

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

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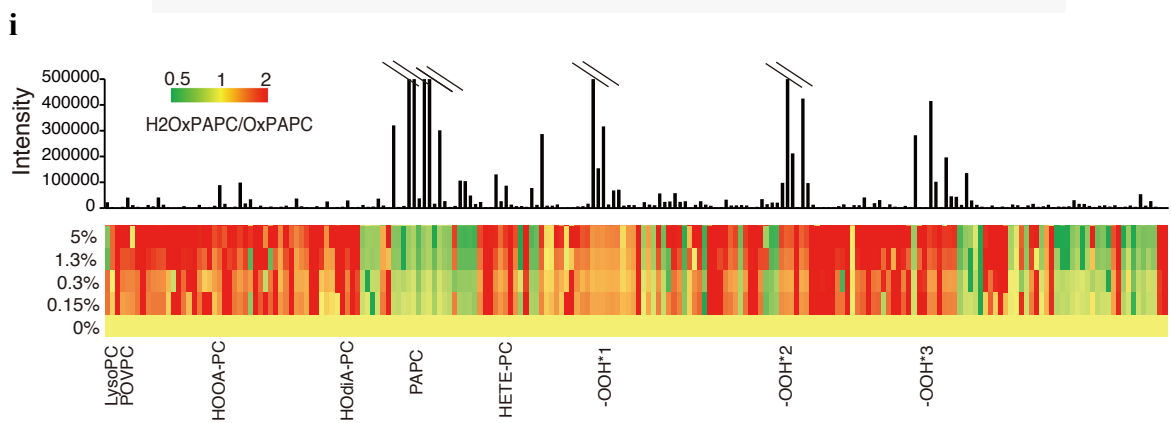
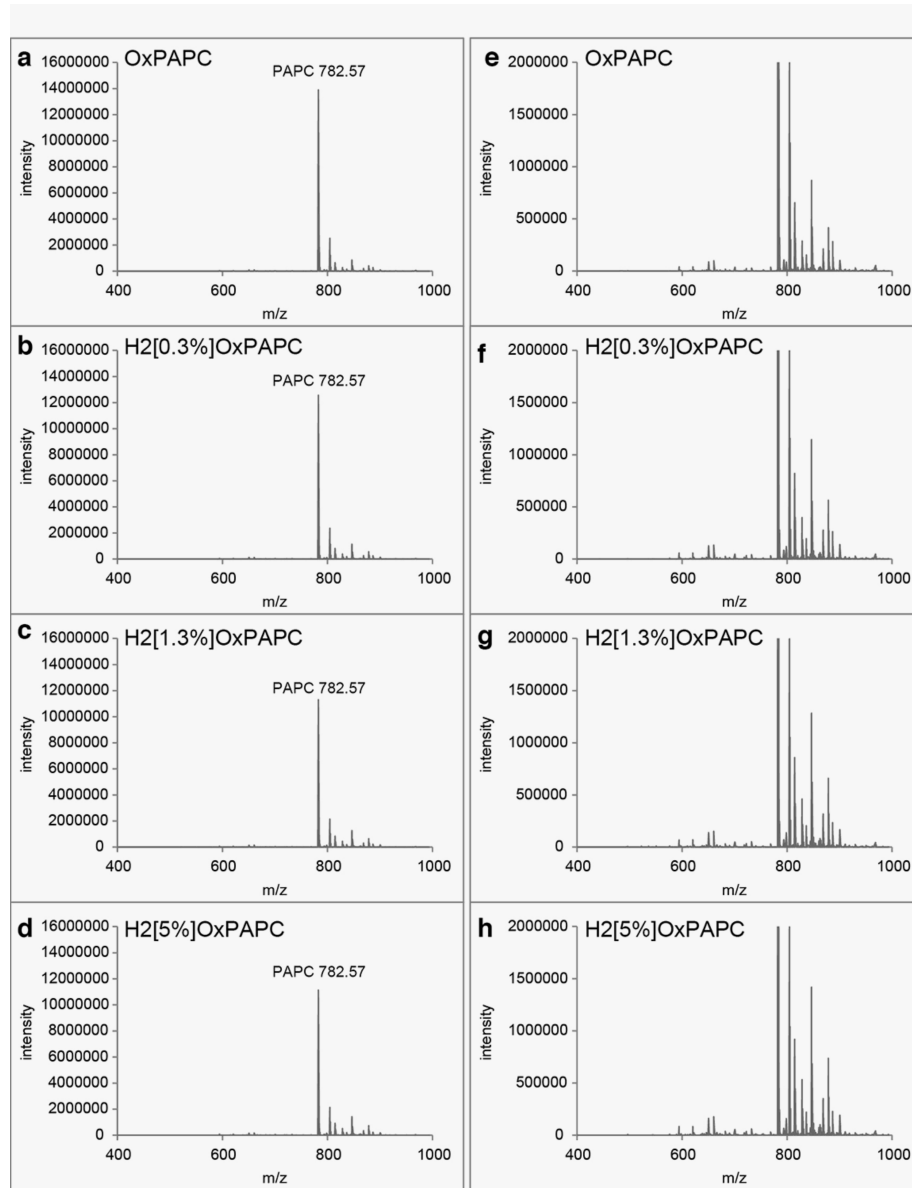
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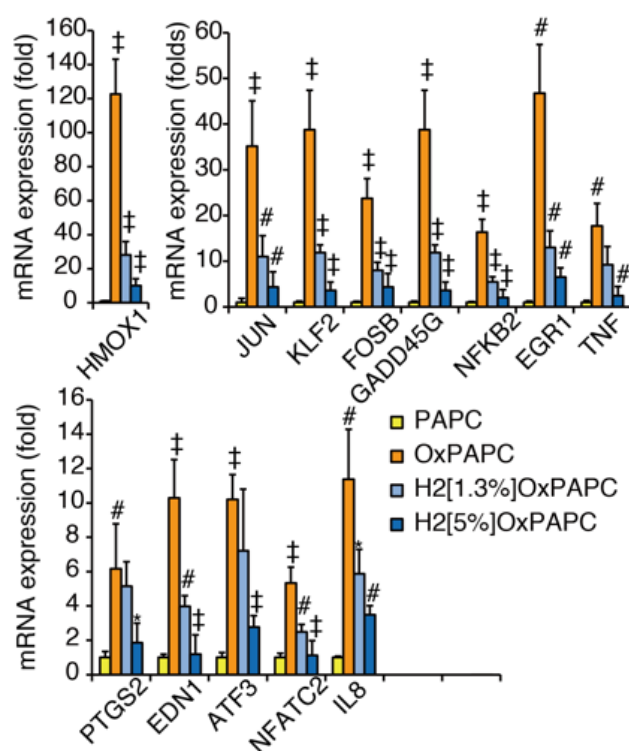
Supplementary Figure 1.



## Supplementary Information

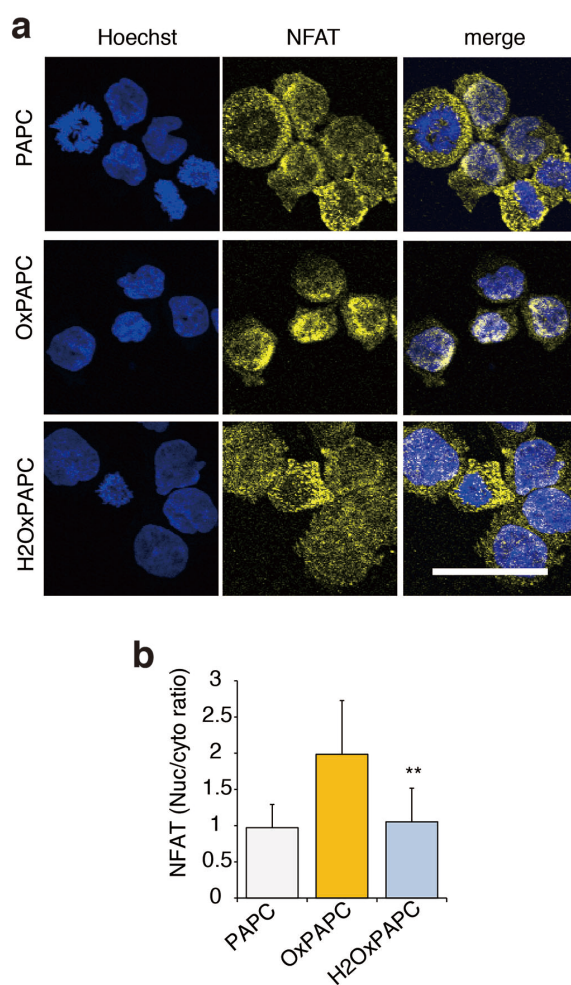
**Supplementary Figure 1. Profiles of mass spectrometry.** PAPC was autoxidized, and subjected to mass spectrometry as described in **Methods**. **(a and e)**, **(b and f)**, **(c and g)**, and **(d and h)** indicate the profiles of OxPAPC, H<sub>2</sub>[0.3%]OxPAPC, H<sub>2</sub>[1.3%]OxPAPC and H<sub>2</sub>[5%]OxPAPC, respectively. Vertical and horizontal axes indicate counts, and m/z, respectively. **(e)**, **(f)**, **(g)**, and **(h)** show the expansion in the vertical axes. **(i, upper)** Mass spectrometric profiles show bands corresponding to OxPAPC species. Oxidized PAPCs are identified according to the molecular mass. Lyso, -OOH\*1, -OOH\*2, and -OOH\*3 indicate lysophosphatidylcholines derived from PAPC, mono-peroxide, di-peroxide, and tri-peroxide of PAPC, respectively. **(i, lower)** Each resulting band (the average of two experiments) is shown in a heat map. Bands representing increased and decreased levels (H<sub>2</sub>OxPAPC vs. OxPAPC) are shown as red and green, respectively, according to the color gradient.

Supplementary Figure 2.



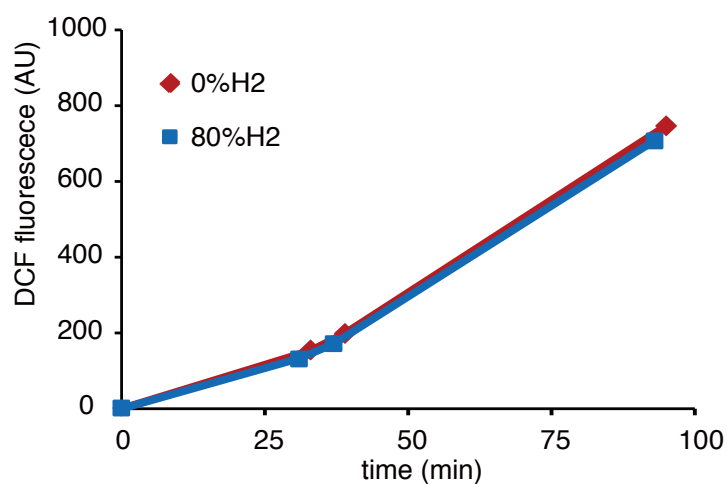
**Supplementary Figure 2. Validation of the expression of the genes selected by microarray analysis by using RT-PCR.** The results of microarray, including marginal genes, were validated by RT-PCR coupled with TaqMan probes. The names of the genes and primer sets are listed in **Supplementary Tables 1** and **2**, respectively. \*, #, and ‡ indicate  $P = 0.03$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively, for OxPAPC vs. PAPC, and H<sub>2</sub>[1.3%]OxPAPC or H<sub>2</sub>[5%]OxPAPC vs. OxPAPC.

## Supplementary Figure 3.



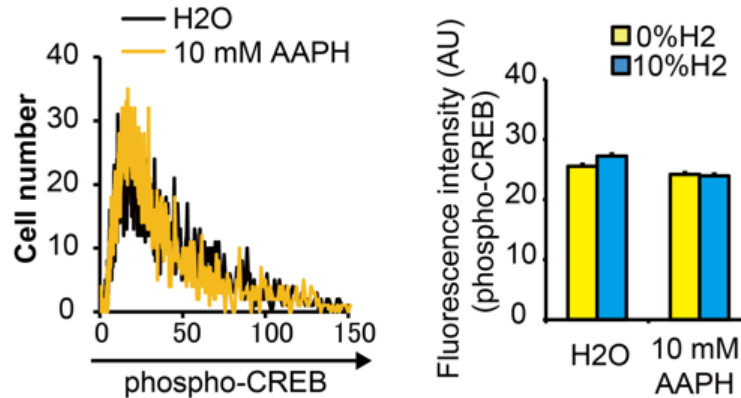
**Supplementary Figure 3. Translocation of NFAT to the nucleus is visualized by immunostaining.** OxPAPC and H<sub>2</sub>OxPAPC were obtained in the absence or presence of 2.5% H<sub>2</sub> as performed in **Figure 3**, and then exposed to THP-1 for 1.5 h. **(a)**The cells were immunostained with anti-NFAT1 (yellow) and counterstained with Hoechst 33342 (blue) as described in **Methods**. Scale bar: 50  $\mu$ m. **(b)**The NFAT-expressing areas were semi-quantified. \*\*  $P < 0.01$  vs. OxPAPC ( $n = 10$ ).

Supplementary Figure 4.



**Supplementary Figure 4. H<sub>2</sub> does not react with AAPH** 2',7' dichlorofluorescein diacetate (DCFH-DA) was used for measuring the radical activity of AAPH. Briefly, DCFH was obtained by hydrolyzing DCFH-DA with 0.01 M aqueous NaOH for 30 min at 37°C in the dark. AAPH (1 mM) and DCFH (10 μM) were added to sodium phosphate buffer (0.1 M, pH 7.2) in the absence or presence of 80% H<sub>2</sub>. The fluorescence intensity of DCFH was measured at 520 nm with excitation at 500 nm.

Supplementary Figure 5.



**Supplementary Figure 5. CREB was not activated by a free radical inducer in THP-1.** THP-1 cells ( $1 \times 10^5$  cells/mL) were treated with or without 10 mM AAPH for 3 h in the absence or presence of 10% H<sub>2</sub>. The cells were fixed for 30 min with 10% neutral buffered formalin. After washing, the cells were permeabilized and blocked with 5% bovine serum albumin in Tris buffered saline (pH 7.4) for 30 min. The cells were incubated with anti-phospho-CREB (Ser133) antibodies (1:400 dilution; #9198; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C, followed by incubation with Alexa Fluor 488-conjugated anti-rabbit antibodies (1:400 dilution; A-11008; Molecular Probes) for 1 h at 25°C. The cells (5,000 cells) were analyzed using flow cytometry using a Cell Lab Quanta flow cytometer (Beckman Coulter, Miami, FL, USA). **(Left)** Representative profiles of flow cytometry in the absence and presence of 10 mM AAPH. **(Right)** Quantification of flow cytometry results.

**Supplementary Table 1 Genes whose expressions were increased by OxPAPC and decreased by H<sub>2</sub>OxPAPC**

Gene Symbol	Description and Gene accession number	*Expression by PAPC **Increase by OxPAPC (vs. PAPC)    **Decrease by H2[1.3%]OxPAPC (vs. OxPAPC)    ***Decrease by H2[5%]OxPAPC (vs. OxPAPC) (fold)				Transcription factor and KEGG Pathway (involved in signal transduction)	NFA T target Ref.
		*	**	***	****		
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2) [NM_000963]	40	5.7	0.25	0.34	NF-kappa B signaling pathway // VEGF signaling pathway // TNF signaling pathway // Retrograde endocannabinoid signaling // Oxytocin signaling pathway	1,2,3
HMOX1	heme oxygenase 1 (HMOX1) [NM_002133]	310	110	0.25	0.17	HIF-1 signaling pathway // TNF signaling pathway // Melanogenesis	
ATP1B2	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, beta 2 polypeptide (ATP1B2) [NM_001678]	31	31	0.30	0.17	cGMP-PKG signaling pathway // cAMP signaling pathway // Adrenergic signaling in cardiomyocytes // Thyroid hormone signaling pathway	
PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent) (PLA2G4C), [NM_003706]	185	5.6	0.31	0.25	MAPK signaling pathway // Ras signaling pathway // VEGF signaling pathway // Fc epsilon RI signaling pathway // GnRH signaling pathway // Oxytocin signaling pathway	
SOST	sclerostin (SOST) [NM_025237]	22	13	0.34	0.09	Wnt signaling pathway	
JUN	jun proto-oncogene (JUN) [NM_002228]	155	15	0.34	0.27	Transcription factor // MAPK signaling pathway // ErbB signaling pathway // cAMP signaling pathway // Wnt signaling pathway // Toll-like receptor signaling pathway // T cell receptor signaling pathway // B cell receptor signaling pathway // TNF signaling pathway // Neurotrophin signaling pathway // GnRH signaling pathway // Estrogen signaling pathway // Oxytocin signaling pathway (American trypanosomiasis)	
HSPA1A	heat shock 70kDa protein 1A (HSPA1A) [NM_005345]	2550	3.8	0.35	0.48	MAPK signaling pathway // Estrogen signaling pathway	
PLK2	polo-like kinase 2 (PLK2) [NM_006622]	235	6.21	0.36	0.21	FoxO signaling pathway	
KLF2	Kruppel-like factor 2 (KLF2), [NM_016270]	3240	16	0.39	0.21	Transcription // FoxO signaling pathway	4
HSPA1B	heat shock 70kDa protein 1B (HSPA1B), [NM_005346]	6710	6.9	0.40	0.30	MAPK signaling pathway // Estrogen signaling pathway	
FOSB	FBJ murine osteosarcoma viral oncogene homolog B (FOSB) [NM_006732]	132	21	0.40	0.31	transcription factor	
CAMKK1	calcium/calmodulin-dependent protein kinase kinase 1, alpha (CAMKK1), [NM_032294]	524	3.6	0.43	0.22	AMPK signaling pathway // Adipocytokine signaling pathway // Oxytocin signaling pathway	
ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (ID3), mRNA [NM_002167]	317	9.3	0.43	0.44	TGF-beta signaling pathway	5
JUND	jun D proto-oncogene (JUND), [NM_001286968]	1495	4.2	0.45	0.25	Transcription factor // MAPK signaling pathway // Osteoclast differentiation	
SOCS1	suppressor of cytokine signaling 1 (SOCS1) [NM_003745]	350	4.0	0.46	0.31	Jak-STAT signaling pathway // Insulin signaling pathway // Prolactin signaling pathway	6
JUNB	jun B proto-oncogene (JUNB), mRNA [NM_002229]	157	6.1	0.47	0.36	Transcription factor // TNF signaling pathway	
SGK1	serum/glucocorticoid regulated kinase 1 (SGK1) [NM_005627]	159	10	0.48	0.35	FoxO signaling pathway // PI3K-Akt signaling pathway	
FIGF	c-fos induced growth factor (vascular endothelial growth factor D) (FIGF), mRNA [NM_004469]	27	72	0.51	0.29	Ras signaling pathway // Rap1 signaling pathway // Cytokine-cytokine receptor interaction // PI3K-Akt signaling pathway // Focal adhesion // Pathways in cancer	
GADD45G	growth arrest and DNA-damage-inducible, gamma (GADD45G), [NM_006705]	113	11	0.52	0.39	MAPK signaling pathway // FoxO signaling pathway // Cell cycle // p53 signaling pathway	7
FGF3	fibroblast growth factor 3 (FGF3), mRNA [NM_005247]	274	15	0.54	0.26	MAPK signaling pathway // Ras signaling pathway // Rap1 signaling pathway // PI3K-Akt signaling pathway	8
RELB	v-rel avian reticuloendotheliosis viral oncogene homolog B (RELB) [NM_006509]	1627	4.3	0.54	0.37	MAPK signaling pathway // NF-kappa B signaling pathway	
CTSK	cathepsin K (CTSK), [NM_000396]	5560	3.3	0.56	0.35	Toll-like receptor signaling pathway	
ATF3	activating transcription factor 3 (ATF3) [NM_001674]	215	7.6	0.56	0.27	Transcription factor	9
BBC3	BCL2 binding component 3 (BBC3) [NM_014417]	9388	3.1	0.59	0.42	p53 signaling pathway // Hippo signaling pathway	
RASA4B	RAS p21 protein activator 4B (RASA4B) [NM_001277335]	557	2.8	0.59	0.31	Ras signaling pathway	
DGKA	diacylglycerol kinase, alpha 80kDa [Source:HGNC Symbol;Acc:2849] [ENST00000546995]	148	7.3	0.60	0.45	Phosphatidylinositol signaling system	



## Supplementary Information

CYP27A1	cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27A1) [NM_000784]	668	2.9	0.60	0.51	PPAR signaling pathway	
ATP2B4	ATPase, Ca <sup>++</sup> transporting, plasma membrane 4 (ATP2B4), [NM_001684]	215	2.6	0.57	0.46	Calcium signaling pathway // cGMP-PKG signaling pathway // cAMP signaling pathway // Adrenergic signaling in cardiomyocytes	10
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) (NFKB2) [NM_001261403]	205	3.7	0.61	0.38	MAPK signaling pathway // NF-kappa B signaling pathway	
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 (NFATC2) [NM_173091]	386	3.6	0.61	0.25	Transcription factor// cGMP-PKG signaling pathway // Wnt signaling pathway // VEGF signaling pathway // T cell receptor signaling pathway // B cell receptor signaling pathway // Oxytocin signaling pathway	
ITGA10	integrin, alpha 10 (ITGA10), mRNA [NM_003637]	254	3.3	0.62	0.32	PI3K-Akt signaling pathway	
CD86	CD86 molecule (CD86), transcript variant 2, mRNA [NM_006889]	4229	5.5	0.64	0.11	Toll-like receptor signaling pathway	
RASGRP2	RAS guanyl releasing protein 2 (calcium and DAG-regulated) [Source:HGNC Symbol;Acc:9879] [ENST00000377494]	678	2.7	0.67	0.34	MAPK signaling pathway // Ras signaling pathway // Rap1 signaling pathway // Chemokine signaling pathway // Platelet activation	
EDN1	endothelin 1 (EDN1) [NM_001955]	88	5.1	0.53	0.25	HIF-1 signaling pathway // TNF signaling pathway	3,11,12,13
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) [NM_021127]	7459	9.1	0.69	0.39	p53 signaling pathway	
PPARG	peroxisome proliferator-activated receptor gamma (PPARG), [NM_138711]	457	3.0	0.71	0.38	Transcription factor// PPAR signaling pathway // AMPK signaling pathway	
CPT1B	carnitine palmitoyltransferase 1B (muscle) (CPT1B), [NM_152246]	4652	3.1	0.71	0.36	PPAR signaling pathway // AMPK signaling pathway	
PPP1R12B	protein phosphatase 1, regulatory subunit 12B (PPP1R12B), [NM_002481]	431	3.1	0.73	0.19	Oxytocin signaling pathway	
FLNA	filamin A, alpha (FLNA), transcript variant 1, mRNA [NM_001456]	386	2.5	0.74	0.32	MAPK signaling pathway	14
FBXO32	F-box protein 32 (FBXO32), transcript variant 1, mRNA [NM_058229]	501	3.3	0.74	0.49	FoxO signaling pathway	
MAPK8IP3	mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3), [NM_015133]	3580	2.6	0.75	0.41	MAPK signaling pathway	
EGR1	early growth response 1 (EGR1), [NM_001964]	2210	5.2	0.84	0.58	Transcription factor	15
TNF	tumor necrosis factor (TNF), [NM_000594]	2850	5.7	0.95	0.60	MAPK signaling pathway // NF-kappa B signaling pathway // mTOR signaling pathway // Apoptosis // TGF-beta signaling pathway // Toll-like receptor signaling pathway // NOD-like receptor signaling pathway // RIG-I-like receptor signaling pathway // Fc epsilon RI signaling pathway // TNF signaling pathway // Adipocytokine signaling pathway	16,17,18,19,20
IL8	interleukin 8 (IL8) [NM_000584]	91	2.3	PCR	PCR	Chemokine signaling pathway // NF-kappa B signaling pathway // Toll-like receptor signaling pathway // NOD-like receptor signaling pathway // RIG-I-like receptor signaling pathway	16,21,22,23

Gene expression was examined by comprehensive microarray analysis as described in **Methods** (SurePrint G3 Human GE 8x60K v2 Microarray, Agilent Technologies, Santa Clara, CA, USA). Genes that OxPAPC up-regulated (more than 2.5-fold vs. PAPC) and that H<sub>2</sub>[1.3%]OxPAPC and H<sub>2</sub>[5%]OxPAPC down-regulated (less than 0.75-fold and 0.5-fold, respectively, vs. OxPAPC) were selected. When the difference obtained from three tests was not significant ( $P > 0.05$ ), the genes were excluded. Functionally important genes that marginally unfitted the above criteria were added to this table after the validation by semi-quantitative PCR analysis. Transcription factors and target genes to NFAT are listed according to the literature. The contribution to signal transduction was according to the KEGG Pathway Database (<http://www.genome.jp/kegg/pathway.html>).

In the first line of the table; (\*) expression levels treated with PAPC; (\*\*) increase (fold) vs. PAPC; (\*\*\*) decrease (fold) by H<sub>2</sub>[0.3%]OxPAPC vs. OxPAPC and (\*\*\*\*), and decrease (fold) by H<sub>2</sub>[5%]OxPAPC vs. OxPAPC.

**References cited in Supplementary Table 1 suggesting the NFAT targets**

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**Supplementary Table 2 Nucleotide sequences of primer sets and TaqMan probes**

Gene	Forward primer	Reverse primer	TaqMan probe
Human			
HMOX1	CCACCAAGTTCAAGCAGCTC	CTGACTGCGGGAGTCATCTC	ACCGTCCC GCATGAACTCCC
JUN	CAAGAACGTGACAGATGAGCAG	AGCGTGTTCTGGCTGTGC	CACGAAGCCCTCGGCGAAGCCC
KLF2	AAGTGGCATCTTCTCTCCCA	AATGCCGCAGACAGTACAAA	CAAGGCATCACAAGCCTCGATCC
FOSB	GTGAGTGTGTGAGCGCTTCT	AGACAGGCTGAGCCAGAGTT	CACGTTGGCCCTCAAAGCGA
GADD45G	GCGAGAACGACATCGACATA	TTCGAAATGAGGATGCAGTG	ACGATAGCCGCCAGCCGCT
NFKB2	TGATAGCGACTCGGACTCTG	GAGCAGCATTTAGCAGCAAG	ACACCCGAAGCAGCTCCGG
EGR1	GAGATGGCCATAGGAGAGGA	AAGTGGGCAGAAAGGATTGT	TGGCTCTGAGAACCTCCATCTGACC
TNF	TCTTCTCGAACCCGAGTGA	CCTCTGATGGCACCACCAG	TAGCCCATGTTGTAGCAAACCTCAAGCT
PTGS2	GCTCAAACATGATGTTTGCATTC	GCTGGCCCTCGCTTATGA	TGCCCAGCACTTCACGCATCAGTT
EDN1	ACATTGGTGACAGACCTTCG	GTCAGGAACCAGCAGAGGAT	CCCTGTGGCCGACTCTGCAC
ATF3	GCCGAAACAAGAAGAAGGAG	GCTCCTCAATCTGAGCCTTC	CCGACTCTTCTGCAGGCACTCC
NFATC2	AATGGAATGGATGCAAATGA	TGCTAAGCTTGTCCTCAACAG	AGCCTCGGTCTGCTCAGGCC
IL8	GCCAACACAGAAATTATTGTAAAGCTT	AATTCTCAGCCCTCTTCAAAACTT	AGAGCTCTGCTGGACCCCAAGGAAAAC
GAPDH	GGGAAGGTGAAGGTCGGA	GCAGCCCTGGTGACCAG	CAACGGATTGGTCGTATTGGGCG
Mouse			
EGR1	GAGGAGATGATGCTGCTGAG	TGCTGCTGCTGCTATTACC	AGTTCCTCGGTGCTGCCGAA
ATF3	CAACAGAGGATGGACGACAC	TGTAGCCAAGGACAAAGCTG	TGCCCAGGGTCTTCTCTGCTGC
PTGS2	AGAACCTGCAGTTTGTGTG	GCTCCTGCTTGAGTATGTCG	TCTGTTGTGCTCCCGAAGCCA
TNF	TCGTAGCAAACCAAGTG	TTGAGATCCATGCCGTTG	AGGAGCAGCTGGAGTGGCTGAGC
GAPDH	CATCACTGCCACCCAGAAGA	ATGTTCTGGGCAGCC	TGGATGGCCCTCTGGAAAGCTG