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Aldehyde oxidase 1 gene is regulated by Nrf2 pathway

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Abstract:

Aldehyde oxidase is a member of the molybd-flavo enzyme family that catalyzes the hydroxylation of heterocycles and the oxidation of aldehydes into corresponding carboxylic acids. Aldehyde oxidase-1 (*AOX1)* is highly expressed in liver and is involved in the oxidation of a variety of aldehydes and nitrogenous heterocylic compounds, including anti-cancer and immunosuppressive drugs. However, the physiological substrates of AOX1 have not been identified, and it was unknown how the expression of *AOX1* is regulated. Here, we found that the *AOX1* gene is regulated by the Nrf2 pathway. Two Nrf2 binding consensus elements (anti-oxidant responsive element, ARE) are located in the 5' up stream region of the rat *AOX1* gene. Molecular analyses using reporter transfection analysis, EMSA, and ChIP analysis show that Nrf2 binds to and strongly activates the rat *AOX1* gene.

Highlites

- *AOX1* gene has two AREs, a Nrf2 binding consensus sequence
- Both two AREs are essential for AOX1 gene activation
- Nrf2 interacts with ARE *in vitro* and i*n vivo*.
- *AOX1* gene is activated by drug via Nrf2 pathway

Key words

- Aldehyde oxidase 1
- Nrf2-Small Maf heterodimer
- Antioxidant responsive element (ARE)
- Drug metabolism

1. Introduction

Aldehyde oxidase (AOX) catalyzes the oxidation of a wide variety of aldehyde compounds with broad substrate specificity. Although its physiological substrates have not been identified, AOX is implicated in the oxidation of indol, pyridoxal, nicotinamide and retinaldehyde (Kitamura et al., 2006; Krenitsky et al., 1972). This enzyme is considered to be an important xenobiotic metabolizing enzymes (Garattini et al., 2008). There is also great interest in AOX since it has the ability to metabolize anti-cancer drugs (e.g., methotrexate), immunosupessive agent (e.g., 6-mercaptpurine) and anti-viral drugs (Garattini et al., 2008; Kitamura et al., 2006). Currently, much attention has been focused on this enzyme as the target of drug development, especially for anti-obesity and anti-cancer drugs (Pryde et al., 2010; Garattini and Terao, 2011). Therefore, it will be critical to identify its substrates and understand how it is regulated. Although a couple of reports suggested that the transcription factor Nrf2 (NF-E2 related factor 2) is responsible for *AOX1* gene regulation (Hu et al., 2006; Suvorova et al., 2009), the molecular mechanism by which *AOX1* gene expression is regulated remains to be elucidated.

Nrf2 is a member of the CNC (cap' n' collar) family of transcription factors and dimerizes with small Maf proteins (i.e., MafK, G, or F), members of the Maf family of transcription factors, through their bZip domain (Andrews et al., 1993). Small Maf proteins lack the transactivation domain present in the large Maf proteins (i.e., in c-Maf, MafB, NRL, and L-Maf/MafA). The Nrf2/small Maf heterodimer binds to ARE (anti-oxidant responsive element) and to MARE (Maf recognition element), and regulates downstream genes, including those of phase II detoxifying enzymes (Ikeda et al., 2004; Ikeda et al., 2002; Jaiswal, 2000; Nioi et al., 2003). Itoh et al. identified Keap1 (Kleh-clike ECH-associated protein) as a key mediator for the induction of phase II detoxifying enzymes by electrophilic drugs (Itoh et al., 1999; Wakabayashi et al., 2003). Keap1 suppresses Nrf2 activity by

specific binding to it in the cytoplasm. An electrophilic agent liberates Nrf2 from Keap1 suppression, allowing Nrf2 to translocate into the nucleus and activate target genes.

We report here that the Nrf2/small Maf heterodimer binds to ARE elements located in the 5'-upstream region of the *AOX1* gene and activates gene expression upon the induction by electrophilic agents.

2. Materials and Methods

2.1. Construction of Plasmids

To construct the *AOX1* luciferase reporter genes, 1.9kb (-1.83 kb/+73 bp relative to the putative cap site) of the rat *AOX1* 5'-flanking region was PCR amplified using the following primers: 5'-GAGCTCAGAAGCCACACAGCCTGTCTTT-3' and 5'-CTCGAGGTCCCTAGCAGGTCCCTAGTAG -3'. The amplified DNA fragment was inserted into the luciferase vector pGVB2 (Nippon Gene, Japan). Various deletion fragments and mutated fragments were prepared using the corresponding PCR primers or restriction sites and inserted into pGVB2. DNA fragments without its own promoter region were joined with the luciferase vector, which contains the minimal promoter sequence of the rat glutathione S-transferase P (GST-P) gene (-50/+36) (Sakai et al., 1988). The Nrf2 expression plasmid was described previously (Ikeda et al., 2004).

2.2. Cell Culture and Transient Transfections.

Rat hepatoma cell line RL-34 was maintained in Dulbecco's modified minimal essential medium (DMEM, Sigma) with 10 % (v/v) fetal bovine serum. For the reporter transfection assay, a total of 0.6 μg DNA (0.2 μg of reporter plasmid, 0.2 μg of the β-galactosidase expression plasmid (pSVβgal, Promega) and 0.2 μg of the Nrf2 expression plasmid (Ikeda et al., 2002) or empty expression vector (pcDNAver.3.1, Invitrogen)) were transfected into cells using the transfection agent FuGENE (Roche Applied Science) according to the supplier's protocol. At 45 hr post-transfection, cells were harvested and assayed for luciferase activity using a luciferase assay kit (Nippon Gene, Japan) and for β-galactosidase activity. The luciferase activity was normalized to the β-galactosidase activity and all experiments were repeated at least twice.

2.3. RNA Analyses

Total RNA was prepared from the rat hepatoma cell line RL-34 with or without diethylmalate (DEM, 50μM, 24hr) treatment by RNA isolation reagent (ISOGEN, Nippon Gene, Japan). 5'-RACE analysis was performed using a SMARTer RACE cDNA amplification kit (Clontech). An *AOX1* gene specific anti-sense primer (5'-GAACCCACACTGGGTGCTGTGACAC-3') was designed based on the mouse *AOX1* gene (Demontis et al., 1999).

For quantification of *AOX1* mRNA by RT-PCR, the following primers were used: (5'-CTGGACCAGGTGAAGGACAT-3' and 5'-GTCCAGATGTCTGCTCACGA-3'). The reference mRNA, Glyceraldehyde-3-phosphodehydrogenase (GAPDH), was amplified with the primers (5'GGCATTGCTCTCAATGACAA-3' and 5'-TGTGAGGGAGATGCTCAGTG-3'). Real-time PCR was performed using the Sso0Eva-Green Supermix (BioRad) and Opticon2 thermal cycler (BioRad).

2.4. Electrophoretic Gel Mobility Shift (EMSA) Assay

The cDNA fragments encoding the DNA-binding domain of transcription factors (amino acids 318-598 for Nrf2 and 1-156 for MafK) were fused to the *E. coli* maltose-binding protein (MBP) gene of a pMAL-c2 vector（New England Biolabs）. The fusion proteins were expressed in *E. coli* and purified as described previously (Ikeda et al., 2004). The ARE-1 and ARE-2 probes for EMSA were prepared by PCR, labeled with digokisigenin-11-ddUTP (DIG). EMSA was performed using MBP-fused Nrf2 and MafK proteins and DIG-labeled probes by Gel shift kit (DIG Gel Shift Kit, 2nd generation, Roche) according to the supplier's protocol. DIG-probe and Nrf2/MafK complexes were analyzed by low-salt polyacrylamide gel electrophoresis and blotted on positive-charged nylon membrane (GE Health Sciences). DNA-protein complexes were detected by an anti-DIG antibody and visualized by a chemiluminescence imaging system (Vesa Doc5000, Bio-Rad)

2.5. Chromatin Immunoprecipitation（ChIP）

ChIP analysis was carried out using a ChIP-kit (ChIP-IT Express, Active Motif) according to the supplier's protocol. Briefly, RL-34 hepatoma cells treated with or without dietylmalate were fixed by 1% formaldehyde for 10 min and were harvested. Nuclei were isolated and treated with nuclease cocktail for shearing DNA to around 500-bp long. Chromatin was immunoprecipitated using anti-Nrf2 antibody (Ikeda et al., 2004), reverse cross-linked and DNA was isolated. The promoter region of the *AOX1* gene was amplified by the primers: (5'-TACCCAGAGAAATCCTGTAT-3' and 5'-GGTCCCTAGCAGGTCCCTAGTAG-3').

3. Results and Discussions

3.1. Sequencing of the 5'-flanking region of *AOX1* and identification of the *AOX1* gene cap site.

The 5'-up-stream region (-1830 bp to +73 bp, relative to putative cap site) of the rat *AOX1* gene was amplified from rat genomic DNA (Sparague-Dawley strain) and cloned. Compared to the GenBank accession No. NW_001084882 sequence, a -TA- repeat-like insertion at -1288 was found in our clone, as shown in Fig 1 (sequence was submitted to GenBank, accession No. JQ965812).

To identify the 5'-end of the rat *AOX1* mRNA, we performed 5'-RACE analysis. Sequence analysis of the clones derived from the 5'-RACE amplified fragment indicates that the 5'-end of *AOX1* mRNA is located at 78, 73 and 48 nucleotides upstream of the translation initiation codon. Thus, we designated 5'-end of the longest clone as the putative cap site (Fig 1, +1 nucleotide).

Two anti-oxidant responsive element consensus sequences (ARE, -G(A)TGACNNNGCA-) were found at 321 nucleotides and 208 nucleotides upstream from the cap site. "TRASFAC" analysis, a computer search of transcription factor binding elements, did not identify any significant binding sites in this region other than ARE or related sequences (i.e. binding consensus of AP-1, c-Fos, Maf).

When some gaps were introduced, the sequence homology between rat and mouse *AOX1* gene promoter region (-400 to ATG) was more than 85% and the two AREs were completely conserved. Although the promoter sequence of the human *AOX1* gene diverges from that of the rodent gene, ARE-like sequences were located in similar positions (-GTGACN$_3$GAC-, -ATGAAN$_3$GTC- and -GTGACN$_3$CAC- at 315, 282 and 226 nucleotides upstream from ATG, respectively).

3.2. Reporter transfection analysis of the 5'-upstream region of *AOX1*

A series of 5'- deletion constructs of the *AOX1*-luciferase reporter gene were transfected with the Nrf2 expression vector into the rat hepatoma cell line, RL-34 (Fig. 2A). Most of the constructs (up to -329Luc constructs) were strongly activated when the Nrf2 expression vector was co-transfected. This suggests that the *AOX1* gene is targeted by the Nrf2-Keep1 pathway. Deletions of up to -180 bp (-180Luc) completely abolished the Nrf2 response, indicating that the -180 to 329 bp region likely contains the Nrf2-responsive element (anti-oxidant responsive element, ARE). As mentioned above, two ARE-consensus sequences (-G(A)TGACNNNGCA-) were located within this region (ARE-1:-GTGACTCAGCA- at -321 bp and ARE-2:–ATGACTCAGGA- at -208 bp). The deletion construct, -243 bp (-243Luc) did not result in stimulation and the construct containing a point mutation in the ARE-1 consensus sequence (-329-1mLuc) also lost stimulation (Fig. 2B). These results clearly indicate that the ARE-1 sequence at -321 bp is responsible for Nrf2 stimulation. However, when we introduced the mutation in ARE-2 sequence, stimulation was significantly decreased (Fig. 2B, -329-2mLuc). This result suggests that the ARE-2 was also important for Nrf2 responsiveness. To analyze the Nrf2-responsive activity of each region, we next constructed several reporter plasmids containing the ARE-1 and/or ARE-2 regions and their mutated fragments, which were joined to a heterologous promoter (rat GST-P gene promoter (Sakai et al., 1988)). The reporter plasmid containing both ARE-1 and ARE-2 (ARE-12/GstLuc) was strongly stimulated by Nrf2, similar to the reporter gene with its own promoter (Fig. 2B, -329Luc). When a mutation was introduced into ARE-1 consensus sequence (ARE-1m2/GstLuc), the stimulation by Nrf2 was markedly reduced. The ARE-2 mutant (ARE12m/GstLuc) also led to a lack of stimulation, although the ARE-1 sequence was intact. These observation were reproduced by the constructs containing each ARE region alone. The construct containing ARE-1 region (ARE-1/GstLuc) had no stimulation and the

ARE-2/GstLuc, containing ARE-2 region, also lost stimulation (Fig. 2B). These results indicate that both ARE-1 and ARE-2 are important for Nrf2 stimulation and that the two ARE sequences act synergistically. As described below (Fig. 3), the Nrf2/MafK heterodimer binds to both the ARE-1 and ARE-2 sequences, *in vitro*.

This is an interesting observation since most of the Nrf2 target genes have a single ARE in its promoter (Ikeda et al., 2004; Ikeda et al., 2002; Jaiswal, 2000; Rushmore et al., 1991), which is sufficient for Nrf2 activation. It remains unclear why *AOX1* gene requires two AREs for the Nrf2 responses. Thus, the mechanism by which the two AREs within *AOX1* gene interact with the Nrf2/small-Maf heterodimer remains to be determined.

Without the Nrf2 expression vector, luciferase activity was not significantly affected by a series of deletion constructs up to -329 bp, but greatly decreased in the presence of the -180 bp deletion construct (Fig. 2A). These results suggest that the ARE sequences are the main enhancer elements of this gene, at least in hepatoma and likely in liver cells.

3.3. The Nrf2/small Maf heterodimer binds to the *AOX1* gene *in vitro* and *in vivo*

To confirm that the Nrf2/MafK hetero-dimer directly binds to the AREs of the *AOX1* gene *in vitro* and *in vivo*, we performed EMSA and ChIP analyses (Fig. 3). Digokisigenin-labeled ARE-1 or ARE-2 fragments were mixed with *E. coli* synthesized Nrf2-MBP and MafK-MBP fusion proteins, and analyzed by polyacrylamide gel electrophoresis as described in the Materials and Methods. As shown in Fig. 3A, Nrf2 alone resulted in no bands (Fig. 3A, lane 3) but the Nrf2+MafK hetero-dimer strongly bound to both ARE-1 and ARE-2 probes (Fig. 3A, lanes 4 and 8). The MafK homo-dimer (Fig. 3A, lane 2) was weakly bound to ARE and very faint shifted bands were observed just below the Nrf2/MafK hetero-dimer band. The binding specificity was confirmed by adding cold probes as a competitor (Fig. 3A, lane 5, 6 and 9).

Although the Nrf2/MafK hetero-dimer can bind to either ARE in EMSA, reporter transfection analysis showed that Nrf2 stimulation is lost when the *AOX1* gene contains either ARE-1 or ARE-2 alone, or a mutated version of one of the two ARE sequences (Fig. 2B). As discussed above, both elements ware required for *AOX1* gene activation.

We used ChIP analysis to further confirm that Nrf2 binding to the *AOX1* ARE sequences *in vivo*. Chromatins from RL-34 rat hepatoma cells treated with or without drug (DEM) were immunoprecipitated and the Nrf2 bound to the *AOX1* promoter region was analyzed by PCR as described in the Materials and Methods (Fig. 3B). The promoter region of the *AOX1* gene was highly amplified in the immunoprecipitated chromatin, but not in the pre-immune control. This result clearly indicates that Nrf2 binds to the *AOX1* promoter *in vivo*. However, due to the close proximity of the ARE-1 and ARE-2 sequences, we were unable to compare Nrf2 binding to each element.

3.4. AOX1 expression is induced by drugs

We found that Nrf2 binds to and activates *AOX1* gene. Therefore, the activation of the Nrf2 pathway by drugs should stimulate *AOX1* gene expression, which we examined *in vivo*. Using semi-quantitative RT-PCR and real-time PCR, we analyzed *AOX1* mRNA expression in hepatoma cells treated with diethylmalate (DEM), an electrophilic reagent. The mRNA was prepared from RL-34 cells, treated with 50 μM DEM for 24hr, and analyzed. As shown in Fig. 4A, the level of *AOX1* mRNA increased in response to DEM treatment. The $\Delta\Delta Ct$ calculations from the real-time PCR results show that 3-4 times RNA was expressed in drug-treated RL-34 cells (Fig. 4B). In the reporter transfection analysis, *AOX1* reporter gene (-329Luc) was also stimulated by drug treatment (Fig. 4C). Although ChIP analysis was not exactly quantitative, it seems that the DEM-treated chromatin was more efficiently precipitated than non-treated chromatin in the ChIP analysis (Fig. 3B, DEM(+)).

Based on our data above, we found that the Nrf2-Keap1 pathway regulates the expression of the rat *AOX1* gene. Since *AOX1* is mainly expressed in the hepatocytes (Garattini et al., 2003; Kurosaki et al., 1999; Wright et al., 1999), this enzyme seems to function in both detoxification and drug metabolizing. Understanding the mechanisms by which the *AOX1* gene is regulated will greatly facilitate future studies of drug metabolism and development.

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Figure Legends

Fig. 1

The nucleotide sequence of the rat *AOX1* gene promoter. The putative cap site is designated as +1. The insertion sequences were boxed. The ARE-1 and -2 regions are shadowed. The ATG translation initiation site is underlined.

Fig. 2

Reporter transfection analysis of the *AOX1* promoter. (A) The deletion constructs were transfected into RL-34 cells, with Nrf2 expression vector (+) or empty expression vector (pcDNA ver3.1) (-), and the relative luciferase activity is shown. Constructs are indicated schematically. (–) numbers show the position of each fragment, relative to cap site.

(B) Fold activation of the indicated constructs by Nrf2 is shown. -329-1m and ARE-1m2/GstLuc constructs have mutations in ARE-1. Similarly, -329-2m, -243m and ARE-12m/ GstLuc were mutated in ARE-2 as indicated. Nucleotide sequence of the ARE consensus (AREcon.), ARE-1 and ARE-2, and the mutated forms of ARE-1 (ARE-1m), ARE-2 (ARE-2m) are shown (mutated bases are indicated by small letters).

Fig. 3

Binding analysis of Nrf2/MafK with ARE *in vitro* and *in vivo*. (A) EMSA of ARE-1 and ARE-2. Nrf2-MBP and MafK-MBP proteins were incubated with DIG-labeled ARE-1 or ARE-2 probes. 20 x (+) or 50 x (++) non-labeled probes were added in the competition assay (lanes 5, 6 and 9). The long and short arrows indicate the Nrf2-MafK complex and the MafK-MafK complex, respectively. The positions of the ARE-1 and ARE-2 probes are indicated. (B) ChIP analysis of Nrf2 binding to the *AOX1* gene. Chromatin from DEM-treated (+) or non-treated (-) RL-34 cells were immunoprecipitated with the anti-Nrf2 antibody, and the

AOX1 promoter region was amplified. Total chromatin DNA (Input) and DNA immunoprecipitated with pre-immune serum (Pre-immune) were also analyzed as a control. The primers used for PCR are indicated. The PCR products were analyzed by 6% polyacrylamide gel electrophoresis.

Fig. 4

Activation of *AOX1* gene in DEM-treated hepatoma cells. (A) RT-PCR of RL-34 cells treated with (+) or without (-) 50 μM DEM. The isolated RNA was also used for PCR without reverse transcription (RT(-)). Glyceraldehyde-3-phosphodehydrogenase mRNA (GAPDH) was amplified as a control. (B) Real-time PCR of *AOX1* mRNA. $\Delta\Delta Ct$ calculation for *AOX1* mRNA is shown. (C) Reporter transfection analysis of the *AOX1* promoter with DEM treatment. The -329Luc plasmid was co-transfected with Nrf2 expression plasmid (Nrf2 +) or empty vector (Nrf2 -). After 15hr of transfection, 50 μM DEM (DEM +) or DMSO (DEM -) was added and incubates further 20hr.

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-1830                                   ctctcagaag ccacacagcc tgtcttttct
-1800  caagactcct cccttgcact cctctttaga aatctcaggt ctagctcttg aaagtctctc
-1740  tcctgaggta ttggcaaaga ctgagcagtg cactccctca caggcttccc agatatctaa
-1680  gttttaatta attcaacccc ctccctttgt taccacagtt gcacattgct agaattttca
-1620  tttttgcatc ttcagcagaa tgtgctgagt gcatggctaa aaagacttgt gaaaagtctt
-1560  aaaggtccac agttttattg ttactggagt actgatgctc ttgtttttaa gtagatttat
-1500  gggcaaaata aaatagcttc gtttttttt tatatttttt gtgtatgagt gttttgcttg
-1440  tgtgtgtctg tatgcctgtg tatgtgtgtg cacatgtgta tatacatgtg aatgcatgtg
-1380  tatatgtatg tgtgtgcata tgtatatgta tgtgtgtgtg tatatatata aatatatata
-1320  tatatatata tatatatata tatatatata tatgtaggta agcgtgtgtt atgtgtgtgc
-1260  ctggggacct gggccattag aagaaggaaa tcagattccc cggaactgga gttgtggaag
-1200  actgtgaatg ttgggaattg aacctgttag aaacaagtgc tctgaaccac tgagccatct
-1140  ctacagcctc tactctaaga ttttagaatc agaattattg gtgtcagggg aatgacagac
-1080  ttccagctct taatctgctg actattactc agcagaatgt ccaaaactgg ctactttata
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-960   ctagaccctg tcaaagaaaa tgtttgccag gcatggaggt tcacacacac ccttgattcc
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-720   gaggccagga ggtgatgtgc tttttttctt ctccatatac aaacttgaga agttttcgca
-660   atgaaaatgt cctgtagtgg tgcacacctt taatcctagc aatcaggagg cagaggcagg
-600   tggagatctg tgagttagag accagtctgg tctacaaaga gttctaagac agccagagct
-540   gttacccaga gaaatcctgt ataattgggg gatgtggtat ggggaggggg aaggcgatgt
-480   tgttttgcat taataaaata atctagggag atatactaag tgaaacgagg tctgaaaaat
-420   aatttggtct cgtttgagac ttgccaggtt ttataaagca gattttaaaa tgtaaaaaaa
-360   ggccaaaaaa taacgttaag ggtgcaggac tcttggcatg tgactcagca gtacagccag
                                                      ARE-1
-300   gctgccctcc cacccccctaa ataaacagag aggattcaaa cgaatcggaa agcacatcca
-240   acaaacaagc actggtacag gctattaaag caatgactca ggattttctg aggatatctg
                                                ARE-2
-180   cagagctgcg ggacgggaac cgtccttccc atcgatgcca aaaaccgaag atttcagttt
-120   gtctcctgcg ctggcacact cgatagtcag gacagttttc cctttaggac tttggcagag
-60    aactttctgg gagggtggag acctagaggc tcaggcaagc cccgccctgt ggcctcgggc
+1     aatttattct ctccaaggac tggctctgct agggacccgc tagggatcta ctagggacct
       gctagggacc tcgtcgtcat ggatcccccg  +90
                             ATG
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Fig. 1

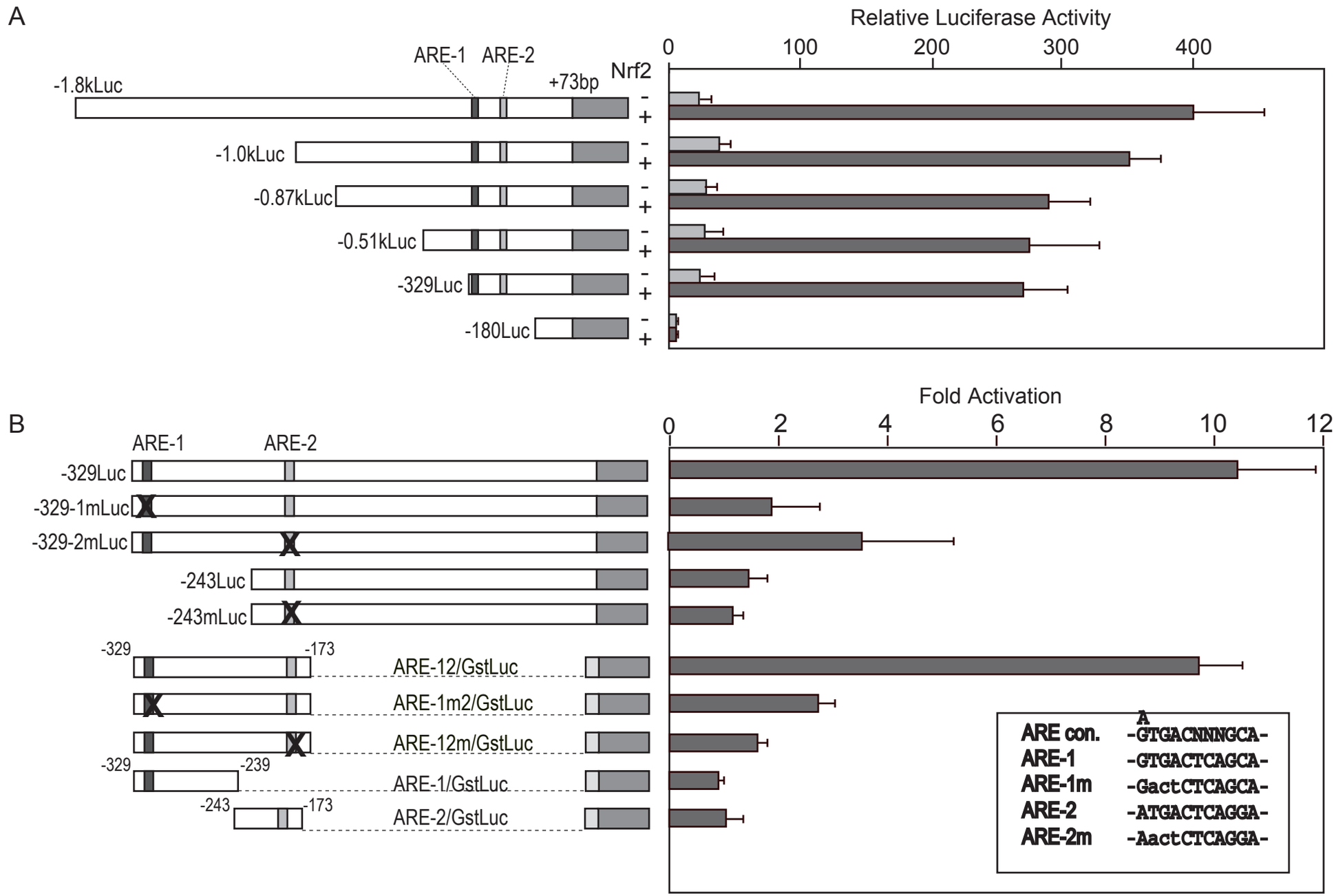
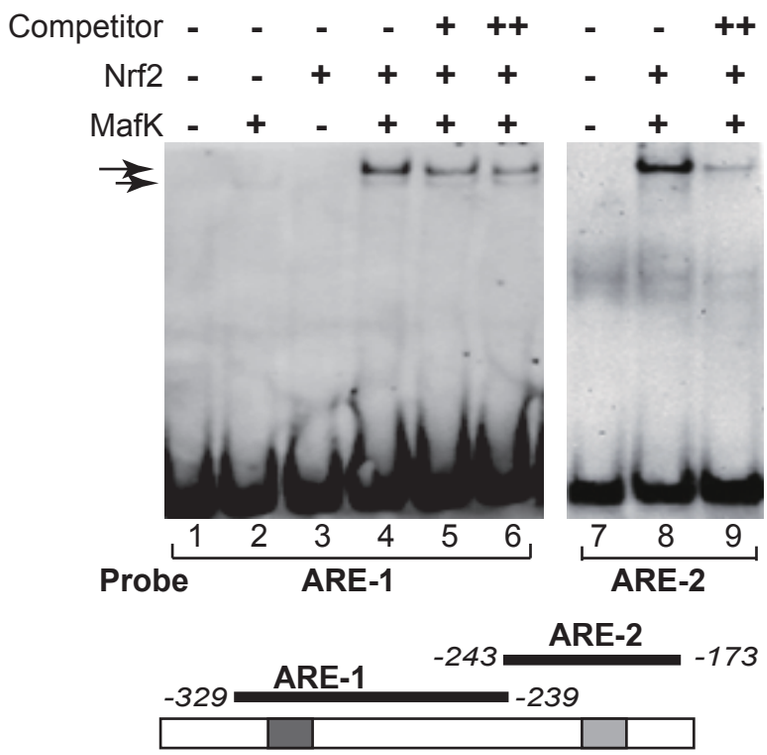

A
Relative Luciferase Activity
0
100
200
300
400
ARE-1
ARE-2
+73bp
Nrf2
-1.8kLuc
-1.0kLuc
-0.87kLuc
-0.51kLuc
-329Luc
-180Luc
-
+
B
Fold Activation
0
2
4
6
8
10
12
ARE-1
ARE-2
-329Luc
-329-1mLuc
-329-2mLuc
-243Luc
-243mLuc
-329
-173
ARE-12/GstLuc
ARE-1m2/GstLuc
ARE-12m/GstLuc
-329
-239
ARE-1/GstLuc
-243
-173
ARE-2/GstLuc
ARE con. -GTGACNNNGCA-
A
ARE-1 -GTGACTCAGCA-
ARE-1m -GactCTCAGCA-
ARE-2 -ATGACTCAGGA-
ARE-2m -AactCTCAGGA-

Fig. 2

A EMSA



B ChIP Analysis

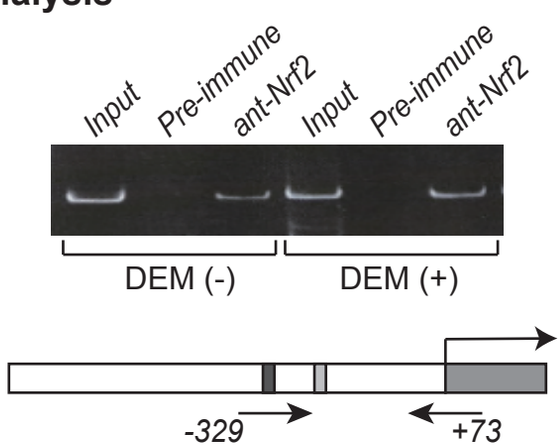


Fig. 3

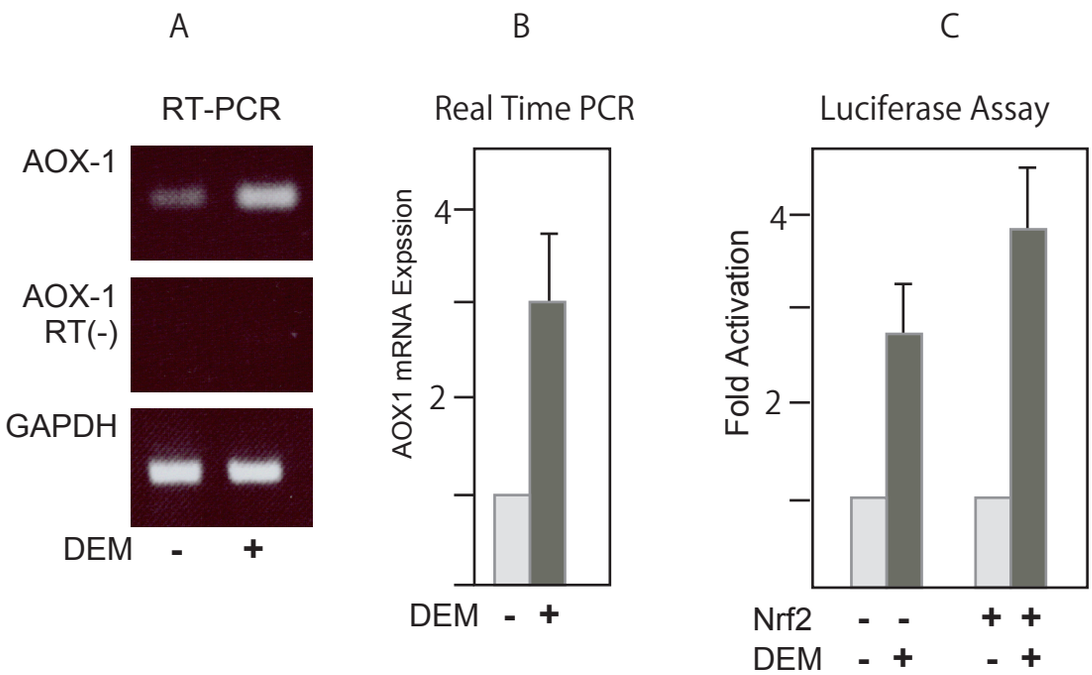

A
RT-PCR
AOX-1
AOX-1
RT(-)
GAPDH
DEM - +
B
Real Time PCR
AOX1 mRNA Expssion
4
2
DEM - +
C
Luciferase Assay
Fold Activation
4
2
Nrf2 - - + +
DEM - + - +

Fig. 4