

SCIENTIFIC REPORTS



OPEN

Molecular hydrogen regulates gene expression by modifying the free radical chain reaction-dependent generation of oxidized phospholipid mediators

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We previously showed that H₂ acts as a novel antioxidant to protect cells against oxidative stress. Subsequently, numerous studies have indicated the potential applications of H₂ in therapeutic and preventive medicine. Moreover, H₂ regulates various signal transduction pathways and the expression of many genes. However, the primary targets of H₂ in the signal transduction pathways are unknown. Here, we attempted to determine how H₂ regulates gene expression. In a pure chemical system, H₂ gas (approximately 1%, v/v) suppressed the autoxidation of linoleic acid that proceeds by a free radical chain reaction, and pure 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), one of the major phospholipids, was autoxidized in the presence or absence of H₂. H₂ modified the chemical production of the autoxidized phospholipid species in the cell-free system. Exposure of cultured cells to the H₂-dependently autoxidized phospholipid species reduced Ca²⁺ signal transduction and mediated the expression of various genes as revealed by comprehensive microarray analysis. In the cultured cells, H₂ suppressed free radical chain reaction-dependent peroxidation and recovered the increased cellular Ca²⁺, resulting in the regulation of Ca²⁺-dependent gene expression. Thus, H₂ might regulate gene expression via the Ca²⁺ signal transduction pathway by modifying the free radical-dependent generation of oxidized phospholipid mediators.

Molecular hydrogen (H₂) was originally thought to behave as an inert gas in mammalian cells; however, our previous studies showed that this is not always the case¹, demonstrating that H₂ neutralizes the hydroxyl radical (·OH) and peroxynitrite (ONOO⁻) inside cells and acts as a novel antioxidant to protect the cells against oxidative stress^{1,2}. Inhalation of 1%–4% (v/v) H₂ gas is effective for the treatment of ischemia/reperfusion injuries^{1,3,4}. Recently, inhalation of 1.3% H₂ gas from a premixed gas was shown to protect neurons in a cardiac arrest model⁵. However, the mechanism of how such a low concentration of H₂ exerts the positive effects is not known.

Numerous studies have strongly suggested that H₂ has the potential for a variety of therapeutic and preventive applications^{6,7}. In addition to extensive animal experiments, more than 10 clinical studies examining the efficacy of H₂ have been reported^{6,7}, including double-blinded clinical studies in patients with Parkinson's disease and rheumatism^{8,9}. Based on these studies, the field of hydrogen medicine is rapidly growing.

Subsequently, H₂ was shown to exhibit multiple functions, including anti-inflammatory, anti-apoptotic, anti-allergic, and antioxidant activities, as well as regulation of differentiation and energy metabolism^{6,7}. To exert multiple functions in addition to anti-oxidative roles, H₂ regulates various signal transduction pathways and the expression of many genes^{6,7}. For examples, H₂ protects neural cells and stimulates energy metabolism by

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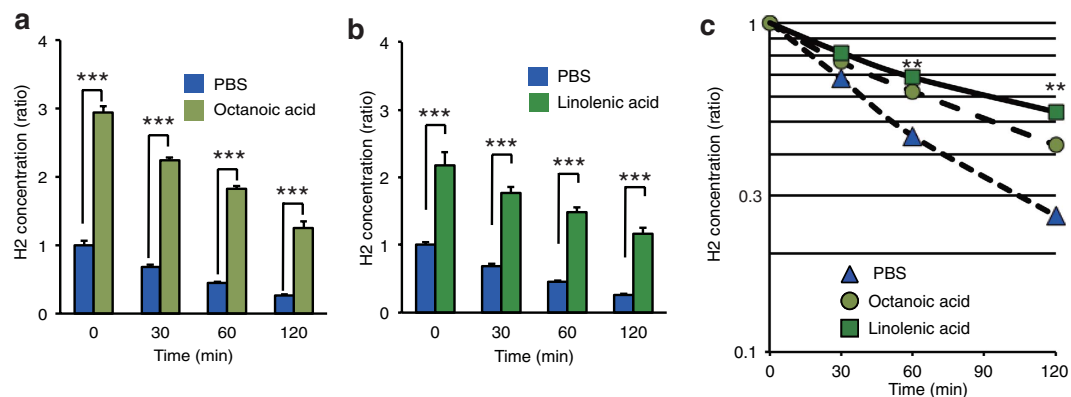


Figure 1. Solubility of H₂ in fatty acids in the presence of an aqueous solvent. H₂-saturated phosphate-buffered saline (PBS) was mixed with the same volume (75 mL) of saturated fatty acid (octanoic acid) (a) or unsaturated fatty acid (linolenic acid) (b) and maintained for 16 h in a closed aluminum bag as described in Methods. The same volume (3 mL) of each phase was transferred to each open tube (ϕ 13 mm), followed by measurement of H₂ at the indicated time ($n = 4$). The experiments were performed at 25 °C. *** $P < 0.001$ vs. PBS. (a,b) Significance was calculated using an unpaired two-tailed Student's *t*-test. (c) Time courses of retention times of H₂ in each phase in the open vessels. ** $P < 0.01$, vs. octanoic acid ($n = 4$).

stimulating the hormonal expression of ghrelin¹⁰ and fibroblast growth factor 21 (FGF21)¹¹, respectively. In contrast, H₂ relieves inflammation by decreasing pro-inflammatory cytokines¹². However, it is difficult to explain the molecular mechanisms by which H₂ exerts these functions by conventional concepts alone. To understand the molecular mechanisms by which H₂ exerts these multiple functions, it is essential to identify the primary targets of H₂ that modulate signal transduction and gene expression.

Therefore, in this study, we aimed to elucidate one of the molecular mechanisms by which H₂ mediates signal transduction and gene expression. Our results suggested that low concentrations of H₂ modulated Ca²⁺ signal transduction and regulated gene expression by modifying the production of oxidized phospholipid species.

Results

H₂ accumulated in the lipid phases. To understand the difference between intracellular conditions and aqueous solutions, we focused on the lipid phases to determine the intracellular localization of H₂ accumulation at room temperature. H₂ incorporation was two- or three-fold higher in the liquid fatty acid phases than in the aqueous phase in the presence of both water and fatty acids, and was retained longer in the fatty acid phases than in the aqueous phase in open vessels (Fig. 1a,b). In particular, H₂ seemed to be retained significantly longer in the unsaturated fatty acid (linolenic acid) than in the saturated fatty acids (octanoic acid) (Fig. 1c), although this difference in retention time might be attributed to the difference in the number of carbons. Since unsaturated fatty acids are the primary targets for initiating a free radical chain reaction, we assumed that H₂ could efficiently suppress this reaction in biomembranes, even at low concentrations.

Autoxidation of unsaturated fatty acids was suppressed by low concentrations of H₂ gas.

Autoxidation of unsaturated fatty acids proceeds by a free radical chain reaction in air¹³. Thus, we measured autoxidation of a filmy di-unsaturated fatty acid (linoleic acid: R-CH = CH-CH₂-CH = C-R') at 37 °C for 20 h in the dark in the presence of various concentrations of H₂ gas. A conjugated diene, which should be formed by autoxidation, was estimated by the absorption at 234 nm (Fig. 2a). The absorption at 234 nm was increased depending on the formation of the conjugated diene [R-CH = CH-CH = CH-CH(-OOH)-R' or R-CH(-OOH)-CH = CH-CH = CH-R'] accompanied by peroxidation in a pure chemical system (H₂, O₂, and N₂ were supplied from gas cylinders) (Fig. 2b). O₂ was essential for autoxidation (Fig. 2c). As a result, even only approximately 1% H₂ gas significantly suppressed autoxidation of linoleic acid at 37 °C, even in the absence of any catalysts in the dark in a pure chemical system (Fig. 2c).

Ca²⁺ signal transduction by H₂-dependent chemical oxidation of phospholipids. Phospholipids are converted into oxidized mediators that modulate various signal transduction pathways by not only enzymatic reactions, but also by chemical oxidation^{14,15}. Oxidized phospholipids, including 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC), are present in oxidatively modified low-density lipoproteins (oxLDLs) and have been found in atherosclerotic lesions¹⁵. These compounds are important as inducers of different cellular responses, including inflammation, proliferation, and cell death. Moreover, autoxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) leads to the chemical production of various bioactive phospholipid species, such as POVPC, PGPC, 1-palmitoyl-2-(5-hydroxy-8-oxooct-6-enoyl)-*sn*-glycero-3-phosphocholine (HOOA-PC), and 5-hydroxy-8-oxo-6-octenedioic acid (HODiA-PC)^{14,15}.

We assumed that low concentrations of H₂ would influence some chemical reactions leading to the production of putative oxidized lipid mediators for the modulation of signal transduction. Because PAPC is one of the major phospholipids in mammalian biomembranes, the role of H₂ in the chemical production of oxidized phospholipid

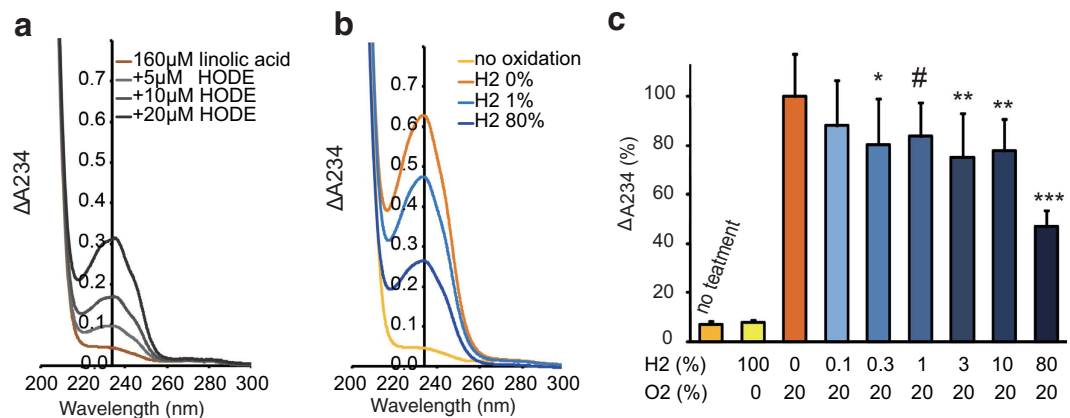


Figure 2. Suppression of autoxidation of linoleic acid-film by H₂ gas. (a) Profile of ultraviolet absorption of 9-hydroxyoctadecadienoic acid; CH₃(CH₂)₄-CH=CH-CH=CH-CH(-OH)-(CH₂)₇-COOH (9-HODE) in cyclohexane, shown as a standard conjugated diene. (b) Linoleic acid-film was autoxidized at 37 °C for 20 h in a glass tube placed in a closed aluminum bag in the presence of various concentrations of H₂ and O₂ as described in Methods. Representative profiles of ultraviolet absorption of the cyclohexane solution of H₂-dependent autoxidized linoleic acid are shown. (c) Linoleic acids autoxidized with various concentrations of H₂ were evaluated by measuring absorption at 234 nm. **P* = 0.034 (0.3% H₂), #*P* = 0.069 (1% H₂), ***P* < 0.01 (3% H₂, 10% H₂), and ****P* < 0.001 (80% H₂) vs. 0% H₂ (*n* = 15).

mediators was determined by conducting autoxidation of pure PAPC (resulting in OxPAPC) in the absence of any catalysts in the dark. The peroxidation of PAPC in air was confirmed by an increase in the signal for the fluorescent dye specific to lipid peroxides, Liperfluor (Fig. 3a). A previous study indicated that OxPAPC activates transcription factors involved in Ca²⁺ signaling¹⁶. Indeed, when THP-1 cells (a human monocytic cell line derived from a patient with acute monocytic leukemia) were exposed to OxPAPC, a transient increase in cellular Ca²⁺ was observed when a Ca²⁺-sensitive fluorescent dye, Fluo4-AM was used (Fig. 3b). This Ca²⁺ signaling depended on OxPAPC in an oxidation time-dependent manner (Fig. 3c).

Next, the H₂-dependent production of OxPAPC, which leads to the activation of Ca²⁺ signaling, was investigated by autoxidizing PAPC for 3 days at 25 °C in air at various concentrations of H₂ (designated as H₂OxPAPC, and the notation of H₂[x%]OxPAPC was used when autoxidized in the presence of x% H₂). H₂ suppressed the generation of total peroxides as revealed by Liperfluor fluorescence intensity (Fig. 3d). Ca²⁺ signaling was observed when PAPC was autoxidized with less than 0.3% H₂, whereas more than 1.3% H₂ significantly disrupted this signaling (Fig. 3e).

In order to investigate the molecule(s) influenced by H₂, we analyzed H₂OxPAPCs by using mass spectrometry on autoxidation day 3. In all, 209 bands were detected, with molecular masses ranging from 126.3754 to 991.6494 Da; this was consistent with the findings of a previous report¹⁵ (Supplementary Fig. 1). The differences in the production of H₂OxPAPC and OxPAPC species were presented using a heat map (Supplementary Fig. 1i). The levels of many bands were increased or decreased with differences in concentrations of H₂. For examples as the relatively increased species, the levels of the Ca²⁺ signaling inducers POVPC¹⁶, HOOA-PC, HODiA-PC, and hydroxyeicosatetraenoic acid-3-phosphocholine (HETE-PC)¹⁷ were slightly increased in response to H₂ (Supplementary Fig. 1i).

Because the reduced form of POVPC was reported to function as an antagonist¹⁸, it is possible that increased levels of the reduced form(s) of some OxPAPC species, rather than the decreased levels of putative agonists (such as POVPC), might have disrupted Ca²⁺ signaling as a putative antagonist(s). Further studies are warranted to identify the H₂-dependent bioactive mediator(s).

Comprehensive analysis of H₂-dependent regulation of gene expression. Next, we investigated how H₂OxPAPC influences gene expression. PAPC was autoxidized in the absence or presence of various concentrations of H₂ for 3 days and then administered to cultured THP-1 cells. In a preliminary experiment, the change in the expression level of tumor necrosis factor (TNF)- α gene in response to OxPAPC from that to H₂OxPAPC peaked at 4 h. Thus, by using microarray analysis, we comprehensively analyzed the change in gene expression in response to the H₂-dependent mediators at 4 h in three samples under each condition. In all, 86 genes were selected according to the following criteria as described in the legend of Fig. 4a: a significant increase in OxPAPC (vs. PAPC), and a significant decrease in H₂[1.3%]OxPAPC and H₂[5%]OxPAPC (vs. OxPAPC) (Supplementary Table 1). The gene expression profile was presented in a heat map (Fig. 4a). The selected genes were validated by semi-quantitative real-time polymerase chain reaction (RT-PCR), and marginal changes in the expression levels of some genes were confirmed (Supplementary Fig. 2).

In addition, the regulatory expression of TNF- α and IL-8 by H₂OxPAPC was investigated using THP-1 and a different cell type (human aortic endothelial cells: HAEC), respectively (Fig. 4b,c).

According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (<http://www.genome.jp/kegg/pathway.html>), the functions of 7,143 genes were identified and classified (Fig. 4d, upper). We

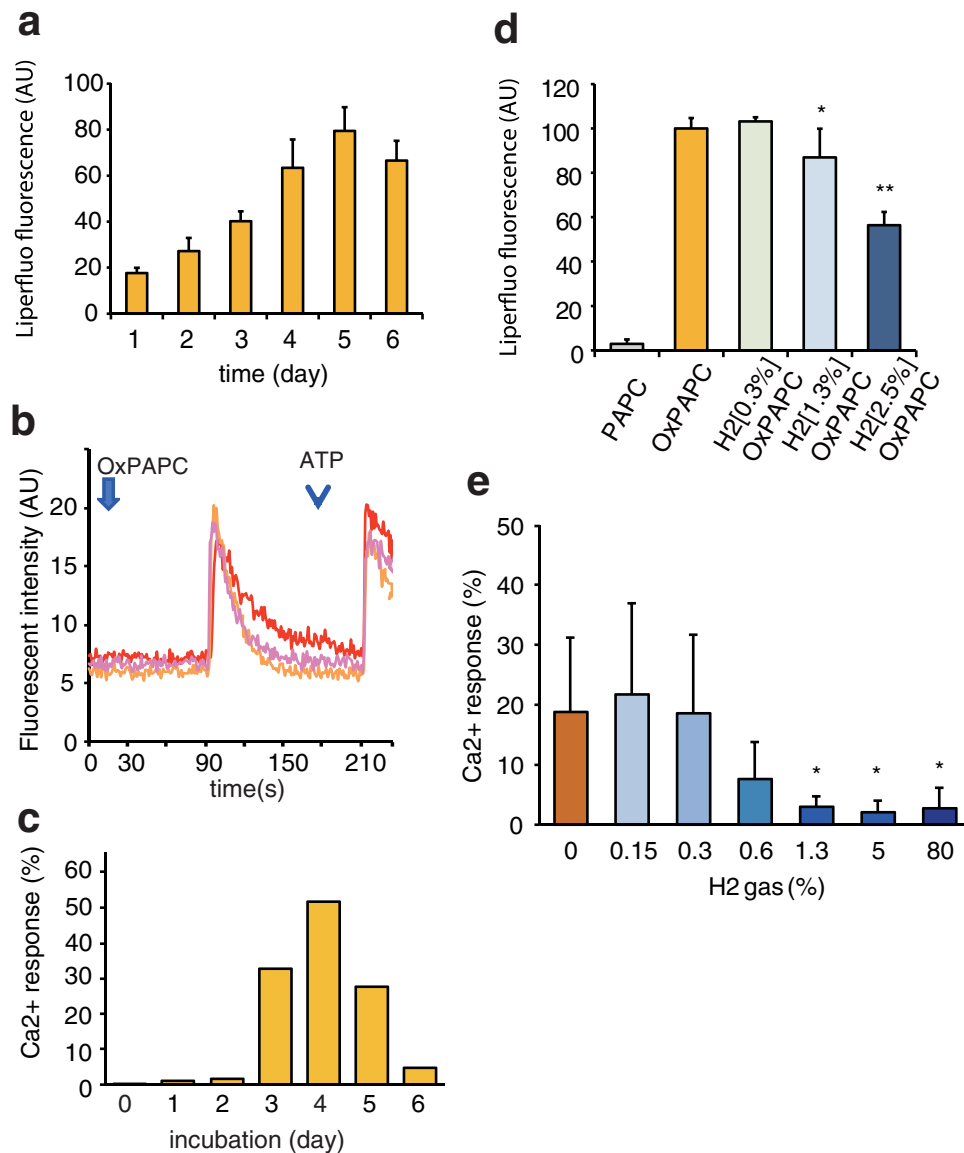


Figure 3. PAPC autoxidized with H₂ modulated Ca²⁺ signaling. (a) Chemically pure PAPC was autoxidized in air with 100% humidity at 25 °C in a closed aluminum bag for the indicated periods, and time-dependent production of peroxides in air from chemically pure PAPC was estimated using Liperfluo fluorescence, where wavelengths of excitation and emission were set at 488 and 535 nm, respectively, as described in Methods. (b) Representative responses in THP-1 by OxPAPC with the fluorescent Ca²⁺ indicator Fluo4-AM are shown as described in Methods. The arrow and arrowhead indicate the addition of OxPAPC and ATP, respectively. ATP (a ligand of the Ca²⁺ channel P2X7) was used as a positive control. (c) PAPC was autoxidized for the indicated periods at 25 °C, and subjected to the Ca²⁺-signaling assay in THP-1 cells. The OxPAPC-induced Ca²⁺ response depended on autoxidizing period of OxPAPC. (d) PAPC was autoxidized in air for 3 days in the absence or presence of the indicated concentrations of H₂ (H₂OxPAPC), and the peroxide of OxPAPC or H₂OxPAPC was estimated using Liperfluo as described in (a) (*n* = 3–6). **P* = 0.044, ***P* < 0.01. (e) PAPC was autoxidized in air for 3 days with the indicated concentrations of H₂ (H₂OxPAPC) and then subjected to Ca²⁺ signaling assays as described in Methods (*n* = 6). **P* = 0.021 (1.3% H₂), **P* = 0.022 (5% H₂), and **P* = 0.030 (80% H₂) vs. no H₂.

classified the 86 selected genes (Fig. 4e, lower). Of these 86 genes, 46.5% belonged to those involved in signaling pathways (Fig. 4d, upper), whereas 25.8% of the total number of 7,143 genes is involved in signaling pathways (Fig. 4d, lower). Genes encoding factors involved in signal transduction and transcription factors are indicated by blue and black, respectively, on the right in the heat map (Fig. 4a).

Among the genes involved in signaling pathways, the proportion of those belonging to Ca²⁺ signaling were lower in the selected genes than in those in the entire genome, indicating that H₂ regulates fewer components of the Ca²⁺ signaling pathways (Fig. 4e, lower). This was consistent with the finding that H₂OxPAPC decreased Ca²⁺ signaling. In contrast, the proportion of genes belonging to the mitogen-activate protein kinase (MAPK)

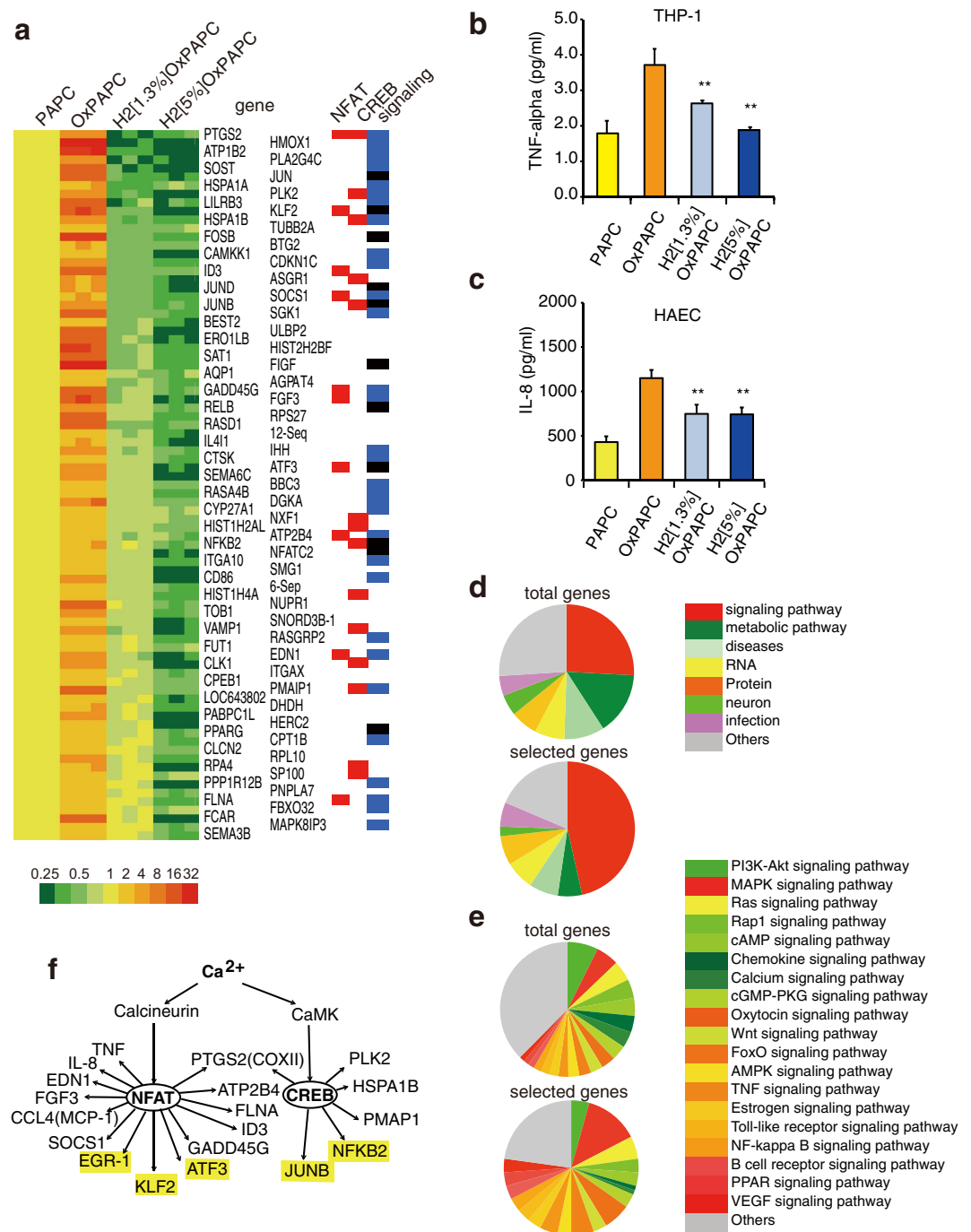


Figure 4. Changes in gene expression regulated by H₂OxPAPC. (a) Three samples of PAPC, OxPAPC, and H₂OxPAPC were exposed to THP-1 cells for 4 h, and the gene expression was comprehensively analyzed using microarray. Eighty-six genes were selected according to the following criteria; genes up-regulated by OxPAPC (more than 2.5-fold, vs. PAPC) and those down-regulated by H₂[1.3%]OxPAPC and H₂[5%]OxPAPC (less than 0.75-fold and 0.5-fold, respectively, vs. OxPAPC) are shown in a heat map (red and green indicate the up-regulation vs. PAPC treatment, and the down-regulation vs. OxPAPC treatment, respectively, as shown in the color gradient). Possible target genes of NFAT and CREB are marked with red on the right. Genes encoding factors involved in signal transduction and transcription are indicated by blue and black, respectively, on the right. The release of TNF- α (b) (from THP-1) and IL-8 (c) (from HAEC) was investigated using ELISA as described in Methods. (d, upper) Ratio of genes belonging to each category for a total of 7,142 genes identified by the KEGG database. (d, lower) Ratio of genes belonging to each category in the 86 selected genes listed in a. (e, upper) Ratio of genes belonging to each signaling pathway identified by the whole KEGG database. (e, lower) Ratio of genes belonging to each signaling pathway in the selected genes listed in (a). (f) The H₂OxPAPC-dependent expression of genes transcribed by CREB and NFAT. Transcription factors are indicated in yellow.

signaling was higher (Fig. 4e, lower), indicating that H₂ regulates more components of MAPK signal transduction pathways (Fig. 4e, lower).

The signal transduction pathways that were regulated by H₂ are shown in Supplementary Table 1 according to the KEGG Pathway Database. These data suggested the possibility that low concentrations of H₂ contribute to various signal transduction pathways via oxidized phospholipid species.

cAMP response element binding protein (CREB)-target genes were selected according to the CREB Target Gene Database (<http://natural.salk.edu/CREB/>), and nuclear factor of activated T cells (NFAT) target genes were selected by referring to Medline, as shown in Supplementary Table 1. The target genes of CREB and NFAT are marked by red on the right in the heat map panel as NFAT or CREB (Fig. 4a). A considerable number of the selected genes were targets of CREB or NFAT (Fig. 4f). These data are consistent with the findings of previous studies showing the Ca²⁺-dependent regulation by these transcription factors: CREB is activated via phosphorylation by a calmodulin-dependent kinase (CaMK)¹⁹ in a Ca²⁺-dependent manner, and NFAT is dephosphorylated by calcineurin (CN) in a Ca²⁺-dependent manner, translocates to the nucleus, and then functions as a transcription factor with its partner proteins, e.g., activator protein 1 (AP-1), CREB, or nuclear factor-kappa B (NF-κB)²⁰. Indeed, exposure of THP-1 to OxPAPC, but not to H₂OxPAPC, stimulated the nuclear translocation of NFAT (Supplementary Fig. 3).

Thus, H₂-dependent oxidized mediators or putative antagonists could be associated with transcriptional regulation via Ca²⁺ signaling.

Free radical inducers contributed to the NFAT pathway in cultured cells. Autoxidation of unsaturated fatty acids, including PAPC, proceeds by a free radical chain reaction¹³, and ·OH is the primary trigger for this reaction^{13,21,22}. We previously showed that H₂ reduces ·OH levels inside cultured cells by using the spin trapping method and a specific fluorescent indicator¹. Thus, in this study, we investigated the effects of H₂ on the lipid free radical chain reaction by using cultured cells. To initiate a free radical chain reaction inside the cells, we used 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH)²³, which is not affected by H₂ (Supplementary Fig. 4) and is suitable for the slow generation of free radicals by a spontaneous chemical reaction. The lipid free radical chain reaction results in the production of lipid peroxides (LPOs)^{21,24}, which can be detected using the fluorescent dye Liperfluo²⁵. Thus, we exposed cultured THP-1 cells to AAPH and estimated LPO production based on the Liperfluo signal. The Liperfluo signal significantly decreased in the presence of low levels of H₂ gas (e.g., 1.3%; Fig. 5a,b). Thus, even at such low concentrations, H₂ has the potential to reduce the generation of LPOs by suppressing the initiation and/or propagation of free radical chain reactions in cultured cells.

Next, we determined whether the responses induced by chemically produced H₂OxPAPC (Figs 3 and 4) could simulate the effects induced by the free radicals in cultured cells. When THP-1 cells were exposed to AAPH, the cellular Ca²⁺ levels increased (Fig. 5c) in a time-dependent manner (Fig. 5d), as shown by the analysis of Fluo-3, and the Ca²⁺ signaling was suppressed by H₂ (Fig. 5c,e). NFAT was also activated, as shown by the translocation of NFAT into the nucleus (Fig. 5f,g), and the nuclear translocation of NFAT were recovered by H₂ (Fig. 5f,g). Moreover, the free radical inducer stimulated the expression of some target genes of NFAT, including *TNF-α*, early growth response protein 1 (*EGR1*), and activating transcription factor 3 (*ATF3*), which have been shown in Supplementary Table 1, and H₂ decreased their expressions (Fig. 5h), suggesting that H₂ regulates these genes via the NFAT pathway.

In contrast, AAPH-mediated activation of CREB was not observed (Supplementary Fig. 5) in this cultured cell line, regardless of the stimulation of cellular Ca²⁺. In particular, the expression of the CREB-target gene *NFKB2* (NF-κB, subunit 2 gene) was not affected by AAPH (Fig. 5i), and the expression of *HMOX1* (Heme Oxygenase 1 gene), a nuclear factor-E2-related factor 2 (Nrf2)-target, was slightly but not significantly increased by H₂ (Fig. 5i). This result was consistent with those of a previous study²⁶. Thus, the NFAT pathway could mainly contribute to the H₂-dependent transcriptional response induced by free radicals at least in THP-1 cells.

Taken together, these cellular responses, at least partly, are in agreement with those obtained using the *in vitro* H₂-dependent products of OxPAPC species (Figs 3, 4). Therefore, we proposed a model in which H₂ is linked to the modulation of Ca²⁺ signal transduction and the NFAT pathway via oxidized phospholipid species, as illustrated in Fig. 6.

Discussion

While the biological effects of H₂ have been evaluated in more than 300 animal studies and 10 clinical analyses in humans^{6,7}, the molecular mechanisms by which H₂ at low concentrations exerts its multiple effects on signal transduction remained unknown. Therefore, in this study, we aimed to examine how H₂ regulates signal transduction pathways that mediate gene expression. Our results suggested that low concentrations of H₂ modulated Ca²⁺ signal transduction and regulated gene expression by modifying the production of oxidized phospholipid species. Hence, these data provide important insights into one of the molecular mechanisms by which H₂ mediates gene expression.

H₂ can be ingested via several methods. Drinking of H₂-infused water (H₂-water) has been shown to be efficacious in the treatment of various diseases in animal models and humans^{6,7}; however, H₂ can be infused up to only 0.8 mM under atmospheric pressure, and drinking saturated H₂-water provides a blood concentration up to only ~10 μM, with a short dwelling time in the body^{11,27}. Moreover, inhaling 1%–4% (v/v) H₂ gas was shown to be effective, reaching concentrations of 8–32 μM H₂ in the blood^{1,4,5}. However, initiation of cellular signals by these low concentrations of H₂ may be difficult to be explained because H₂ should be too inert to react with most molecules. To activate H₂ for reaction with the other molecules, a sufficient level of a putative catalyst must be present; however, it is unlikely that such a putative catalyst would be abundant inside cells. Moreover, H₂ is very small and is unlikely to bind to a putative H₂-binding receptor because its intra-molecular fluctuation would be

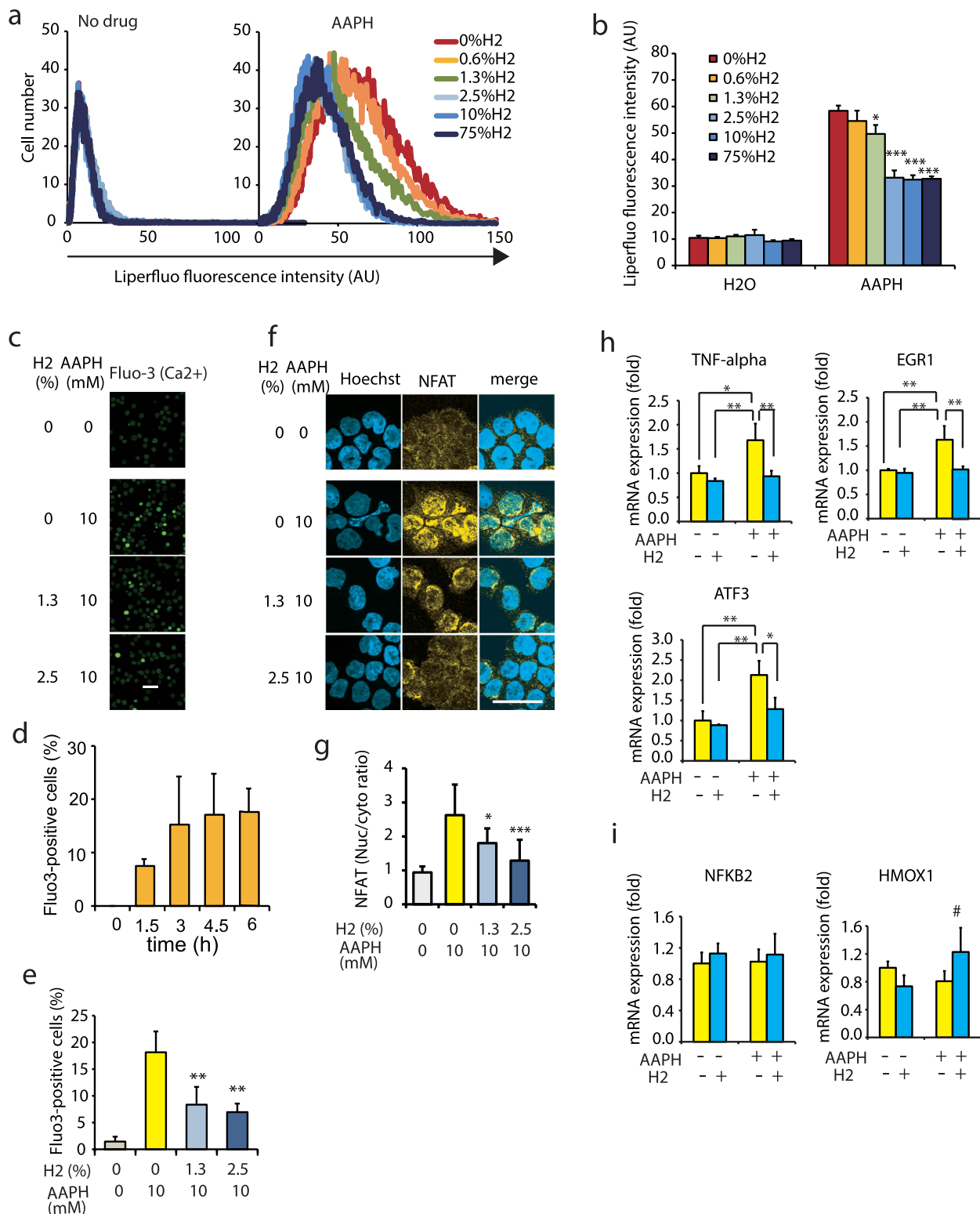


Figure 5. H₂ suppressed free radical inducer-dependent fatty acid peroxidation and Ca²⁺ and NFAT signaling. (a) THP-1 was exposed to a free radical inducer (10 mM AAPH) in the absence or presence of the indicated concentrations of H₂ for 4.5 h. Representative flow cytometric profiles are shown to demonstrate lipid peroxides with Liperfluor signals. (b) The Liperfluor signals were quantified. **P* = 0.015, ****P* < 0.001 vs. 0% H₂ (*n* = 6). (c) THP-1 cells were treated with 10 mM AAPH for 3 h in the presence of the indicated concentrations of H₂. Intracellular Fluo-3 fluorescence intensity was observed using a laser scanning confocal microscope. Scale bar: 50 μ m. (d) THP-1 cells were treated with 10 mM AAPH for the indicated periods in the absence of H₂ and then time dependent increase in Ca²⁺-signal was monitored by intracellular Fluo-3 fluorescence intensity as described in (c). (e) Fluo3-positive cells were semi-quantified after the treatment with 10 mM AAPH for 3 h in the absence or presence of the indicated concentrations of H₂. ***P* < 0.01 vs. no H₂ (*n* = 3). (f) THP-1 was

treated with 10 mM AAPH for 3 h in the presence of the indicated concentrations of H₂. The translocation of NFAT into the nucleus was examined as described in Methods and shown by immunostaining in yellow. The nucleus was counter-stained with Hoechst 33342 as shown in blue. Scale bar: 50 μm. (g) The NFAT-expressing areas were semi-quantified and shown by the ratio of NFAT in the nucleus with that in cytosol. **P* = 0.023 and ***P* < 0.01 vs. no H₂ (*n* = 10). (h,i) The expressions of the NFAT-target genes (*TNF-α*, *EGRI*, and *ATF3*) (h) and non-NFAT target genes (*NFKB2* and *HMOX1*) (i) were estimated using RT-PCR coupled with a TaqMan probe (the probes are listed in Supplementary Table 2). The names of the genes are described in Supplementary Table 1. **P* = 0.015 (for *ATF3*) (+AAPH and +H₂ vs. +AAPH and -H₂). #*P* = 0.14 (for *HMOX1*) (+AAPH and -H₂ vs. +AAPH and +H₂), and ***P* < 0.01 (*n* = 3)

expected to lead to instability in terms of thermodynamics, as previously discussed²⁸. Thus, it was unknown how low concentrations of H₂ regulate signal transduction and gene expression.

Since increased oxidative stress involving ·OH triggers free radical chain reactions, we assumed that the chemically produced mediators derived from phospholipids could contribute to various pathogenic conditions. In the present study, we verified that a small amount of H₂ (as low as 1.3%) affected free radical-dependent lipid peroxidation, from which oxidized lipid mediators should be derived²².

Generally, H₂ hydrogenates unsaturated fatty acids at higher temperatures with a palladium catalyst. To the best of our knowledge, no studies have examined autoxidation-dependent hydrogenation at approximately 1% (v/v) H₂ gas at 37 °C without any catalysts. Although H₂ was thought to be inert in the absence of a catalyst at body temperature, we demonstrated that approximately 1% (v/v) H₂ suppressed autoxidation of an unsaturated fatty acid in a chemically pure system in this study; thus, our data provided insights into the biological activities of H₂.

There are two possibilities: the effects of oxidized phospholipid species on Ca²⁺ signaling may be explained by decreased levels of a putative agonist that induces Ca²⁺ signaling or by increased levels of a putative antagonist that disturbs Ca²⁺ signaling. Although we could not identify these species in this study, it is likely that H₂ modified the production of reduced forms of oxidized phospholipid species during free radical chain reactions by the following previous findings: POVPC is a bioactive phospholipid-mediator that is produced by chemical oxidation of PAPC, and the reduced form of POPVC has been shown to function as an antagonist for signal transduction¹⁸. Thus, it is possible that during a lipid free radical chain reaction, H₂ contributes to the generation of a reduced form(s) that function(s) as an antagonist(s). Therefore, we proposed a hypothetical model in which H₂ is linked to the modulation of Ca²⁺ signal transduction and the NFAT pathway via oxidized phospholipid species as illustrated in Fig. 6.

Previous studies have shown that 1%–4% was efficacious in inhaling H₂ gas in various animal experiments^{1,3,4,29–31}. Since a mixed gas containing 1.3% H₂, 30% O₂ and 68.7% N₂ is available, the effects of around 1.3% needed to be investigated in further studies, including clinical ones⁵. The effective concentrations of H₂ gas were approximately consistent throughout this study (Figs 2–5).

No receptors involved in Ca²⁺ signaling were identified in the present study; however, a previous study showed that some chemically oxidized phospholipid mediators, such as 9-HODE and 11-HETE, could bind a G-protein coupled receptor (G2A) to induce Ca²⁺ signaling¹⁷. Thus, putative oxidized phospholipid mediators or antagonists might bind to G-protein coupled receptors to modulate signal transduction.

In addition to the anti-oxidative roles of H₂, it has shown to function as an immunosuppressant in allograft transplantation³². This immunosuppressant effect can be explained by the suppression of NFAT activation because an immunosuppressant such as CsA and tacrolimus (FK506) acts through the inactivation of calcineurin. Since pro-inflammatory cytokines are regulated by NFAT-dependent mechanisms²⁰, the anti-inflammatory effects by H₂ can be explained by the suppression of NFAT. Additionally, the anti-allergic effects of H₂ can be explained by the decrease in Ca²⁺/NFAT signaling³³.

A considerable number of the multiple functions of H₂, as shown by previous studies, might be explained by the link between H₂ and NFAT because of the numerous multiple functions of NFAT^{20,34}. For example, decreased expression of inducible nitric oxide synthase (iNOS) by H₂³⁵ can be explained by the inactivation of NFAT³⁶. The suppression of osteoclast differentiation³⁷ and improvement of hypertension^{38,39} by H₂ could involve the NFAT pathway^{40,41}. Moreover, the decreased expression of gene products through an NFAT-dependent pathway might be involved in α-synuclein-induced degeneration of midbrain dopaminergic neurons in Parkinson's disease⁴². This NFAT-dependent pathway might explain the beneficial effects of H₂ in these patients⁸. Further studies are needed to elucidate the mechanisms by which H₂ exerts multiple functions in terms of the involvement of the NFAT pathway.

In summary, in this study, we investigated the link among H₂, oxidized phospholipids, and Ca²⁺ signaling. Further studies are warranted to identify the H₂-dependent bioactive mediator(s). Our data provided important insights into one of the mechanisms by which H₂ regulates signal transduction and gene expression; however, H₂ might contribute to other types of signaling pathways as well because H₂ regulates many genes belonging to various signaling pathways. A more detailed understanding of the molecular mechanisms of H₂-dependent signal transduction and gene expression is expected to facilitate the application of H₂ in a wide range of medical applications.

Methods

Measurement of H₂. Gases containing H₂ were prepared by mixing H₂, O₂, N₂, and CO₂ at various concentrations from each gas cylinder equipped with a flow meter. The H₂ concentration in the mixed gas or air was tested in each experiment by using gas chromatography (Breath Gas Analyzer, Model TGA2000; TERAMECS Co. Ltd., Kyoto, Japan) as described previously¹. For the measurement of H₂ in the solvent, H₂ was transferred to the air phase in a closed aluminum bag, and the concentration of H₂ measured by using gas chromatography

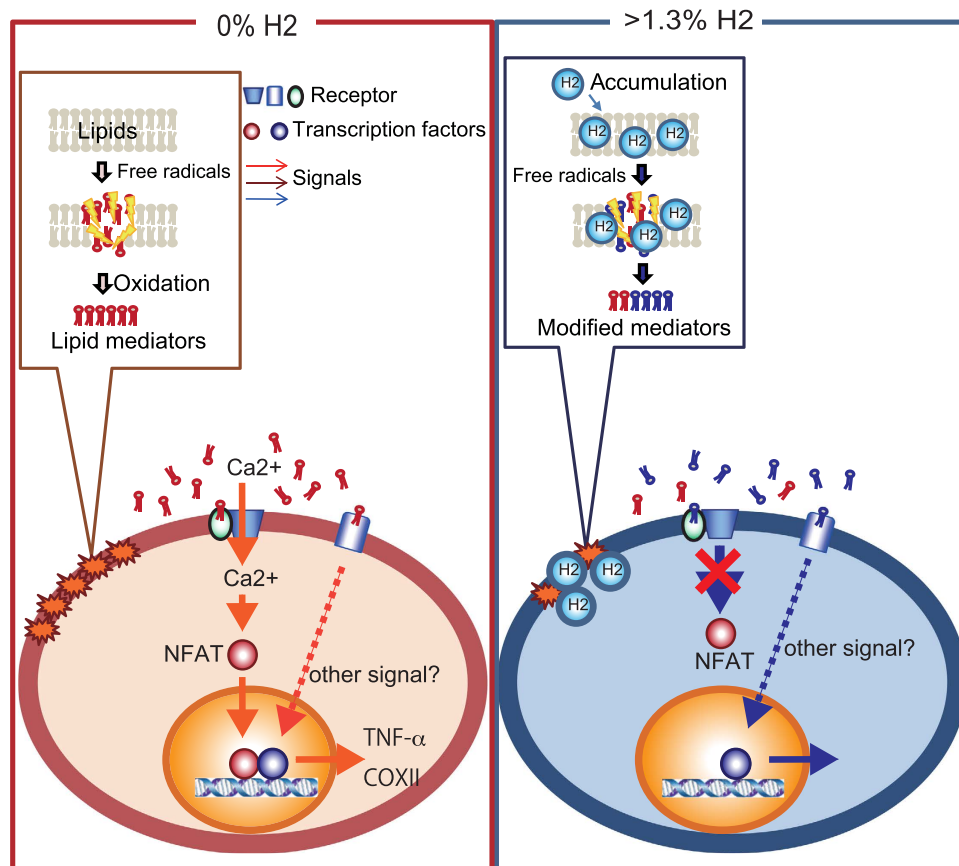


Figure 6. A model of the proposed pathway. When free radical chain oxidation generates oxidized phospholipid mediators, Ca^{2+} signaling is induced, followed by the activation of calcineurin and subsequent induction of the NFAT pathway. On the other hand, H_2 modifies the production of oxidized phospholipids by modulating free radical chain reactions. The putative oxidized phospholipids appear to function as antagonists and lead to a decline in Ca^{2+} signaling.

as described previously¹. The aluminum used in the bag was covered with a plastic film to avoid any influence of aluminum.

Autoxidation of linoleic acid-film. Linoleic acid and (\pm)-9-HODE were purchased from Nacalai Tesque (Kyoto, Japan) and CAY (MI, USA), respectively. Linoleic acid was dissolved in cyclohexane to 16 mM, and 2 μL was dispensed into each glass tube ($\phi 10 \times 50 \text{ mm}$) that had been filled with argon gas; it was allowed to dry up to form a linoleic acid-film at the bottom of a glass tube. The glass tubes were placed into a closed aluminum bag, and the gas in the bag was completely replaced with the indicated mixed gas, where pure H_2 , O_2 , and N_2 were obtained from separate cylinders. The bag was incubated at 37 °C for 20 h for the autoxidation, and 0.2 mL cyclohexane was immediately added to the glass tube to obtain 0.16 mM peroxidized linoleic acid. The concentration of conjugated diene was estimated by measuring the absorption at 234 nm while scanning from 200 to 300 nm.

Autoxidation of pure PAPC in air in the absence or presence of H_2 . Chemically synthesized pure PAPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA). PAPC was autoxidized in air as described previously⁴³. Briefly, 0.5 mg of PAPC in 50 μL of chloroform was transferred to a $\phi 10 \times 50 \text{ mm}$ glass tube and dried up under a gentle stream of nitrogen. The lipid residue was allowed to autoxidize in air with 100% humidity at 25 °C in the presence or absence of the indicated concentrations of H_2 gas in a closed aluminum bag for the indicated periods, and then suspended in PBS at a concentration of 0.5 mg/mL.

Estimation of OxPAPC with Liperflu. OxPAPC was assayed in ethanol with Liperflu as described previously²⁵. Five min after adding OxPAPC to 1 μM Liperflu at room temperature, the fluorescence was measured using a fluorescence spectrophotometer (RF-5300PC; Shimadzu Corporation, Kyoto, Japan), where wavelengths of excitation and emission were set at 488 and 535 nm, respectively.

Measurement of Ca^{2+} signaling. Intracellular Ca^{2+} in THP-1 cells treated with OxPAPC was measured using a Calcium Kit-Fluo 4 (CS22; Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, THP-1 cells were washed with PBS and incubated with 4.5 μM Fluo 4-AM in recording medium (20 mM HEPES,

115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 13.8 mM glucose) containing 0.064% pluronic F-127 and 1.25 mM probenecid for 30 min at 37 °C. The cells were washed with PBS and resuspended in recording medium containing 1.25 mM probenecid. The cells were seeded on 35-mm glass-bottomed dishes and then stimulated with 100 μg/mL OxPAPC or H₂OxPAPC, followed by 25 μM ATP. The changes in Fluo 4-AM fluorescence were monitored using a laser scanning confocal microscope (FV1200; Olympus Corporation, Tokyo, Japan). The strength of each fluorescent signal in 400 cells was examined and judged as positive if there was greater than 30% of the ATP signal.

Intracellular Ca²⁺ of THP-1 cells treated with the free radical inducer AAPH²³ was measured by Fluo-3 (F-23915; Molecular Probes, Eugene, OR, USA). Briefly, THP-1 cells were pre-incubated with 2 μM Fluo 3-AM in HBSS containing 0.02% pluronic F-127 for 30 min at 37 °C, resuspended in RPMI1640 (with 10% FBS) containing 2.5 mM probenecid, seeded in 24-well plates, and then treated with AAPH in the presence or absence of H₂. Changes in Fluo-3 fluorescence signals were observed using a laser scanning confocal microscope (FV1200; Olympus).

Mass spectrometric analysis and presentation of data using heat maps. OxPAPC (dissolved in chloroform at 2.5 mg/mL) was analyzed using by electrospray ionization-mass spectrometry (ESI-MS) by using an LTQ ORBITRAP XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a nitrogen sheath gas flow rate of 40 AU at 300 °C. The sample was directly infused. The scanning range was from *m/z* 250 to 1000 in the positive ion detection mode. The ion spray voltage was set to 4 kV. OxPAPC species were identified according to their *m/z* values and confirmed using mass spectrometric analysis as described previously^{14,44,45}.

Two independent experiments were performed. The average of the data was used for construction of a heat map and displayed in mass spectrometric profiles. In the heat map, bands were arranged according to molecular mass from small to large, and the strength of each band obtained from H₂OxPAPC was compared with those by OxPAPC. Red and green bands represented increased and decreased levels as compared with those of OxPAPC, respectively. The mass spectrometric display indicates the average band from two experiments. Only when bands were detected by all of 10 experiments (two experiments at 0%, 0.2%, 0.3%, 1.3% and 5% of H₂), they were adopted.

Comprehensive analysis of gene expression. THP-1 cells were exposed for 4 h to PAPC or OxPAPC, H₂[1.3%]OxPAPC, and H₂[5%]OxPAPC that had been autoxidized for 3 days with 0%, 1.3%, or 5% H₂, respectively. Total RNA was extracted using an RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA) and labeled using a Low-Input QuickAmp Labeling Kit, One-Color (Agilent Technologies, Santa Clara, CA, USA). Gene expression analysis was performed on samples from three independent experiments using a microarray (SurePrint G3 Human GE 8 × 60 K v2 Microarray; Agilent Technologies). The raw microarray data were deposited in the Gene Expression Omnibus (GEO; accession number, GSE62434; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62434>). CREB target genes were selected according to the CREB Target Gene Database (<http://natural.salk.edu/CREB/>), while NFAT target genes were selected by reference to Medline, as listed in Supplementary Table 1. Signal transduction pathways associated with each gene were identified according to the KEGG Pathway Database (<http://www.genome.jp/kegg/pathway.html>).

Quantitative real-time PCR. To quantify mRNA levels, quantitative real-time PCR was carried out using TaqMan Probe and Premix Ex Taq (Probe qPCR; TaKaRa Bio Inc., Shiga, Japan) in a TaKaRa PCR Thermal Cycler Dice TP960 (TaKaRa Bio) according to the manufacturer's protocols. To normalize mRNA expression levels, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous internal control. Primers and probes used for RT-PCR are described in Table 2.

ELISA (Enzyme-linked immuno-sorbent assay) HAEC and THP-1 cells were treated with PAPC, OxPAPC or H₂OxPAPC for 22 h. The IL-8 (HAEC) and TNF-α (THP-1) contents in the culture media were determined using Human CXCL8/IL-8 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) and Human TNF-α Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), respectively, according to the manufacturer's protocol.

Detection of lipid peroxidation in cultured cells. THP-1 cells (1 × 10⁵ cells/mL) were stained with 5 μM Liperfluo²⁵ for 30 min and then treated with 10 mM of AAPH²³ for 4.5 h in the absence or presence of the indicated concentrations of H₂ gas in a closed vessel. The cells were analyzed using a Cell Lab Quanta flow cytometer (Beckman Coulter, Miami, FL, USA).

Detection of the translocation of NFAT into the nucleus by immunofluorescence. THP-1 cells (1 × 10⁵ cells/mL) were treated with OxPAPC (0.1 mg/mL), or H₂[2.5%]OxPAPC (0.1 mg/mL) for 1.5 h, which were used for the Ca²⁺ signaling assay, and then the translocation of NFAT was determined using immunofluorescence as follows. The cells were fixed for 20 min with 10% neutral buffered formalin (3.8% formaldehyde), and then permeabilized with 0.2% Triton X-100 in Tris-buffered saline (TBS-T) for 10 min. After the cells were washed, and blocked with 5% nonfat milk in TBS-T, they were incubated with anti-NFAT1 antibodies (1:100 dilution; 25A10.D6.D2; Abcam, Cambridge, MA, USA) overnight at 4 °C, followed by incubation with Alexa Fluor 488-conjugated anti-mouse antibodies (1:400 dilution; A-11029; Life Technologies, Carlsbad, CA, USA) for 1 h at 25 °C. The cells were counterstained with Hoechst 33342. Immunofluorescence was observed using a laser scanning confocal microscope (FV1200; Olympus).

THP-1 cells (1×10^5 cells/mL) were treated with 10 mM AAPH for 3 h in the absence or presence of indicated concentrations of H₂, and the NFAT translocation was investigated using immunofluorescence as described above.

Cell culture. THP-1 cells (ATCC) were cultured in RPMI1640 containing 10% FBS. Human aortic endothelial cells (HAEC) were obtained from Lonza and maintained in endothelial cell growth medium [EBM medium + growth supplements + FCS (Lonza)]. Cells were cultured at 37 °C in a 5% CO₂ humidified atmosphere and were used for experiments from passage 4 to 8.

Statistical analysis. Statistical differences between groups were assessed by one-way analysis of variance (ANOVA) with Tukey-Kramer post hoc analysis unless otherwise mentioned. Statistical analyses were performed with IBM SPSS21 software. Results were considered significant at $P < 0.05$. When $0.01 < P < 0.05$, the actual P values were noted. Data are presented as means \pm standard deviations.

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Acknowledgements

We thank Ms. Mayumi Takeda and Ms. Suga Kato for technical assistance and secretarial work, respectively. Financial support for this study was provided by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (23300257, 24651055, and 26282198 to S.O.; 23500971 and 26350129 to N.K.; and 25350907 to K.N.).

Author Contributions

S.O. conceived of and directed the project, analyzed data, and wrote the manuscript. N.K. planned each experiment. K.I., A.I., N.K., K.N., T.Y. and H.I. performed experiments on cell culture (by K.I.), Ca²⁺ signaling (by A.I.), gene expression (by N.K.), and chemical reactions (by K.N.), supported by H.I. and T.Y.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: S.O. holds the right of a patent regarding a medical use of hydrogen gas.

How to cite this article: Iuchi, K. *et al.* Molecular hydrogen regulates gene expression by modifying the free radical chain reaction-dependent generation of oxidized phospholipid mediators. *Sci. Rep.* **6**, 18971; doi: 10.1038/srep18971 (2016).



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