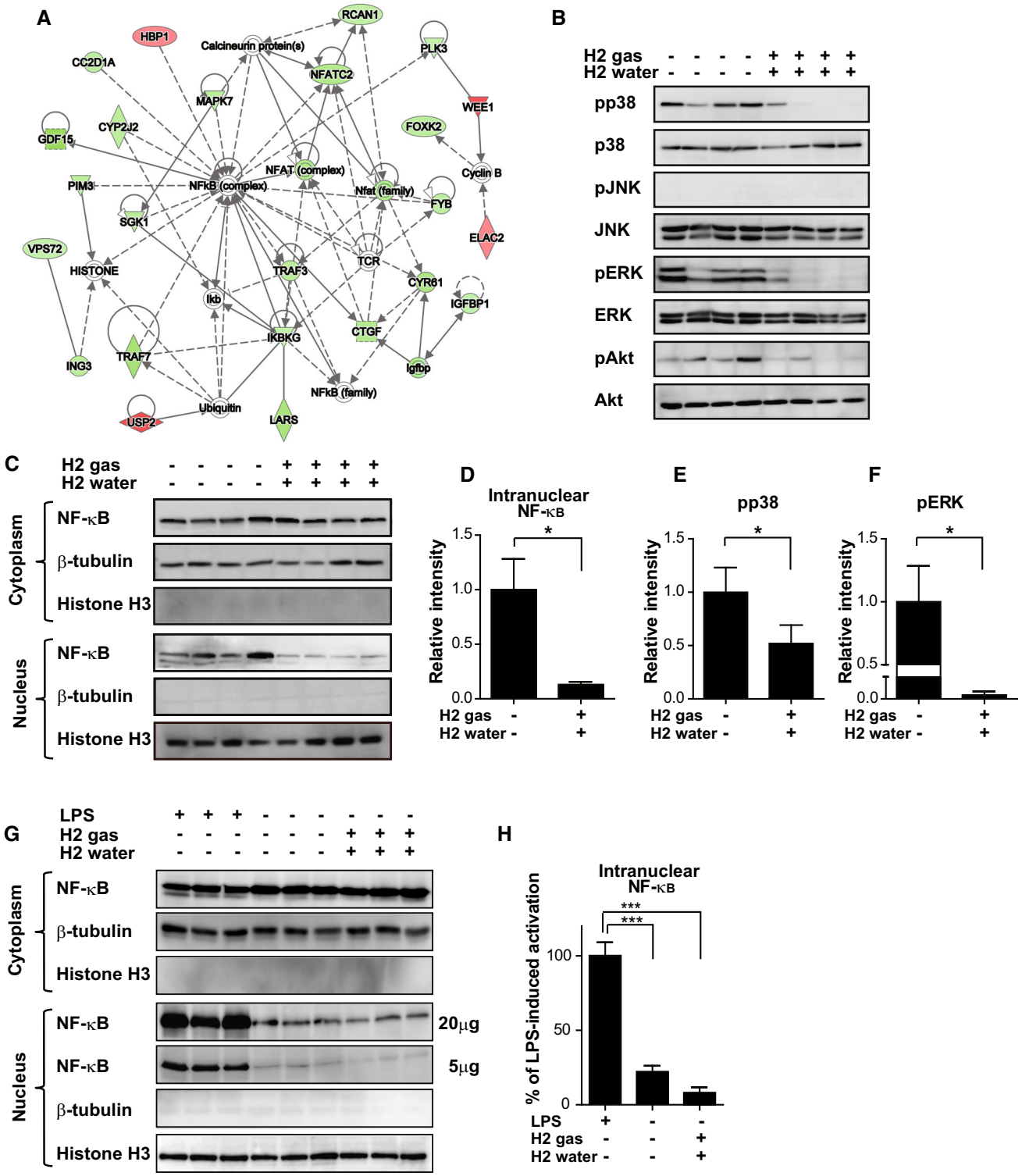
We have previously investigated the differences between HRW and HCA using Parkinson's disease model rats [12]; however, neither other report has compared the therapeutic effects between HRW and HCA nor demonstrated their different molecular actions. To explore this, we selected nine genes from Supplementary Fig. 2 (*Ctgf*, *Gdf15*, *Bcl6*, *G6pc*, *Egr1,* and *Dusp1* as NF-κB target genes and *Cry61*, *Wee1,* and *Ucp1* as NF-κB non-target genes which were statistically ranked within the top six most differentially expressed genes in Supplementary Fig. 2) and compared their expression levels during the administration of HCA, HRW, or both. To check which molecules in Supplementary Fig. 2 are NF-κB targets, we referred to IPA results for *Ctgf* and *Gdf15* (direct interaction in Fig. 2A and Supplemental Fig. 3A) and to reports for *Bcl6*, *G6pc*, *Egr1,* and *Dusp1* [17–23]. We found that HCA and HRW additively changed gene expression in the liver regardless of whether a molecule was a NF-κB target or not (Fig. 3). Western blot analysis revealed that H_2 treatment with HRW and/or HCA suppressed signaling in the NF-κB, p38, and ERK pathways (Fig. 4). These results suggest that combined administration of HRW and HCA may enhance the physiological effects of H_2, partially by changing gene expression and signal activation.

Finally, for the individual mice indicated by open circles in the left column of Fig. 3, we also evaluated gene expression in kidney and brain as well as liver. As shown in Fig. 5, we found that the H_2-mediated down-regulation of *Ctgf*, *Gdf15*, *Bcl6*, *Cyr61,* and *Egr1* was similar in the liver, kidney, and brain. As the blood concentrations of H_2 were lower in the kidney and most likely in the brain compared with the liver, we suggest that the effects of H_2 could be easily saturated at a very low concentration or that H_2 exhibited a low dose–response effect in response to other factors, such as secretary proteins from other organs.

Discussion

In this study, we first showed that inhalation of HCA increased the concentration of H_2 in the arteries to the same extent as in the veins and that the H_2 concentrations were consistent with those expected by Henley's law. In fact, the observed H_2 concentrations were similar to those in the study reported by Ohsawa et al. [2].

We next showed that oral intake of HRW temporarily increased the H_2 concentration in atrial and arterial blood. Interestingly, the H_2 concentration in arteries decreased to 10 % of that in the atrium, suggesting that the remainder was lost in the lungs. Furthermore, the H_2 concentrations in the liver and kidney were similar to those in atrial and arterial blood, respectively. Nagata et al. showed that the venous H_2 concentration in rats after administering 3.5 ml of H_2-water was 5 μM, and these results were similar to ours [24]. Moreover, the changes in the liver H_2 concentration reported by Kamimura et al. were similar to ours [16]. Recently, Liu et al. estimated the H_2 concentration in systemic rat organs using gas chromatography after administration of high concentrations of HRW or HCA by a



method similar to ours [25]. They mainly showed H_2 concentrations in blood and organs after administration of H_2 super-rich water or saline from several routes. They also demonstrated H_2 concentrations in arterial blood, liver, and kidney after oral administration of HRW at the concentration of 1.25 ppm, which is similar to the HRW concentration used in our experiments. Since they used different units for the estimation, we converted these values

◀**Fig. 2** H_2 alters signal transduction pathways in the healthy mouse liver. **A** After 3 weeks of H_2 treatment (both H_2-containing air and H_2-rich water), a total of 140 up- (31 genes) and down- (109 genes) regulated genes (Table S1) were identified in the liver and analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems). A network was graphically generated. *Green* (down-regulated genes) and *red* (up-regulated genes) *nodes* indicate input genes. The biological relationship between two nodes is represented as a *line*. *Bold lines* indicate a direct interaction, and *dotted lines* indicate an indirect interaction. **B**, **C**, **D**, **E**, **F**, **G**, and **H** Effects of H_2 on signal transduction in the liver were examined ($n = 4$ animals per group). Tissue lysates (**B**) and cytosolic and nuclear fractions (**C** and **G**) were subjected to Western blot analysis for the indicated proteins, and the band intensities for intranuclear NF-κB (**D** and **H**), pp38 (**E**) and pERK (**F**) were quantified. The *data* are represented as the mean ± SEM. *Asterisks* indicate statistical significance as determined by Student's *t* test (*$p < 0.05$) for **D**, **E**, **F**, and one-way ANOVA and Tukey's multiple-comparison test (***$p < 0.001$) for **H**

to μM and compared with our results (after personal communication with the author, we confirmed that the Y-axis unit of figure in this report was not ppb/g but ppm/g). After administration of HRW (1.25 ppm), they showed that average H_2 concentrations in arterial blood and liver were 0.05 and 3.59 μM (calculated from Fig. 2A), which were similar to our results (0.39 and 3.63 μM, respectively). On the other hand, they estimated the H_2 concentration in kidney as 2.37 μM, whereas we evaluated it at 0.64 μM, which was closed to that of arterial blood measured by our experiments. The reason for this difference is unclear; however, if their value was correct, almost all H_2 in the kidney would have diffused directly from the gastrointestinal tract, because H_2 concentration in arterial blood was extremely low. If our results are correct, we speculate that their measured value in the kidney might be unreliable because of the high standard error of the mean (SEM) at this point (HRW with 1.25 ppm H_2). In any case, these results indicate that H_2 may be effective at extremely low concentrations in arterial blood. Indeed, both Ohsawa et al. and Fujita et al. investigated the relationship between H_2 concentration and its activity in cell lines and found that H_2 can function at low concentrations [2, 7]. Furthermore, HRW is effective for kidney and brain disease models, in which the blood H_2 concentration will be low after its oral intake. These observations also support the idea that the effects of hydrogen could be easily saturated at very low concentrations.

We used microarray analysis to compare gene expression in mouse livers between control mice and those treated with both HRW and HCA. We found that H_2 treatment altered gene expression even in healthy livers. Nakai et al. also observed changes of gene expression in healthy rats after oral HRW administration alone and concluded that lipid metabolism-related genes were up-regulated [26]. Although we did not observe such changes in gene expression, IPA indicated that NF-κB signaling acted upstream of the down-regulated genes. Therefore, to investigate whether HRW has an effect on NF-κB signaling, we downloaded the microarray data of Nakai et al., from the Gene Expression Omnibus (GEO) Database and performed Gene Set Enrichment Analysis, which is a reliable method to explore statistically significant differences between two biological states (Broad Institute; http://www.broadinstitute.org/gsea/index.jsp). Interestingly, we found that these microarray data also showed that HRW significantly suppressed gene expression related to NF-κB signaling (Supplemental Fig. 5). Furthermore, our Western blot analysis showed that intranuclear translocation of NF-κB decreased, as did the levels of wide-ranging signals, such as p38 and ERK. While many reports have indicated that H_2 attenuates the activation of several signaling molecules in disease processes, there have been few reports investigating their activation in healthy organs. Various signaling events have roles in the liver under physiological conditions. For example, NF-κB is necessary for preventing spontaneous apoptosis and subsequent tumor development through a chronic state of liver inflammation and regeneration [27]. Activation of p38 MAPK maintains adult hepatocytes in cell cycle arrest, and temporary inactivation of p38 MAPK is necessary for hepatic regeneration following partial hepatectomy [28]. ERK activation in the liver can modify pancreatic β cell masses through the neuronal network, which regulates the blood glucose level [29]. Although H_2 may influence the above-mentioned physiological reactions, it may have only a small impact on the adult liver. We observed that the basal activation of NF-κB in the liver was as low as 22 % compared with that following LPS administration, while H_2 treatment only decreased this basal activation by half (Fig. 2G, H). Furthermore, we found no remarkable influences in a positive or negative way under HCA following 2 more years of administration (data not shown). A clinical study in which HRW was actually administered to humans did not report such adverse effects on hepatic functions. However, it will be necessary to investigate the impact of H_2 on normal physiological functions, because the details of the molecular mechanism of H_2 action are still unknown.

Finally, we suggested the possibility that there is a difference in the in vivo mechanisms of action between HRW and HCA. From the expression patterns of nine genes selected from our microarray data, we found that administration of both HRW and HCA results in additive effects on their expression. While the blood H_2 concentration is stably maintained with HCA but not with HRW (Fig. 1), the effects on gene expression are equivalent (Fig. 3), and administration of both HCA and HRW had a

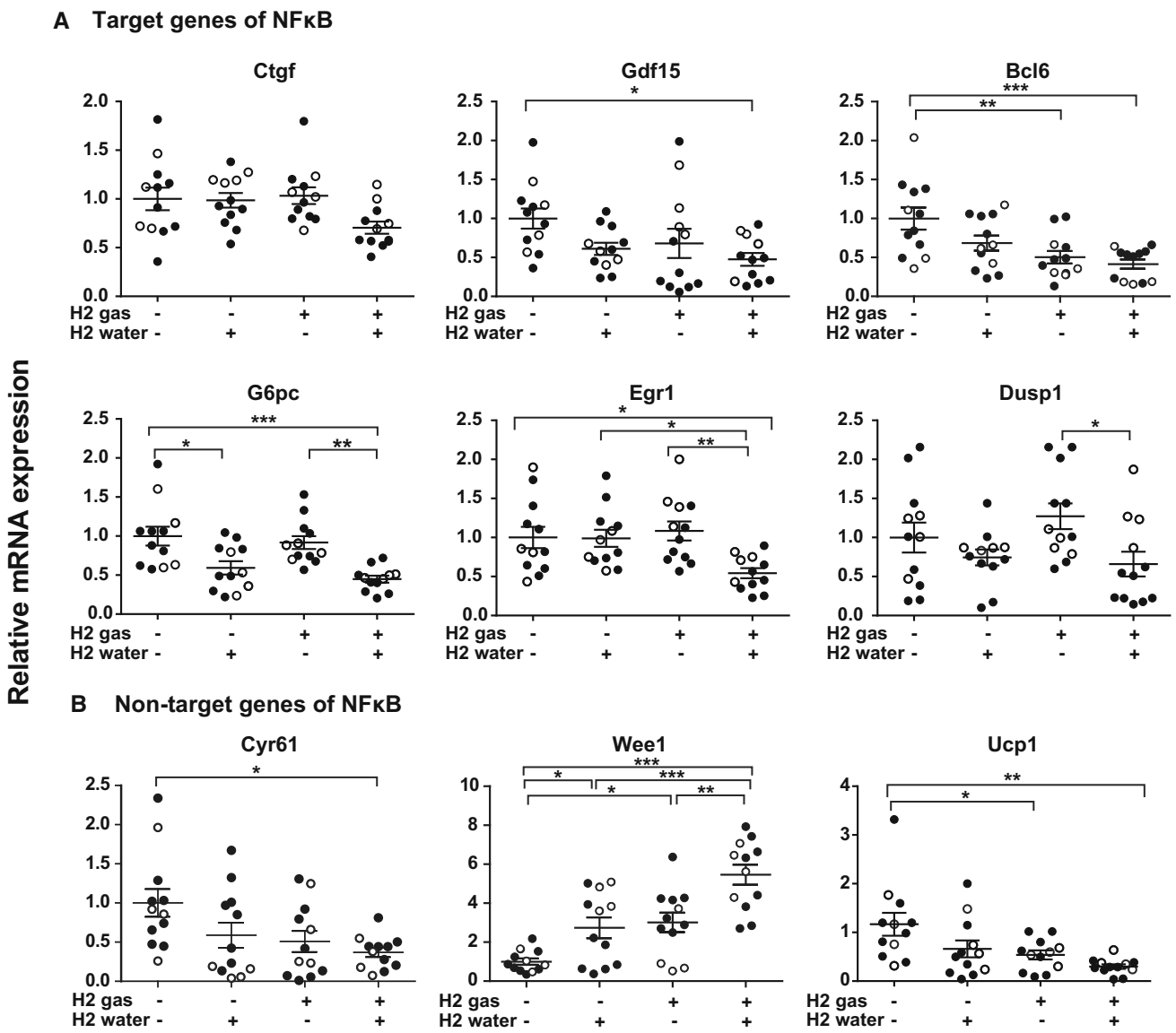


Fig. 3 Effects of H_2 from H_2-rich water (HRW) and/or H_2-containing air (HCA) on gene expression in the liver. Nine NF-κB target (**A**) or NF-κB non-target (**B**) H_2-responsive genes were selected from genes which showed statistically significant differential expression after H_2 treatment by qPCR. After 3 weeks of H_2 administration in the form of HRW and/or HCA (2 % H_2), the expression levels of the selected genes in the liver were quantified by qPCR ($n = 12$ animals per group). The *data* are presented as the mean ± SEM. *Asterisks* indicate statistical significance as determined by one-way ANOVA and Tukey's multiple-comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

stronger effect than administration of either alone. We previously showed that HRW, but not HCA, was effective in a Parkinson's disease rat model; however, by inducing a blood concentration of H_2 similar to that produced by HRW, intermittent administration of HCA resulted in intermediate effects between HRW and the normal HCA treatment [12]. This result suggests that H_2 bioactivity depends not only on its blood concentration but also on changes in the H_2 concentration through an unknown mechanism, by which HRW and HCA show additive effects for gene expression. On the other hand, Matsumoto et al. recently showed that H_2 stimulates ghrelin secretion from the stomach after oral administration and that H_2-induced ghrelin protects nigrostriatal neurons from neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, in Parkinson's disease model rats [30]. This report suggests that, after oral intake, HRW may, presumably dose dependent, induce secreted factors such as ghrelin in the stomach, where H_2 concentration is extremely high. Consequently, the HRW stimulus is transferred to the secreted factors, enabling the HRW stimulus to be transmitted to the whole body. According to

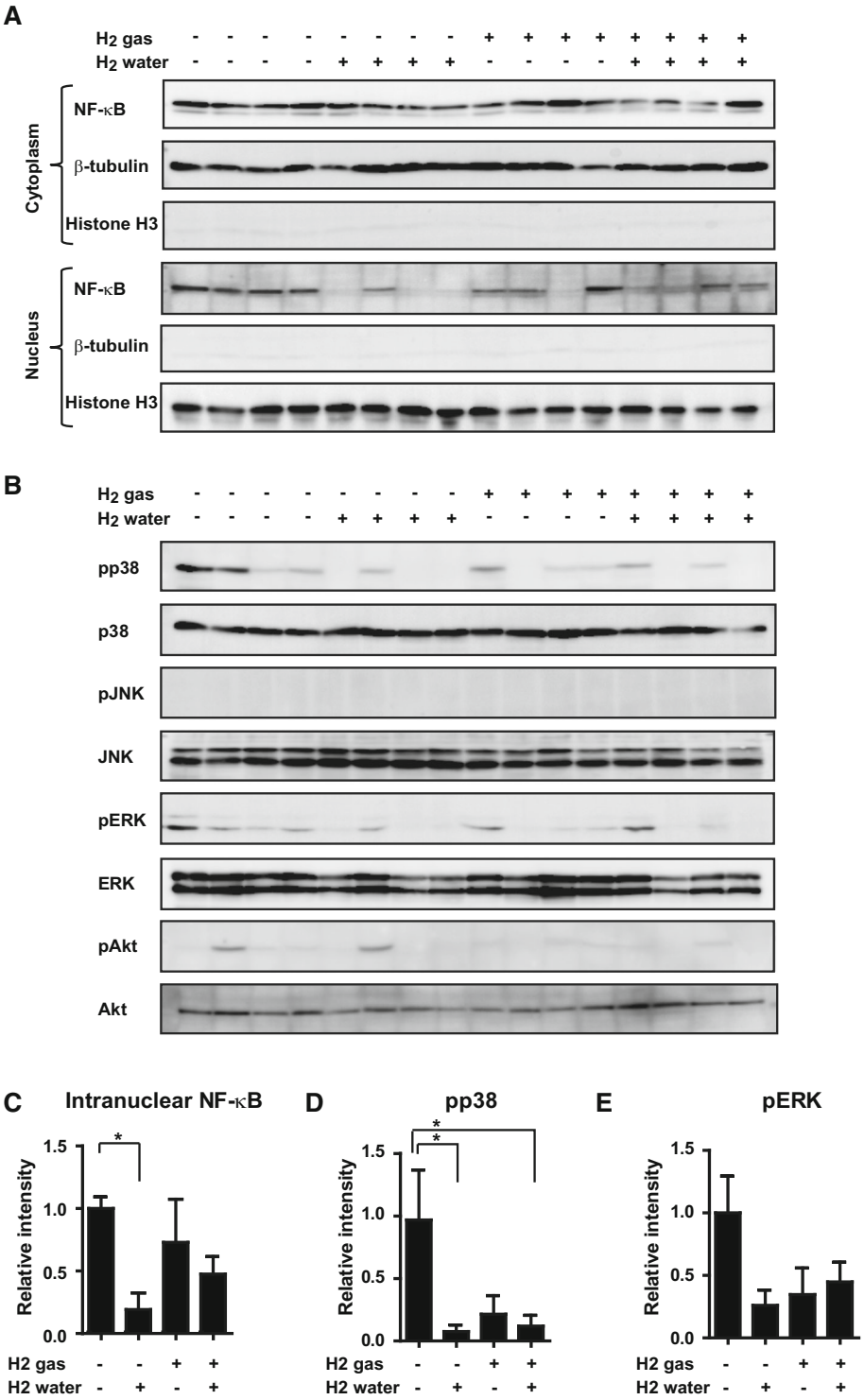


Fig. 4 Effects of H_2 from H_2-rich water and/or H_2-containing air on signaling pathways in the healthy mouse liver. Cytosolic and nuclear proteins (**A**) and whole tissue lysates (**B**) were subjected to Western blot analysis for the indicated proteins. Band intensities for intranuclear NF-κB (**C**), pp38 (**D**), and pERK (**E**) were quantified. *Data* are represented as the mean ± SEM. *Asterisks* indicate statistical significance as determined by one-way ANOVA and Tukey's multiple-comparison test ($*p < 0.05$)

this hypothesis, additive effects of HRW and HCA on gene expression could be explained by additive effects caused by HCA and secreted factors induced by HRW. Our results and those of others suggest that H_2 affects several signal transduction pathways and networks in addition to eliminating hydroxyl radicals. Our result that HRW and

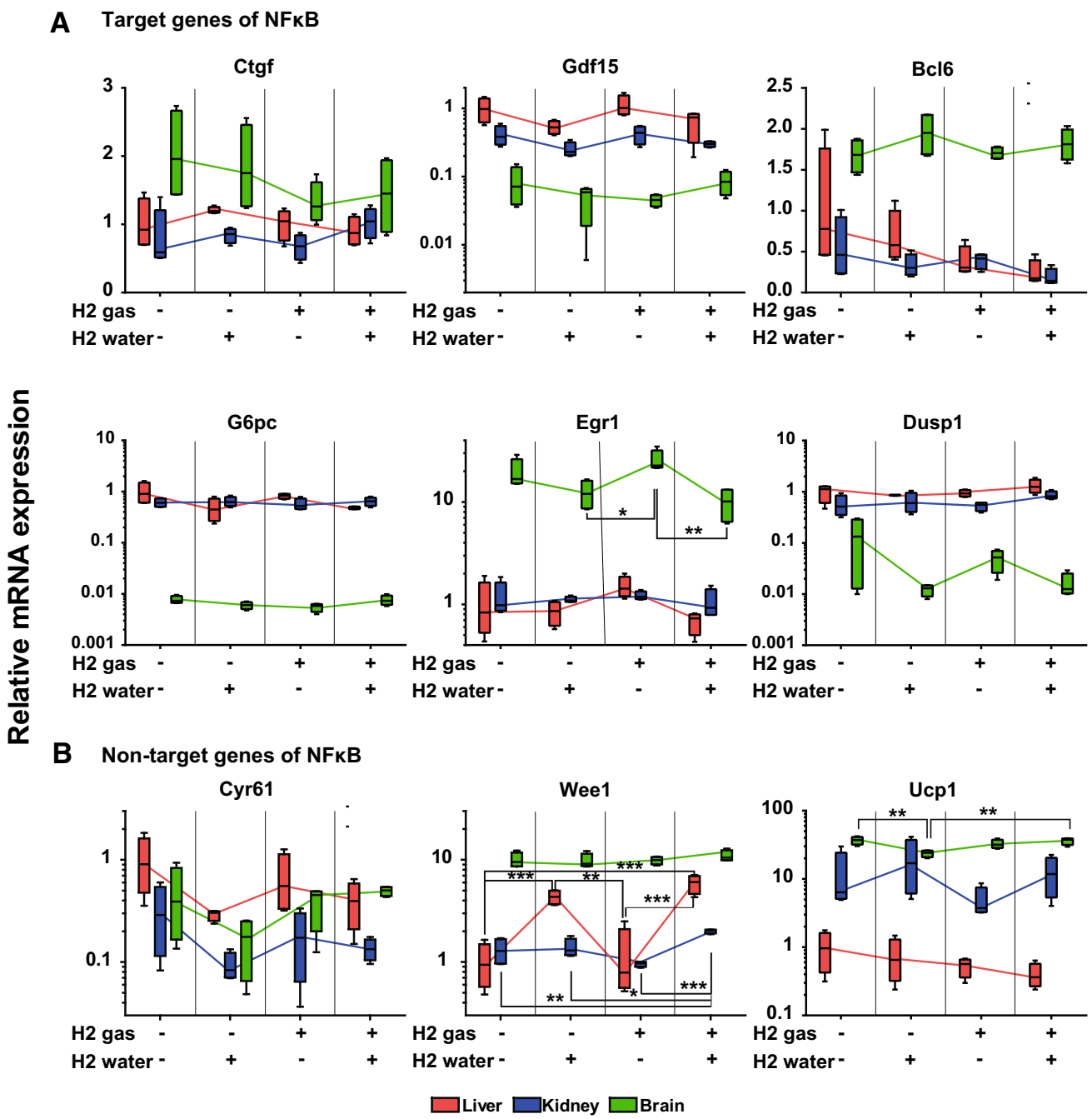


Fig. 5 Effects of H_2 from H_2-rich water (HRW) and/or H_2-containing air (HCA) on gene expression in systemic mouse organs. Nine NF-κB target (**A**) or NF-κB non-target (**B**) H_2-responsive genes were selected from genes that showed statistically significant differential expression after H_2 treatment by qPCR. After 3 weeks of H_2 administration in the form of HRW and/or HCA (2 % H_2), the gene expression levels in the kidney and brain were measured by qPCR in the mice represented by *open circles* in Fig. 3 ($n = 4$ animals per group). Whiskers represent the minimum and maximum values, and boxes represent the interquartile range (25–75th percentiles). *Asterisks* indicate statistical significance as determined by one-way ANOVA and Tukey's multiple-comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

HCA have additive effects may provide clues toward the molecular mechanism of H_2 bioactivity and may lead to drug discovery based on this mechanism.

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