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Nitroalkene fatty acids mediate activation of Nrf2/ARE-dependent and PPARγ-dependent transcription by distinct signaling pathways and with significantly different potencies†

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Abstract

Naturally occurring nitroalkene fatty acids (NAs) derived from oleic (NO_2-OA) and linoleic (NO_2-LA) acids mediate a variety of cellular responses. We examined the signaling pathways involved in NA activation of Nrf2/ARE-dependent versus PPARγ/PPRE-dependent transcription in human MCF7 breast cancer cells. Additionally, we compared the relative potencies of NO_2-OA and NO_2-LA in activating these two transcriptional programs. Here it is demonstrated that, in addition to the direct adduct formation of NA with the Nrf2 inhibitory protein, Keap1, shown by others, NA activation of Nrf2/ARE-mediated transcription results from increased nuclear Nrf2 levels and depends upon activation of the PI3K/AKT and PKC, but not ERK and JNK MAPK, signaling pathways. Examination of the relationship between NA stimulation of the Nrf2/ARE versus PPARγ/PPRE transcriptional programs revealed concentration-dependent activation of distinct signaling pathways that were readily distinguished by selective attenuation of Nrf2/ARE-dependent, but not PPARγ-dependent, transcription by inhibitors of PI3K and PKC. Moreover, measurable, statistically significant activation of PPARγ/PPRE-dependent transcription occurred at nanomolar concentrations of NAs—the 12-NO_2 isomer of NO_2-LA showing the most potent activity—whereas significant activation of Nrf2/ARE-dependent transcription occurred at much higher NA concentrations (≥ 3 micromolar) with the NO_2-OA isomers the most potent. These findings have implications for the physiological roles of NAs suggesting that, at concentrations likely to be encountered in vivo, their direct activation of PPARγ transcription will dominate over their electrophilic activation of Nrf2 antioxidant/protective responses.

Nitroalkene fatty acids (NAs)[1] are naturally occurring electrophilic derivatives of unsaturated fatty acids formed via •NO-dependent oxidative reactions (1). Due to the natural abundance of oleic and linoleic acids in the diet, circulation, and membranes, their nitrated derivatives, NO_2-OA and NO_2-LA, are quantitatively important species that mediate a variety of cellular responses including •NO-dependent vasodilation (2), anti-inflammatory processes (3, 4), heat shock responses (5), and others (1). Additionally, among the more

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[1]Abbreviations: ARE, antioxidant response element; ERK, extracellular signal-regulated kinase; FABP4/aP2, fatty acid-binding protein 4; GAPDH, glyceraldehye 3-phosphate dehydrogenase; JNK, c-Jun-NH_2-kinase; Keap1, Kelch-like ECH-associated protein 1; MAPK, mitogen-activated protein kinase; NA, nitroalkene fatty acid; NO_2-LA and NO_2-OA, nitrolinoleic and nitro-oleic acids; NQO1, NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PPARγ, peroxisomal proliferator-activated receptor gamma; PPRE, PPAR responsive element; RSG, rosiglitazone; SFN, sulforaphane

striking specific properties of NAs are their abilities to activate PPARγ- and Nrf2-dependent transcription of genes containing, respectively, PPRE and ARE promoter elements (6–8). PPARγ, by governing the expression of a large network of genes, is important in the control of lipid and carbohydrate homeostasis, adipocyte differentiation, inflammation/anti-inflammation, and other functions (9–12). Nrf2, on the other hand, is crucial for mediating the expression of a family of genes associated with cellular defense and protection in response to oxidative and electrophilic stress (13, 14). NO_2-LA and NO_2-OA are among the most potent naturally occurring ligand agonists of PPARγ yet identified (6, 7, 15) and, more recently, have been shown to activate Nrf2/ARE-dependent transcription (8). What is not known is the relative potency of NAs and their physiological relevance, at NA levels likely to be achieved in vivo, towards activation of these alternative transcriptional programs.

It is well established that NO_2-LA and NO_2-OA activate PPARγ via direct interaction with the ligand-binding pocket of the transcription factor (15–18). Relatively less is known about the mechanism of NA-activation of Nrf2-dependent transcription. Under basal conditions, Nrf2 associates with its inhibitory partner, Keap1, which serves as an adaptor protein to bridge Nrf2 with a Cul3-based E3 ubiquitin ligase thereby targeting Nrf2 for ubiquitinylation and proteosomal degradation (19, 20). It is widely accepted that a major mechanism by which electrophiles activate Nrf2-dependent transcription is through disrupting Nrf2/Keap1 interactions resulting in Nrf2 stabilization, accumulation, and translocation to the nucleus where it can facilitate transcription of its target genes. This electrophile-mediated disruption of Nrf2/Keap1 interactions often occurs as a consequence of redox modification or direct adduction of key cysteine residues within Keap1 (21–24). Indeed, Kansanen et al. recently demonstrated formation of such adducts between Keap1 and the 9- and 10-*E*-nitro isomers of NO_2-OA (25). While formation of these adducts is likely to contribute significantly to Nrf2 stabilization, other pathways are also implicated in the control of electrophile-stimulated Nrf2/ARE-dependent transcription—pathways that include PI3K/AKT, MAPK, and PKC signaling (26–34).

Accordingly, the studies described herein were designed to: 1) evaluate the roles of these kinase signaling pathways in NA-mediated activation of Nrf2/ARE-dependent transcription, 2) determine whether NA-mediated activation of the two alternative transcription programs (PPARγ- and Nrf2-driven) are independent of each other *or* if they share common signaling pathways or exhibit significant activation pathway cross-talk, and 3) quantitatively compare Nrf2/ARE versus PPARγ/PPRE transcriptional responses to NA over a range of NA concentrations in order to ascertain which transcription program will dominate at NA levels likely to be encountered in vivo.

Experimental Procedures

Materials

Nitroalkene fatty acids (NAs): total (*E*-9, 10, 12, and 13 nitro) nitrolinoleic acid (total NO_2-LA) was synthesized (35) and the *E*-12-NO_2 regioisomer (12-NO_2-LA) was purified from total NO_2-LA by preparative HPLC as described previously (15). *E*-9- and *E*-10-nitrooleic acids were synthesized regio- and stereoselectively using the previously reported procedure (36) and then combined as a 50:50 mixture (9/10-NO_2-OA) for the experiments described herein. Chemicals, agonists and inhibitors: sulforaphane (SFN) was from LKT Laboratories (St. Paul, MN) and rosiglitazone (RSG) was from Cayman Chemicals (Ann Arbor, MI). Kinase inhibitors were from Cell Signaling Technologies, Danvers, MA (LY294002), Sigma-Aldrich, St. Louis, MO (staurosporine, Ro-31-8220), and Promega, Madison, WI (PD98059). Antibodies: antibodies directed against human Nrf2, topoisomerase I, β-actin, JNK, and phospho-JNK were from Santa Cruz Biotechnologies (Santa Cruz, CA) and against AKT, phospho-AKT, ERK, and phospho-ERK were from Cell Signaling.

Expression vectors and reporter genes: Use of the firefly luciferase reporter genes driven by the minimal TK promoter and three tandem PPREs, pPPREx3-tk-LUC (37), and the ARE derived from the murine *gsta1* gene, pARE-TI-LUC (38); the control CMV-*Renilla* reporter gene, pGL4.75; and the human PPARγ1 expression vector, pcDNA3-PPARγ (39) have been described previously (35, 40). Additionally, the *Renilla* luciferase reporter vector driven by a minimal promoter and the murine *gsta1* ARE, pARE-TI-REN, was constructed by replacing the luciferase cDNA in pARE-TI-LUC with the 1235 bp *Hin*dIII/*Bam*HI fragment from pGL4.75 encoding the *Renilla* luciferase cDNA.

Cell culture, inductions, transfections, and assays

All cultures, inductions and transfections were accomplished with MCF7 breast cancer cells in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 10 μg/ml ciprofloxacin. Transfections: all were done using SuperFect Transfection Reagent (Qiagen, Valencia, CA) at a SuperFect:DNA ratio of 5:1 (μl:μg) according to the manufacturer's recommendations. 4×10^4 cells were seeded in 12 well tissue culture plates. Twenty-four hours later cells were transfected with the following vector DNA combinations: Transfection scheme 1) For detection of ARE- or PPRE-dependent transcription in separate transfections, cells were co-transfected with, per well, 0.2–0.4 μg pARE-TI-LUC *or* pPPREx3-tk-LUC plus 0.1–0.2 μg pcDNA3-PPARγ and 20–40 pg pGL4.75 (control to correct for differences in transfection efficiencies). Transfection scheme 2) For simultaneous detection of ARE- and PPRE-dependent transcription, cells were co-transfected with 0.2 μg pARE-TI-REN *and* 0.2 μg pPPREx3-tk-LUC plus 0.1–0.2 μg pcDNA3-PPARγ. Approximately 17–20 hr later, cells were treated with nitroalkene fatty acid (NA), RSG, SFN, or vehicle control according to the induction protocol (below). Cells were harvested at various timed intervals thereafter for Dual Luciferase assay (Promega, Madison, WI). Inductions: all were accomplished by exposing cells to variable concentrations of NAs (9/10-NO_2-OA, total NO_2-LA, or 12-NO_2-LA), SFN, RSG, or vehicle (dimethyl sulfoxide) control for 3 hr in complete medium supplemented with 10% fetal bovine serum. At the end of the 3 hr exposures, medium was replaced (minus inducing agent) and cells harvested at variably timed intervals thereafter for Western blot (40) or Dual Luciferase assay (Promega). For some experiments, kinase inhibitors were added 1 hr prior to induction and were maintained in the medium throughout the subsequent 3 hr exposure and beyond until cell harvesting.

Q-RT-PCR analysis of endogenous gene expression induced by NA

MCF7/PPARG1-4′, a cell line derived from parental MCF7 cells stably expressing PPARγ (15), cells were used to examine the induction of PPARγ/PPRE-responsive (*FABP4/aP2*) and Nrf2/ARE-responsive (*NQO1*) endogenous genes. Cells were treated with variable concentrations of 12-NO_2-LA or vehicle for 3 hr, medium replaced, and cells harvested for total RNA (Trizol Reagent, Invitrogen) 9 hr later. From total RNA, cDNAs were generated by the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and used for Q-RT-PCR with iQ Real-Time SYBR Green Supermix (Bio-Rad) and an ABI Prism 7700 thermocycler as described (41). From reference cDNA preparations, standard curves were generated for NQO1, FABP4/aP2, and GAPDH from which relative mRNA expression levels were determined for unknown samples. Levels of NQO1 and FABP4/aP2 mRNA were expressed in arbitrary units. Levels of GAPDH (housekeeping control) mRNA expression did not differ significantly between cells treated with 12-NO_2-LA and untreated controls. Oligonucleotide pairs used for amplifications were: NQO1, TGCAGCGGCTTTGAAGAAGAAAGG and TCGGCAGGATACTGAAAGTTCGCA; FABP4/aP2, ACTGCAGCTTCCTTCTCACCTTGA and TGGCAAAGCCCACTCCTACTTCTT; and GAPDH, GAAGGTGAAGGTCGGAGTC and GAAGATGGTGATGGGATTTC.

Results

Time and concentration dependent activation of ARE-driven transcription by NAs in MCF7 cells

Treatment of MCF7 breast cancer cells with 9/10-NO_2-OA results in a time-dependent activation of ARE driven transcription which reached a maximum at about 10 hr after the start of induction (Fig. 1A). Activation of ARE-driven transcription with nitrated derivatives of both oleic and linoleic acid is concentration dependent although 9/10-NO_2-OA is somewhat more potent than total NO_2-LA in this capacity (Fig. 1B). Induction of ARE-driven transcription is associated with a striking accumulation of nuclear Nrf2 in both NA-treated (total NO_2-LA and 9/10-NO_2-OA) and positive control (SFN-treated) cells (Fig. 2). For NAs, Nrf2 levels peak at 5 hr post-initiation of induction and decline thereafter. Peak reporter gene activity occurs later (Fig. 1A)—presumably reflecting a lag period during which mRNA and protein are accumulating in the cytosol.

Role of protein kinase signaling pathways in NA-induction of ARE/Nrf2-mediated transcription

It has been recently shown that NO_2-OA forms covalent adducts with key cysteine residues of Keap1 (25). These reactions presumably account, in part, for stabilization and accumulation of Nrf2 and its mediation of ARE-dependent transcription. To determine whether other signaling pathways are involved in Nrf2/ARE-transcription activation, we examined the roles of MAPK, PI3K and PKC signaling—pathways that have previously been implicated in Nrf2-dependent transcription in other systems (26–34). Indeed, PI3K/AKT signaling is activated by NAs (total NO_2-LA and 9/10-NO_2-OA) and, to a lesser extent, SFN as evidenced by a time-dependent phosphorylation of AKT (Fig. 3). That such activation has functional consequence in NA-induction of Nrf2/ARE-driven transcription is supported by the finding that PI3K inhibitors, wortmannin and LY294002, significantly attenuate NA (and SFN) induction of ARE-reporter gene activity (Fig. 4).

Similarly, PKC is implicated in NA-induction of ARE-dependent transcription by experiments shown in Fig. 5: two different pan-PKC inhibitors, staurosporine and Ro-31-8220, significantly attenuate NA and SFN-induced transcription.

In contrast, no significant activation of the ERK or JNK pathways by NA or SFN was evident as shown by the absence of changes in phospho-ERK or phospho-JNK (Fig. 3). Consistent with the lack of ERK/MAPK involvement is the finding that inhibition of the upstream activator, MEK1, by PD98059 had minimal effect on NA- or SFN-induced ARE-reporter gene transcription (Fig. 6).

NAs activate ARE/Nrf2- and PPRE/PPARγ-dependent transcription by distinct signaling pathways and with different potencies

There is considerable opportunity for cross-talk between signaling pathways that have as their endpoints the activation of transcription from ARE- versus PPRE-containing genes. For example, recent results have linked Nrf2 and the induction of PPARγ (42). It was therefore important to establish if activation of these alternative transcriptional programs by NA could be dissociated or, instead, if these pathways were interdependent through the sharing of other common upstream effectors. First, in order to reveal any potential cross-talk between these transcriptional programs, we examined induction of transcription by the nutraceutical activator of ARE/Nrf2-directed transcription, SFN, and the relatively selective PPARγ agonist, RSG. As shown by experiments (Fig. 7) in which cells were co-transfected with PPRE-firefly luciferase and ARE-*Renilla* luciferase reporters, SFN resulted in selective induction of ARE/Nrf2-dependent transcription while RSG treatment exclusively induced

PPRE/PPARγ-dependent transcription—suggesting that, at least in this system, there is little cross-talk between the transcriptional programs. Next, using the PI3K and PKC inhibitors, we asked whether the abilities of NAs to activate the alternative transcription programs could be dissociated. Indeed, as shown in Fig. 8, treatment with either inhibitor significantly attenuated induction of ARE-driven transcription by both NA (12-NO_2-LA) and SFN. In contrast, these kinase inhibitors had very modest effects on activation of PPRE-driven transcription by either 12-NO_2-LA or the control agonist, RSG. Hence, we conclude that the ARE/Nrf2 and PPRE/PPARγ activating properties of NAs involve distinct pathways that are distinguishable by preferential attenuation of ARE-driven transcription by PKC and PI3K inhibitors.

Lastly, we compared the dose-response profiles of NA-activated ARE/Nfr2- versus PPRE/PPARγ-driven transcription. This was important for two reasons: first to ascertain the relative strength of NA towards activation of these alternative transcription programs; and, secondly, to determine at what NA concentrations measurable effects on transcription could be seen and, hence, to determine which of the two transcription programs will dominate upon activation by NAs at concentrations likely to be encountered in vivo. Three NA preparations were used—9/10-NO_2-OA, total NO_2-LA, and 12-NO_2-LA—the latter chosen because, of all NAs previously tested, this isomer has the greatest PPARγ agonist activity at submicromolar concentrations (15, 18). As shown for all three NAs (Fig. 9A-C), significant induction of PPRE/PPARγ-directed transcription occurs at much lower NA concentrations than for induction of ARE/Nrf2-directed transcription. For 12-NO_2-LA (Fig. 9A), significant PPRE-dependent transcription was evident at the lowest concentration tested (30 nM) and, for the other NAs (Fig. 9B & C), at somewhat higher concentrations (≥ 300 nM). In contrast, significant activation of ARE/Nrf2-driven transcription was not observed until considerably higher concentrations, ≥ 3 μM, of NA were added. Finally, as also observed in Fig. 1B, induction of ARE-dependent transcription at higher NA concentrations is more robust for 9/10-NO_2-OA (Fig. 9C) than for the NO_2-LA isomers (Fig. 9A & B). Additionally, we examined the concentration dependent induction of Nrf2-responsive (*NQO1*) and PPARγ-responsive (*FABP4/aP2*) endogenous genes (14, 43) by 12-NO_2-LA using the MCF7/PPARG1-4′ cell line—a cell line derived from MCF7 that stably expresses recombinant PPARγ (15). As shown in Fig. 10 and in agreement with reporter gene findings, induction of the Nrf2-responsive NQO1 mRNA (Fig. 10A) does not occur at low 12-NO_2-LA concentrations (≤ 1 μM); rather, induction reaches significance only at much higher NA concentrations (10 and 20 μM). In contrast, robust induction of PPARγ-responsive FABP4/aP2 mRNA is observed at the lowest concentration, 0.1 μM, of 12-NO_2-LA used (Fig. 10B).

Discussion

The studies described herein establish that NA-mediated inductions of ARE/Nrf2- and PPRE/PPARγ-dependent transcription occur by entirely different pathways in MCF7 breast cancer cells. The body of evidence presented in this work and the work of others are consistent with a mechanism of ARE/Nrf2-transcription activation that involves both direct modification of Keap1 by NA adduction at key cysteine residues (25) and redox activation of PKC plus PI3K/AKT signaling pathways by the NA electrophiles. As summarized in Fig. 11, PKC likely activates Nrf2-mediated transcription by phosphorylation of Nrf2—in particular, serine 40, as described by others (31–33). This modification, along with Keap1 adduct formation with NA, very likely disrupts Keap1-Nrf2 interactions facilitating dissociation, stabilization, and nuclear translocation of Nrf2. Our data would suggest that both processes—adduct formation and PKC activation—are required for full activation of Nrf2-mediated transcription by NAs. Our data would also suggest a crucial role for the PI3K/AKT pathway in NA induction of ARE/Nrf2-directed transcription. The mechanism whereby PI3K/AKT may stimulate this activity is less clear but could occur via indirect

activation of PKC (44) or, alternatively, by facilitating the cytoplasmic-to-nuclear transport of Nrf2 as reported (28, 45).

In contrast, NA activation of PPRE/PPARγ-directed transcription is, in our system, independent of PKC, PI3K/AKT, or the MAPK pathways tested. Based upon previous studies from our laboratory and others showing potent activation of PPARγ (6, 7, 15, 35), avid binding to recombinant PPARγ (15, 18), and specific occupancy of the ligand-binding pocket of PPARγ (16, 17) by NAs, it is most likely that NAs induce PPARγ transcriptional activity directly via agonist-receptor interactions. Importantly, measurable and significant PPARγ activation occurs at much lower concentrations than those required for Nrf2-driven transcription activation. This is especially noteworthy for the naturally occurring 12-NO_2-LA isomer that supports PPARγ activation at concentrations at least as low as 30 nM. This observation is particularly relevant physiologically because, in contrast to earlier estimates (46), under normal conditions, the levels of circulating NAs measured in vivo are considerably lower—at best, in the nanomolar range (47)—than originally thought. While it is true that NA concentrations in circulation or in local cellular compartments and membranes could be much higher under conditions of inflammation and oxidative/nitrosative stress, and it is true that much higher and biologically active levels can be achieved with direct infusion of NAs as pharmaceuticals (48, 49), it is unlikely NAs will exceed nanomolar levels under normal or common pathological conditions. Thus it would appear likely that under such conditions, NA activation of PPARγ and its associated biological effects—anti-inflammation, insulin sensitization, altered lipid and carbohydrate metabolism, and others—will dominate over NA effects upon ARE/Nrf2 associated responses. Therefore, by themselves, the contribution of endogenous NAs to activation of the ARE/Nrf2 cytoprotective response are estimated to be relatively minor; but, considered in combination with other endogenous and nutraceutical electrophiles, the NAs may contribute a meaningful additive effect to overall activation of the ARE/Nrf2 transcriptional program. A final note on NA concentrations: in our experiments, NAs were administered exogenously and in the presence of 10% fetal bovine serum. Given the known propensity of NAs to react with protein sulfhydryls, the actual local concentration of free NAs at their Keap1 and PPARγ targets is unknown and may well be less than the concentration added to the medium. Nevertheless, this possibility does not affect the conclusion that NAs, at low concentrations, are significantly more potent activators of PPARγ-dependent over Nrf2-dependent transcription.

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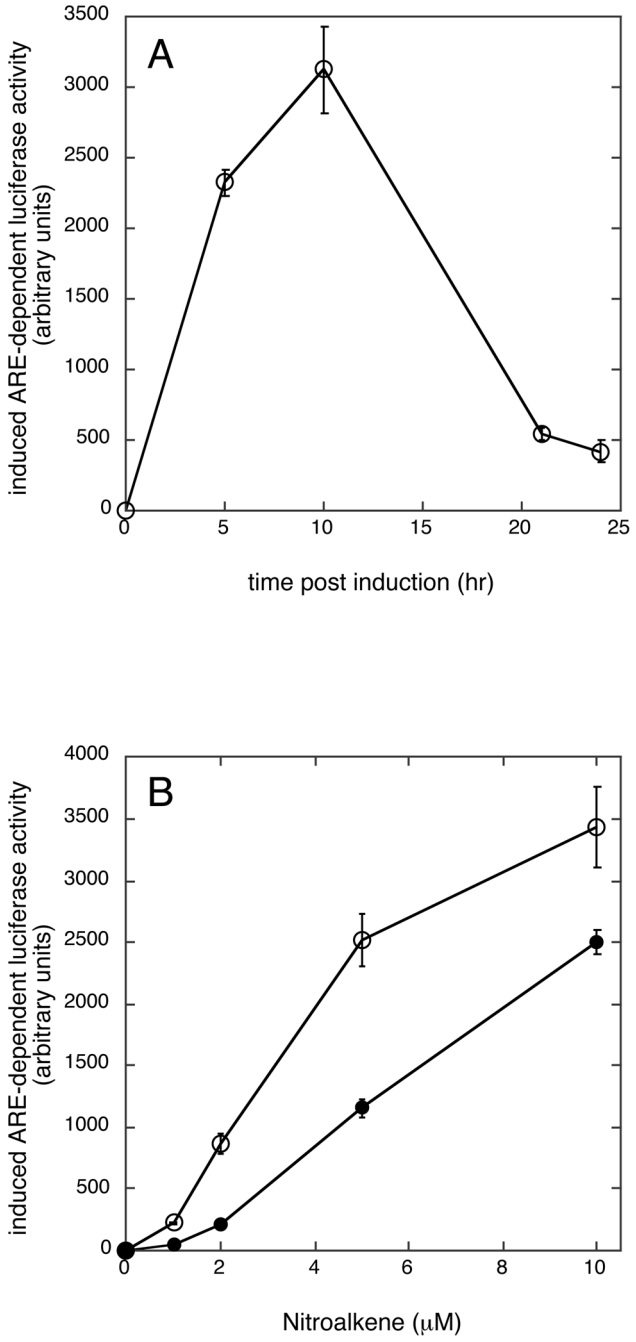


Fig. 1. Induction of ARE/Nrf2-driven transcription by nitroalkene fatty acids (NAs) in MCF7 breast cancer cells is time and concentration dependent

Cells transfected with the ARE-luciferase reporter according to transfection scheme 1 (Experimental Procedures) were exposed to 10 μM 9/10-NO_2-OA (open circles), total NO_2-LA (closed circles), or vehicle control for 3 hr. Medium was changed and incubations (minus NA) were continued for variable time intervals from the start of NA induction (panel A) or for 10 hr from the start of NA induction (panel B). Data was corrected for variations in transfection efficiencies using measurements of activity encoded by the co-transfected CMV-*Renilla* luciferase control, pGL4.75, and was expressed as induced luciferase activity in excess of vehicle controls (arbitrary units) ± 1 sd (n=3).

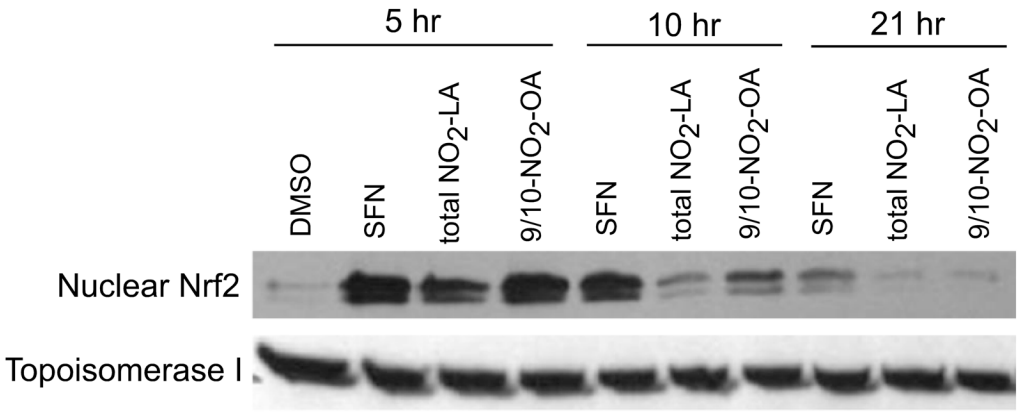


Fig. 2. Accumulation of nuclear Nrf2 in cells treated with NA

Cells were treated for 3 hr with 10 μM total NO_2-LA, 9/10-NO_2-OA, or SFN (positive control). Cells were incubated for variable time periods after removing the inducing agent and harvested for preparation and Western blotting of nuclear protein 5, 10, and 21 hr after the start of NA or SFN induction (40) using antibodies directed against Nrf2 or topoisomerase I loading control.

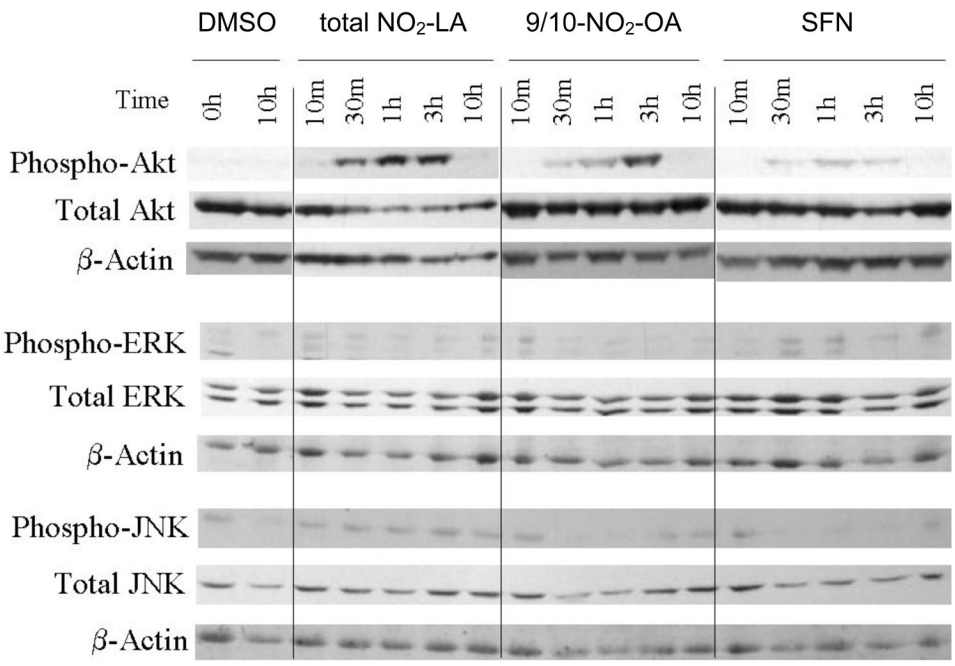


Fig. 3. Protein kinase activation in response to NA treatment

Cells were treated with NAs or SFN for the indicated times up to 3 hr. For 10 hr incubations, medium was changed to remove NA or SFN and incubations continued an additional 7 hr. Cells were harvested at the indicated times after the start of NA or SFN treatment intervals. Total cellular protein was prepared for SDS-PAGE and Western blotting as described (40) using antibodies directed against the indicated total and phosphorylated signaling kinases or β-actin loading control.

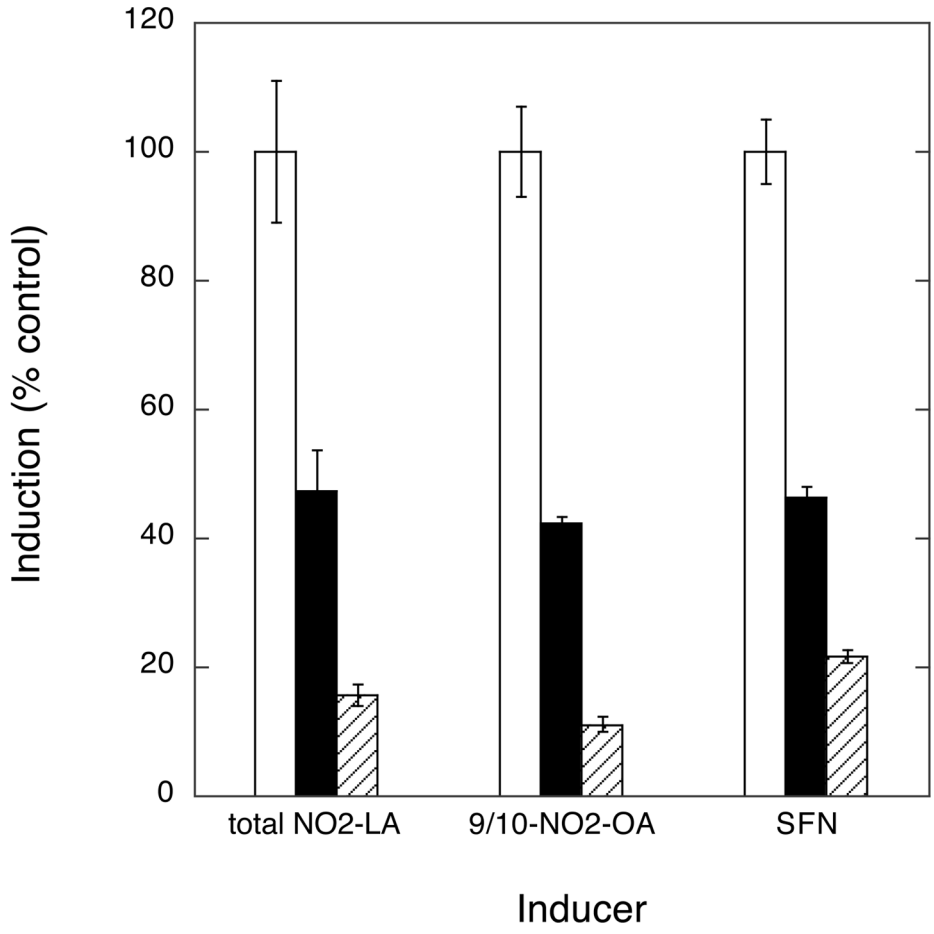


Fig. 4. PI3K activity is required for full activation of ARE/Nrf2-dependent transcription by NAs

Cells were transfected with the ARE-luciferase reporter gene according to transfection scheme 1. One hr prior to the addition of 10 μM inducing agent (total NO_2-LA, 9/10-NO_2-OA, or SFN) or vehicle control, cells were treated with PI3K inhibitors 0.5 μM wortmannin (black bars) or 25 μM LY294002 (cross-hatched bars), or no inhibitor (open bars). Inhibitors remained in the medium throughout the subsequent 3 hr induction and until harvest 10 hr after the start of induction. Data was corrected for variations in transfection efficiencies and expressed as % maximum fold induction of control (minus inhibitors, open bars). Error bars: ± 1 sd (n=3).

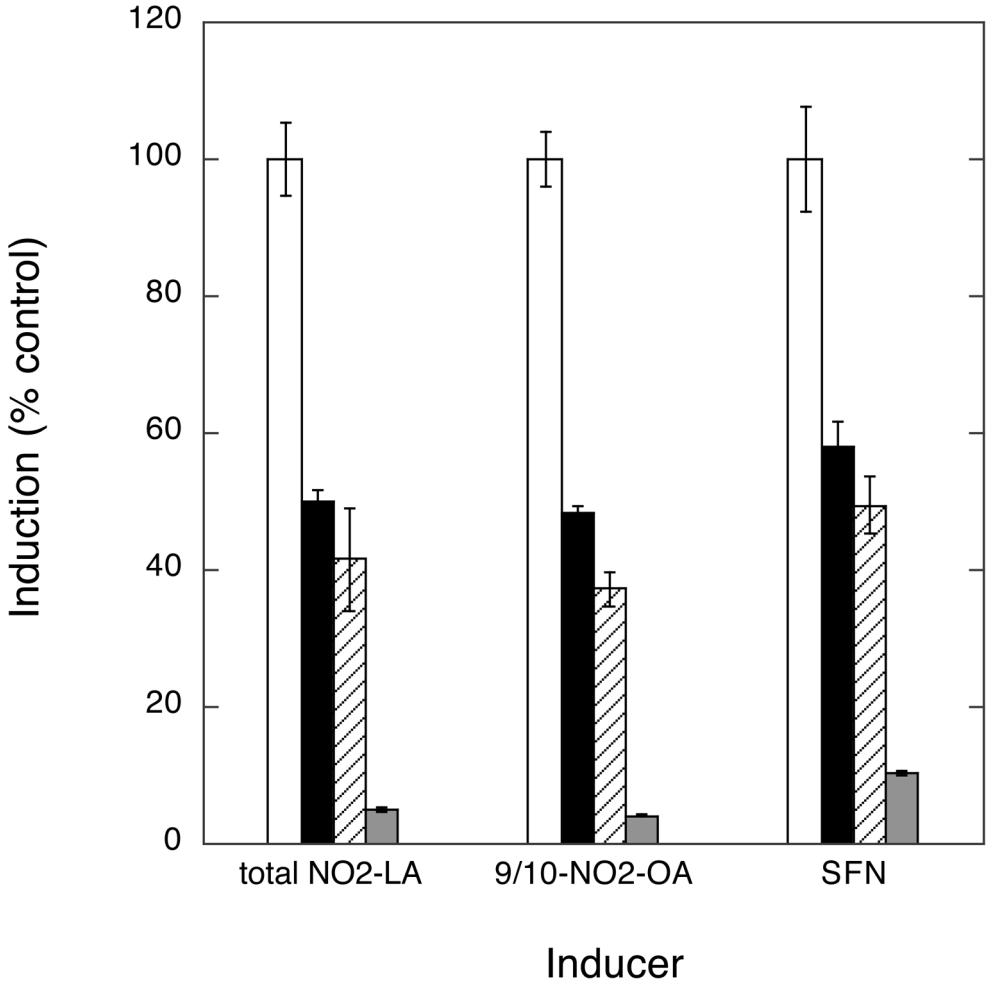


Fig. 5. PKC activity is required for optimal activation of ARE/Nrf2-dependent transcription by NAs

These experiments were conducted exactly as described in Fig. 4 except that PKC inhibitors were used: staurosporine (15 nM, black bars; 50 nM cross-hatched bars), Ro-31-8220 (shaded bars). No inhibitor controls are shown as open bars. Data is expressed as % maximum fold induction ± 1 sd (n=3).

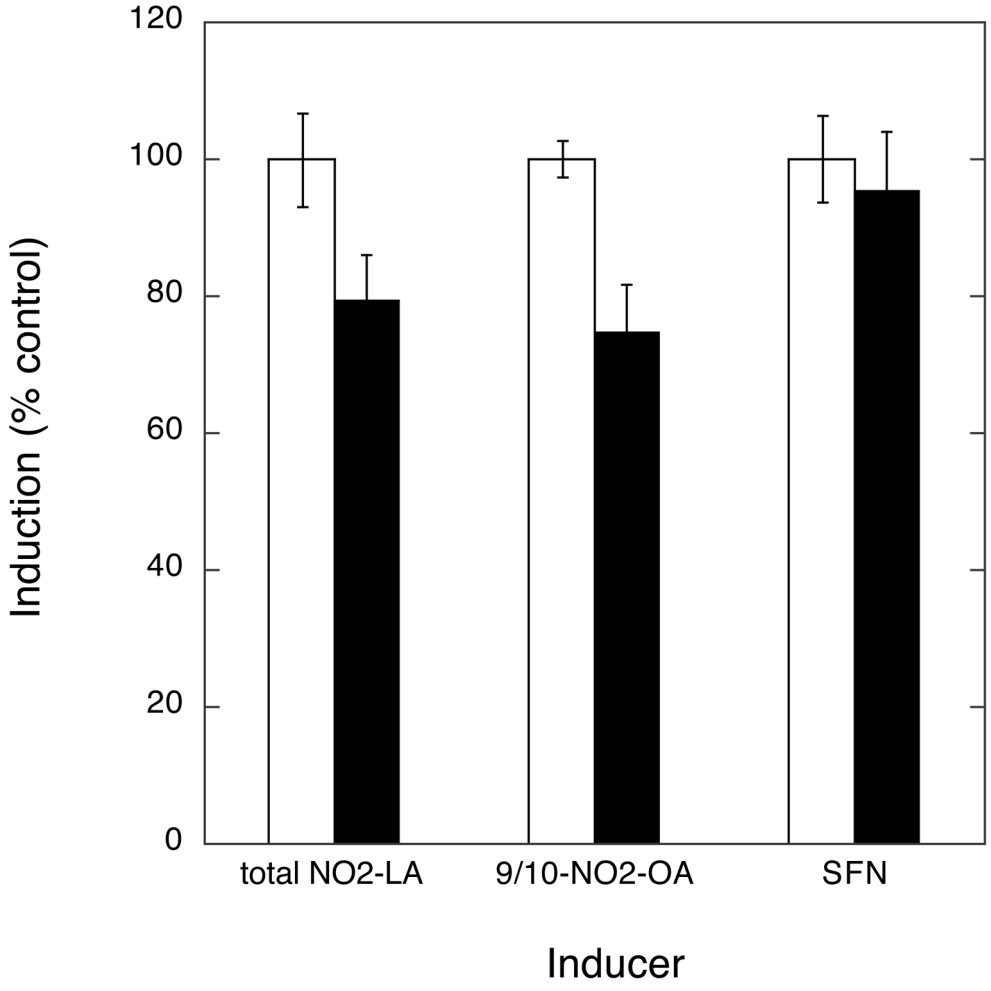


Fig. 6. Role of MAPK pathway in NA-induced ARE/Nrf2-dependent transcription

The effect of the MEK1 inhibitor, PD98059 (20 μM, black bars), on induction of ARE/Nrf2-mediated transcription by 10 μM total NO_2-LA, 9/10-NO_2-OA, or SFN control was accomplished as described (Figs. 4 and 5). Data is expressed as a percent of induction observed in the absence of inhibitor (open bars). Error bars: ± 1 sd (n=3).

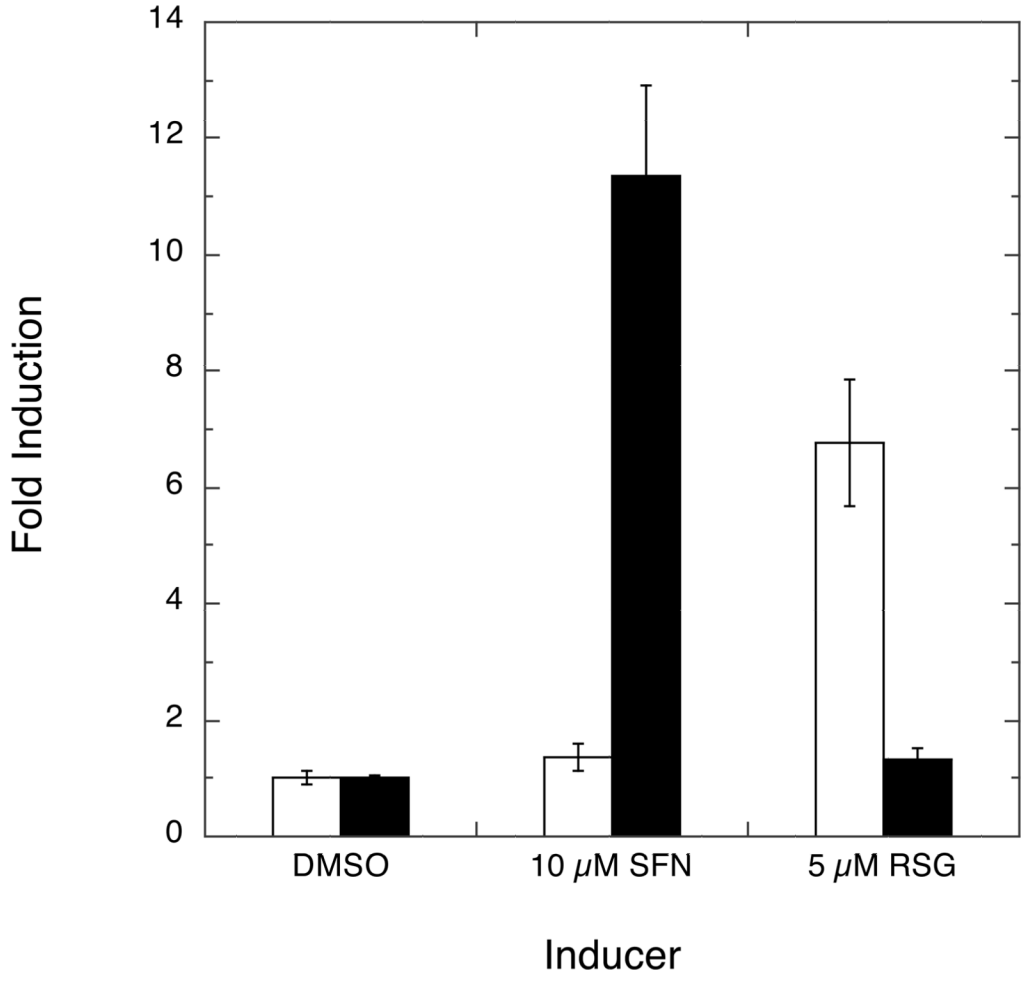


Fig. 7. Absence of cross-talk between ARE/Nrf2- and PPRE/PPARγ-directed transcription in response to treatment with SFN and rosiglitazone (RSG)

MCF7 cells were co-transfected with PPRE-driven firefly luciferase and ARE-driven *Renilla* luciferase reporters according to transfection scheme 2 (Experimental Procedures). Following induction with 10 μM SFN or 5 μM RSG and subsequent incubations, fold induction over vehicle (DMSO) control of PPRE/PPARγ-driven (open bars) and ARE/Nrf2-driven (black bars) was determined. Error bars: ± 1 sd (n=3).

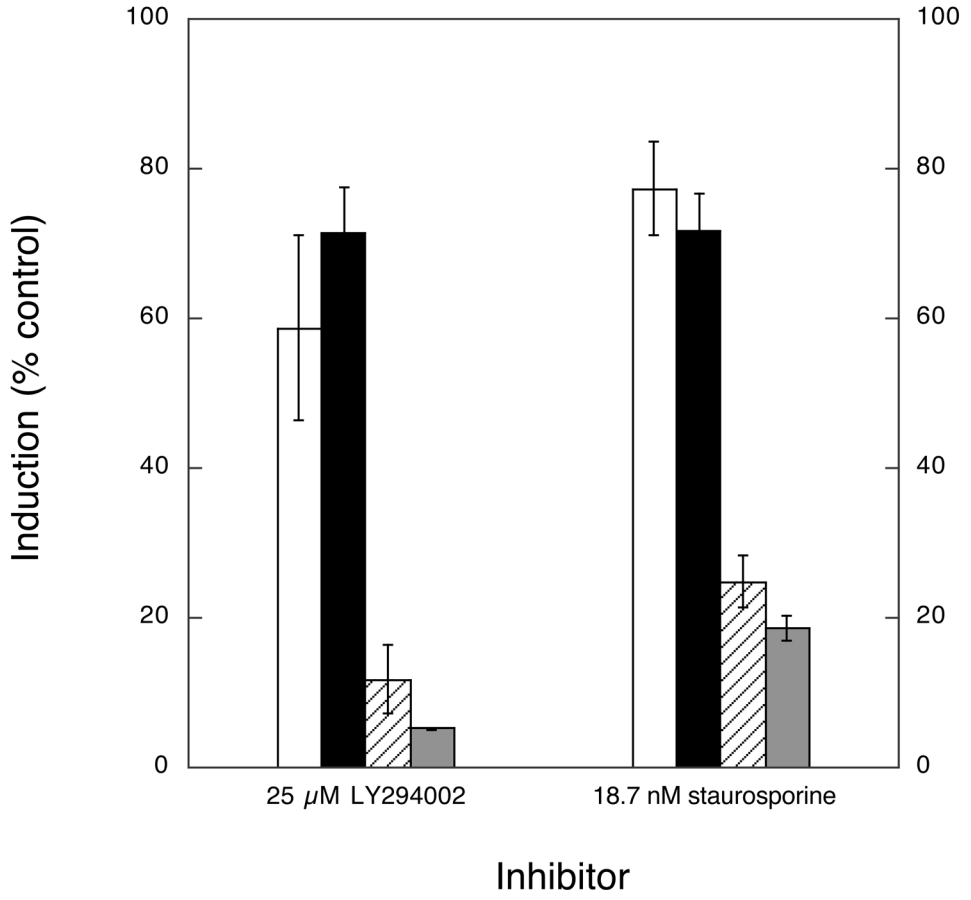
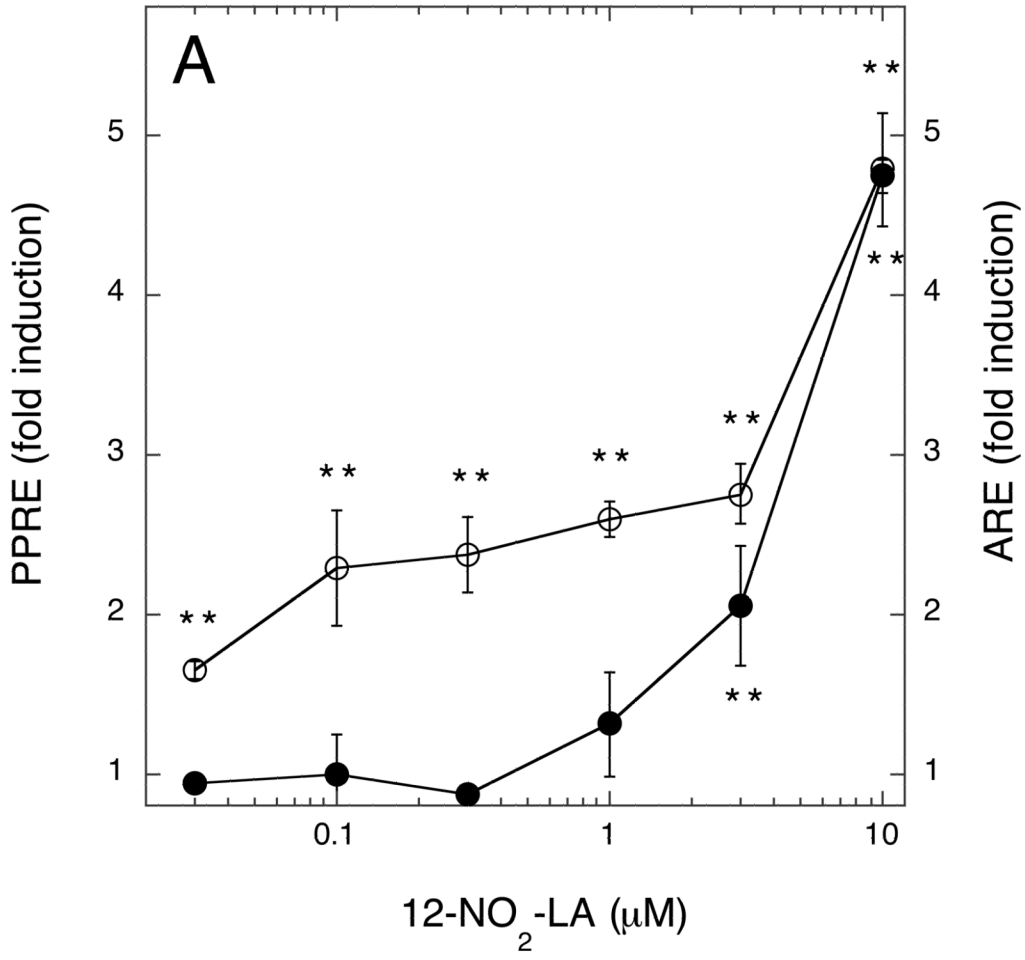
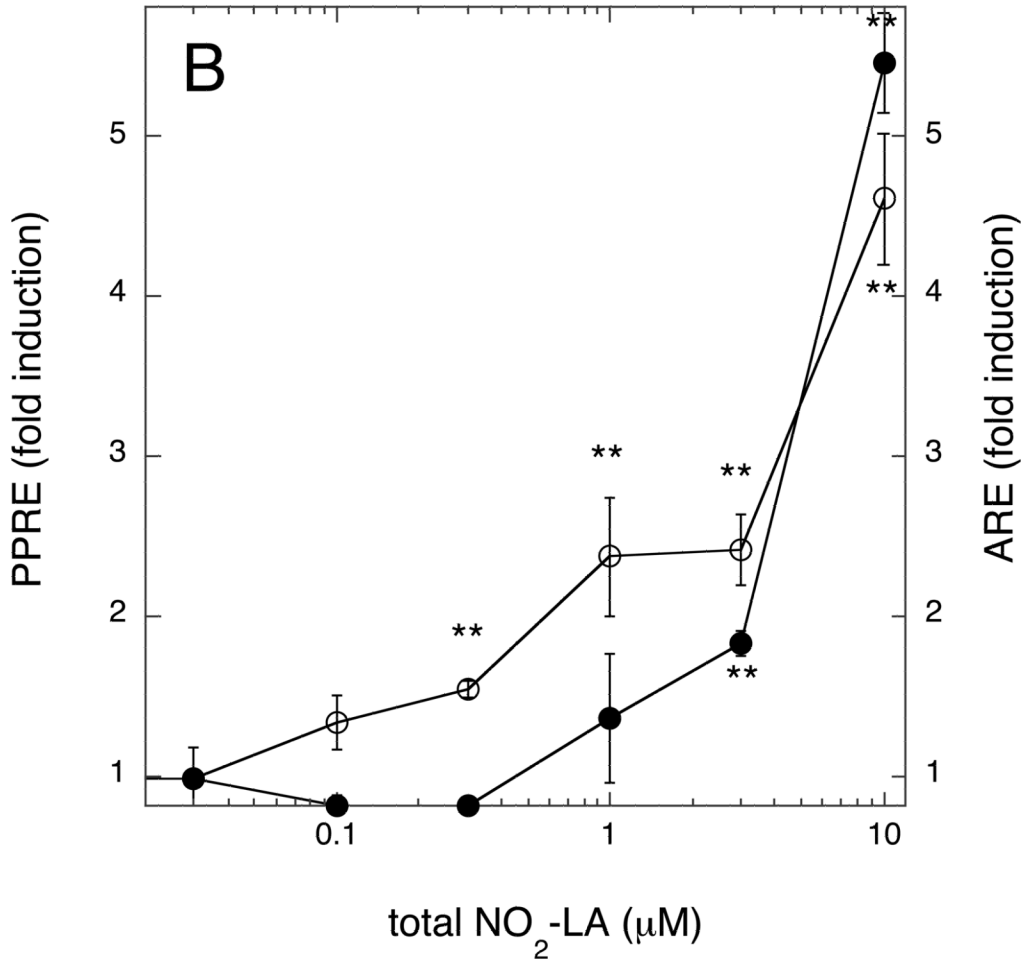


Fig. 8. Resolution of NA-induced ARE/Nrf2 versus PPRE/PPARγ transcription programs by the preferential inhibition of ARE/Nrf2 activity with inhibitors of PI3K and PKC

Cells were co-transfected with PPRE-driven and ARE-driven reporters according to transfection scheme 2 (Experimental Procedures). Transfected cells were treated with 10 μM NA (12-NO_2-LA), 10 μM SFN, or 5 μM RSG ± inhibition with the PI3K or PKC selective inhibitors, LY294002 or staurosporine, as shown. Data is expressed as % maximum fold induction (minus inhibitor) ± 1 sd (n=3). PPRE/PPARγ-driven transcription: shown are cells treated with RSG (open bars) and 12-NO_2-LA (black bars). ARE/Nrf2-driven transcription: shown are cells treated with SFN (cross-hatched bars) and 12-NO_2-LA (shaded bars).





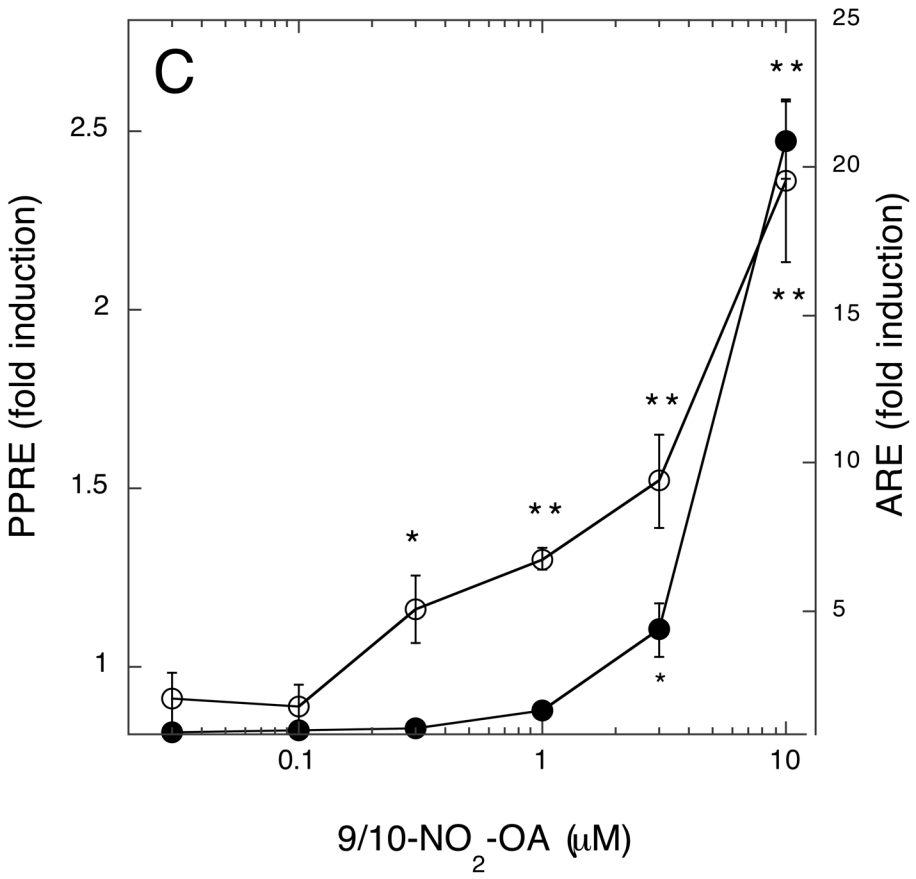


Fig. 9. Concentration-dependence of NA-mediated induction of ARE/Nrf2- versus PPRE/PPARγ-driven transcription programs

Cells were transfected with ARE or PPRE reporter genes according to transcription scheme 1. Transfected cells were treated for 3 hr with the indicated concentrations of NAs—12-NO_2-LA (panel A), total NO_2-LA (panel B), or 9/10-NO_2-OA (panel C). Medium was then replaced (minus NA) and cells harvested for luciferase determinations, corrected for variations in transfection efficiencies, 12 hr later. Data is expressed as fold induction (luciferase activity with NA ÷ luciferase activity without NA) for PPRE/PPARγ-dependent (open circles) or ARE/Nrf2-dependent (closed circles) reporter gene activity ± 1 sd (n=3–4). NA-induced values that are significantly different from un-induced controls are indicated: *, $P<0.05$; **, $P<0.01$ (unpaired, two-tailed t test with Welch correction, GraphPad InStat3).

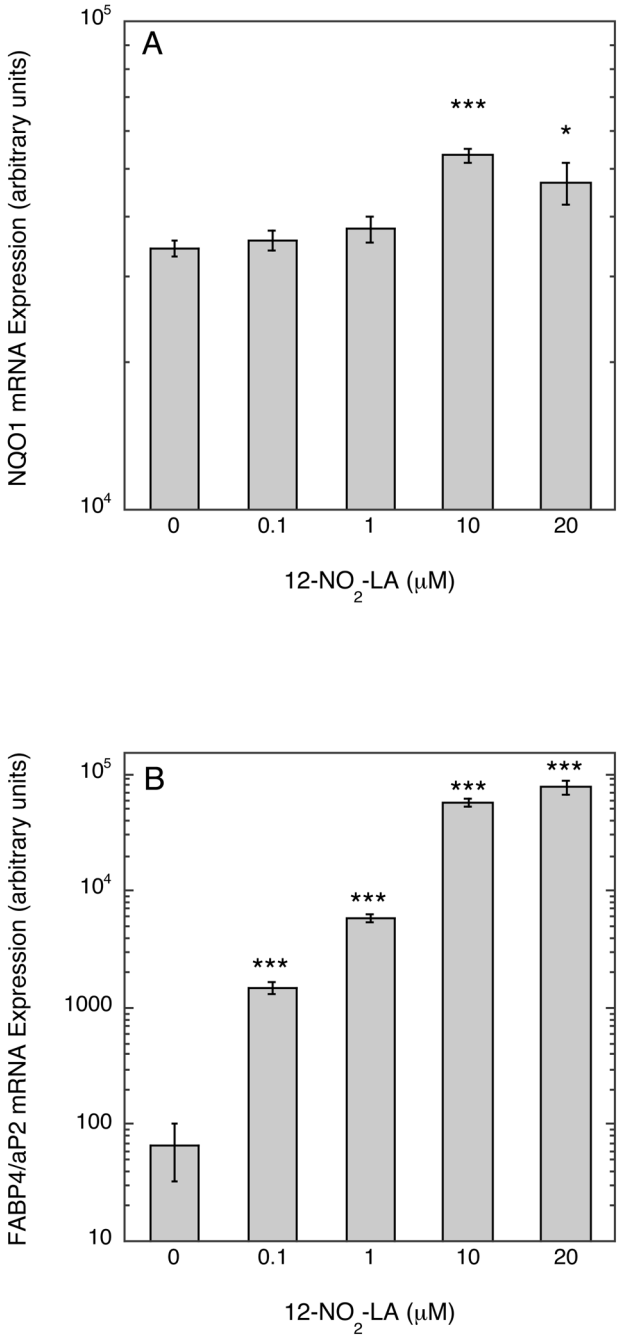


Fig. 10. Induction of Nrf2- (*NQO1*) and PPARγ-responsive (*FABP4/aP2*) endogenous gene expression by 12-NO_2-LA

MCF7-derived cells stably expressing recombinant PPARγ (MCF7/PPARG1-4′) were exposed to variable concentrations of 12-NO_2-LA or vehicle control for 3 hr and harvested 9 hr later (12 hr after the start of 12-NO_2-LA exposures) for mRNA quantification by Q-RT-PCR as described in Experimental Procedures. Shown are levels of NQO1 (A) and FABP4/aP2 (B) mRNAs expressed in arbitrary units. Bars represent the means of replicate independent experiments (n=6) ± 1 sem. * $P<0.05$ and *** $P<0.001$ (t test).

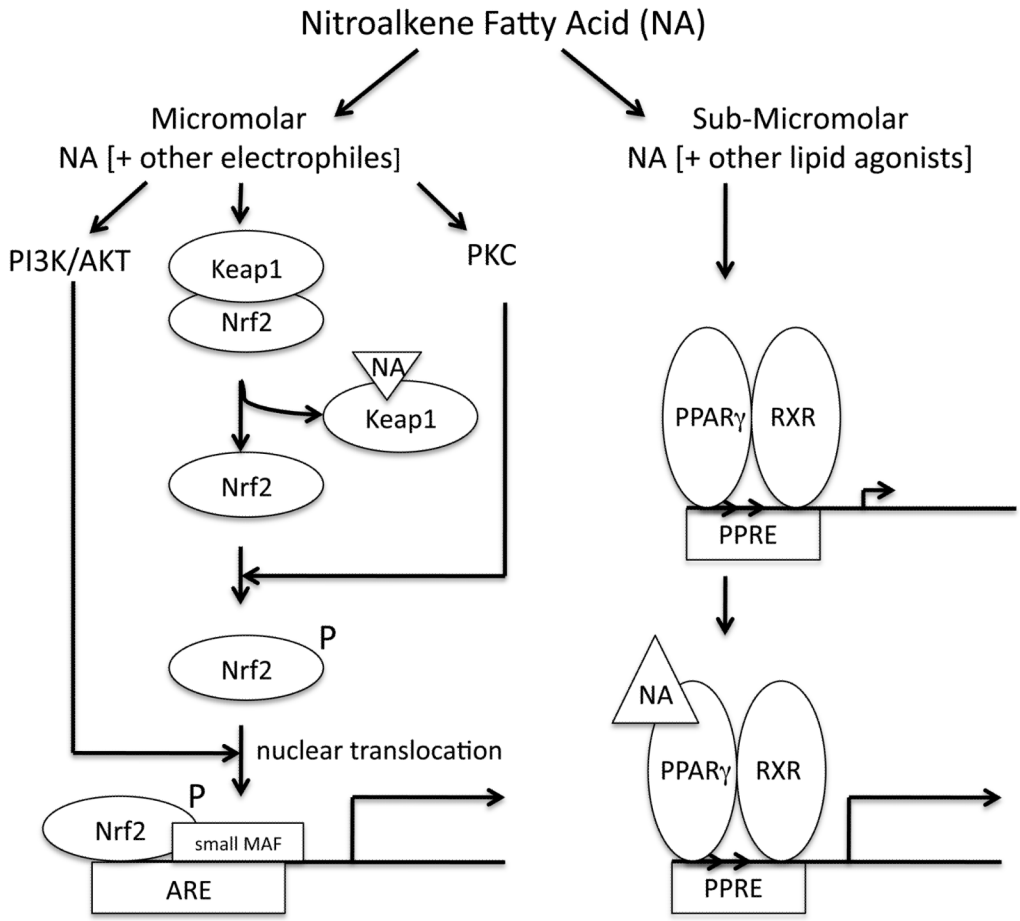


Fig. 11.

Model showing the distinct pathways involved in nitroalkene fatty acid (NA) induction of ARE- and PPRE-dependent transcription programs.