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Oxidative repression of NHE1 gene expression involves iron-mediated caspase activity

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The mechanism of Na^+/H^+ exchanger 1 (NHE1) gene repression upon exposure of cells to non-apoptotic concentrations of hydrogen peroxide (H_2O_2) was investigated. We show that continuous presence of H_2O_2 was not required for inhibition of NHE1 promoter activity. However, the downregulation of NHE1 promoter activity and protein expression was abrogated by the presence of beta mercaptoethanol (βME) and dithiothreitol. The pan-caspase inhibitor zVAD-fmk also blocked the effect of H_2O_2 on NHE1 promoter activity and expression, but unlike βME, caspase inhibition was ineffective in rescuing the early phase of NHE1 repression. Interestingly, the effect of caspase inhibition was observed only after 9 h of exposure to H_2O_2 and completely restored NHE1 promoter activity by 18–24 h. Using tetrapeptide inhibitors of a variety of caspases and siRNA-mediated gene silencing, caspases 3 and 6 were identified as mediators of H_2O_2-induced NHE1 repression, independent of initiator/amplifier caspase activation. Furthermore, incubation of cells with the iron chelator, desferioxamine, not only blocked the activities of caspases 3 and 6, but also affected NHE1 promoter and protein expression in a manner similar to zVAD-fmk. These data show that a mild oxidative stress represses NHE1 promoter activity and expression via an early oxidation phase blocked by reducing agents, and a late phase requiring an iron-dependent increase in caspases 3 and 6 activities.
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We have previously established that apoptotic death in tumor cells is tightly regulated by the ratio of intracellular superoxide (O_2^-) to hydrogen peroxide (H_2O_2).[1–4] An increase in intracellular O_2^- through its direct overproduction,[3] upon exposure to anticancer drugs,[5] following activation of the small GTPase Rac1,[6] or through inhibiting the O_2^- scavenger Cu/Zn superoxide dismutase (Cu/Zn SOD), blocked apoptosis in tumor cells. Contrarily, a tilt towards H_2O_2 primed intracellular milieu for efficient apoptotic execution.[7] More recently, we proposed Na^+/H^+ exchanger 1 (NHE1) as a possible target involved in ROS-mediated regulation of cellular response to apoptosis. We presented evidence that intracellular O_2^- was a signal for the activation of the NHE1 gene promoter, resulting in a significant increase in NHE1 protein expression, which correlated with resistance to apoptotic triggers. In contrast, addition of exogenous H_2O_2 inhibited NHE1 promoter activity and protein expression with a concomitant increase in cell sensitivity to death triggers.[8]

In mammalian cells, the NHE family consists of nine isoforms, NHE1 to NHE-9. Apart from its role as a principal regulator of pHi and cell volume, the ubiquitously expressed subtype NHE1 has been implicated in cell proliferation and transformation (for a review, see Putney *et al*[9]). Owing to the historical focus on gene induction, most reports on the regulation of transcription factors by oxidative stress have described the activation of stress-response genes by factors such as activator protein 1 (AP-1) or nuclear factor κB (NF-κB).[10] In contrast to the wealth of literature on redox-dependent activation of transcription factors, very little is known in terms of the oxidative repression of transcription machinery.[11]

In the present report, we investigated the signaling pathway involved in the inhibition of NHE1 promoter activity and protein expression at concentrations of H_2O_2 that did not activate cell death. We provide evidence linking mild oxidative stress to sustained inhibition of NHE1 promoter activity and protein expression by an iron-dependent activation of caspase 3 and 6 without the need for activation of initiator caspases 8 and/or 9.

Results

H_2O_2 inhibits NHE1 promoter activity, mRNA level, and protein expression. To avoid scavenging of H_2O_2 by the presence of serum in the culture medium, rat muscle cell line L6 stably transfected with a NHE1 reporter gene vector (L6 1.1 kb cells)[12] was grown in 0.5% FBS-containing medium 24 h before exposure to increasing concentrations of H_2O_2. Whereas incubation of L6 cells to 150 μM H_2O_2 for 24 h showed clear signs of cell death, concentrations of H_2O_2 up to 50 μM did not significantly affect cell viability even in the

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presence of 0.5% FBS (Figure 1a). Moreover, Figure 1b and c confirms that cell viability was not affected and no increase in the Sub-G1 fraction of cells could be detected above control-treated cells upon exposure to 25 or 50 μM H_2O_2 in medium containing 0.5% FBS. These results demonstrate that inhibition of NHE1 promoter activity occurs at nontoxic concentrations of H_2O_2 (25–50 μM) (Figure 2a). Note that H_2O_2 also induced NHE1 promoter inhibition if cells were

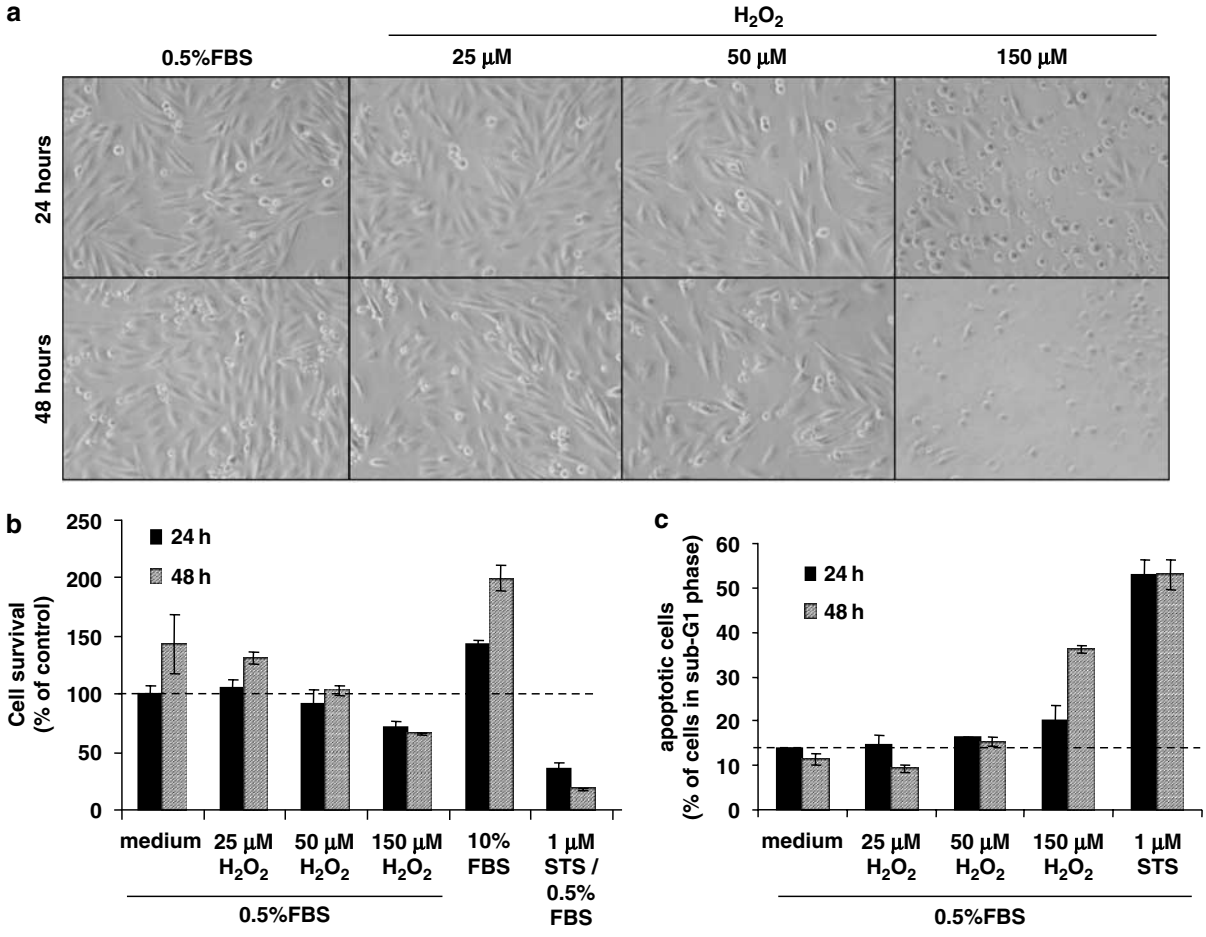
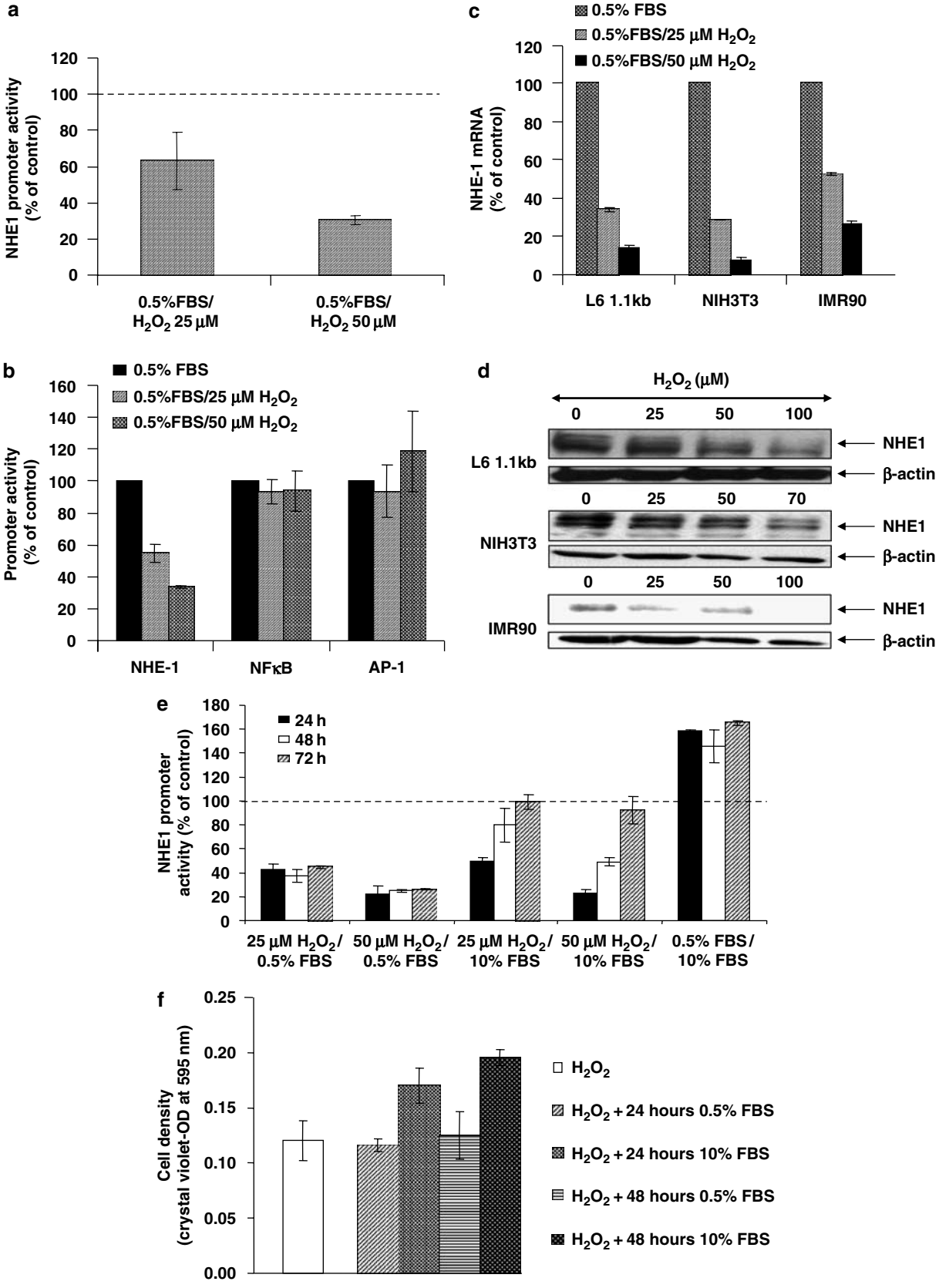


Figure 1 To determine a nontoxic H_2O_2 dose in L6 rat muscle cells, (**a**) L6 1.1 kb cells were treated with 25, 50 or 150 μM H_2O_2 in 0.5% FBS for 24 or 48 h. Photographs were taken using a digital camera attached to a light microscope (Mag $\times$ 200). (**b**) L6 1.1 kb cells were treated with 25, 50 or 150 μM H_2O_2 in 0.5% FBS for 24 or 48 h or as a control cells were left in medium containing 10% FBS or exposed to 1 μM staurosporine (STS). Data represent the average $\pm$ S.D. of two experiments performed in triplicate. (**c**) L6 1.1 kb cells were treated with 25, 50, 150 μM H_2O_2, and 1 μM staurosporine (STS) in 0.5% FBS for 24 or 48 h. Single-cell suspensions of L6 1.1 kb cells were prepared, stained with propidium iodide and analyzed by FACS to quantify the percent of cells with DNA content corresponding to the hypodiploid subG1 phase of the cell cycle as described in Materials and methods. Apoptotic cells was calculated by the percentage of cells in subG1 to the total cells' population. Data represent the average $\pm$ S.D. of two experiments done in duplicate

Figure 2 H_2O_2 represses NHE1 gene expression. (**a**) L6 1.1 kb cells were incubated for 24 h with 25 or 50 μM of H_2O_2 in medium containing 0.5% FBS before NHE1 promoter activity was assessed by luciferase assay as described in Materials and methods. Data were calculated as relative light units (RLU) per microgram total protein and expressed as percentage of the promoter activity measured in cells maintained in medium without H_2O_2 (% of control). (**b**) L6 wild-type cells were transiently transfected with a NHE1, a NFκB or an AP-1 reporter gene construct and a plasmid encoding for the *Renilla* protein before being exposed to 25 or 50 μM of H_2O_2. Data were calculated as luciferase RLU/renilla/μg total protein and expressed as percentage of the promoter activity measured in cells maintained in medium without H_2O_2. (**c**) Cell lines from rat, mouse and human origin were exposed to 25 or 50 μM of H_2O_2 before total RNA was extracted and cDNA generated as described in Materials and methods. NHE1 mRNA expression was quantified by Taqman real-time PCR, normalized to endogenous control (human GAPDH for human cell lines) and (18S RNA for mouse and rat cell lines). Relative NHE1 mRNA expression is expressed as percent of untreated control. (**d**) Cell lines from human (IMR90), mouse (NIH3T3) or rat (L6 1.1 kb) origin were exposed to different concentration of H_2O_2 for 24 h and Western blot analysis was performed as described in Materials and methods. NHE1 protein (97 kDa) was detected using a mouse monoclonal antibody. Anti-β-actin (42 kDa) was used as loading control. (**b**, **c**, **d**) results are represented as mean of at least three experiments carried out in duplicate $\pm$ S.D. (**e**) Following 24 h serum deprivation in DMEM/0.5% FBS, L6 1.1 kb cells were exposed to 25 or 50 μM H_2O_2 for 3 h before releasing in medium containing 0.5% FBS or 10% FBS without H_2O_2 for 24, 48 and 72 h. Control cells not exposed to H_2O_2 were similarly released in medium + 10% FBS. Promoter activity of NHE1 was assessed by luciferase assay, calculated as RLU/μg total protein and expressed as percent of the promoter activity assessed in cells that were not exposed to H_2O_2 and left in medium containing 0.5% FBS for the same period of time. (**f**) L6 1.1 kb were exposed to 50 μM H_2O_2 as described in A and cell density was assessed by crystal violet staining as described in Materials and methods and expressed as OD at 595 nm. Results from (**e**, **f**) are from one representative experiment out of a minimum of three carried out in triplicate $\pm$ S.D.


a
NHE1 promoter activity (% of control)
120
100
80
60
40
20
0
0.5%FBS/ H2O2 25 μM
0.5%FBS/ H2O2 50 μM
c
0.5% FBS
0.5%FBS/25 μM H2O2
0.5%FBS/50 μM H2O2
NHE-1 mRNA (% of control)
L6 1.1kb
NIH3T3
IMR90
b
Promoter activity (% of control)
160
140
120
100
80
60
40
20
0
NHE-1
NFκB
AP-1
d
H2O2 (μM)
0
25
50
100
70
L6 1.1kb
NIH3T3
IMR90
NHE1
β-actin
e
NHE1 promoter activity (% of control)
180
160
140
120
100
80
60
40
20
0
24 h
48 h
72 h
25 μM H2O2/ 0.5% FBS
50 μM H2O2/ 0.5% FBS
25 μM H2O2/ 10% FBS
50 μM H2O2/ 10% FBS
0.5% FBS/ 10% FBS
f
Cell density (crystal violet-OD at 595 nm)
0.25
0.20
0.15
0.10
0.05
0.00
H2O2
H2O2 + 24 hours 0.5% FBS
H2O2 + 24 hours 10% FBS
H2O2 + 48 hours 0.5% FBS
H2O2 + 48 hours 10% FBS

grown in medium with 10% FBS; however, the concentration of H_2O_2 to obtain an inhibitory effect similar to that in 0.5% FBS had to be doubled (data not shown). To quell any doubts that the inhibitory effect of H_2O_2 on NHE1 promoter activity could be an artifact due to the stable integration of the reporter gene in L6 1.1 kb cells' genome or that the inhibition of NHE1 promoter activity by H_2O_2 was nonspecific, parental L6 cells were transiently transfected with either a 1.1 kb NHE1, an NF-κB, or an AP-1 reporter gene construct before exposure to 25 or 50 μM H_2O_2 for 24 h. Results showed that the effect of H_2O_2 was not a nonspecific function of transcription shut down, but a selective effect of H_2O_2 on NHE1 promoter activity, whereas NF-κB and AP-1 promoter activities were not affected (Figure 2b). Furthermore, real-time PCR analysis of NHE1 mRNA level and Western blot analysis of NHE1 protein expression, following incubation of the cells with increasing concentration of H_2O_2 for 24 h confirmed that inhibition of NHE1 promoter activity led to inhibition of NHE1 transcription and protein expression in a variety of cell lines (Figure 2c and d).

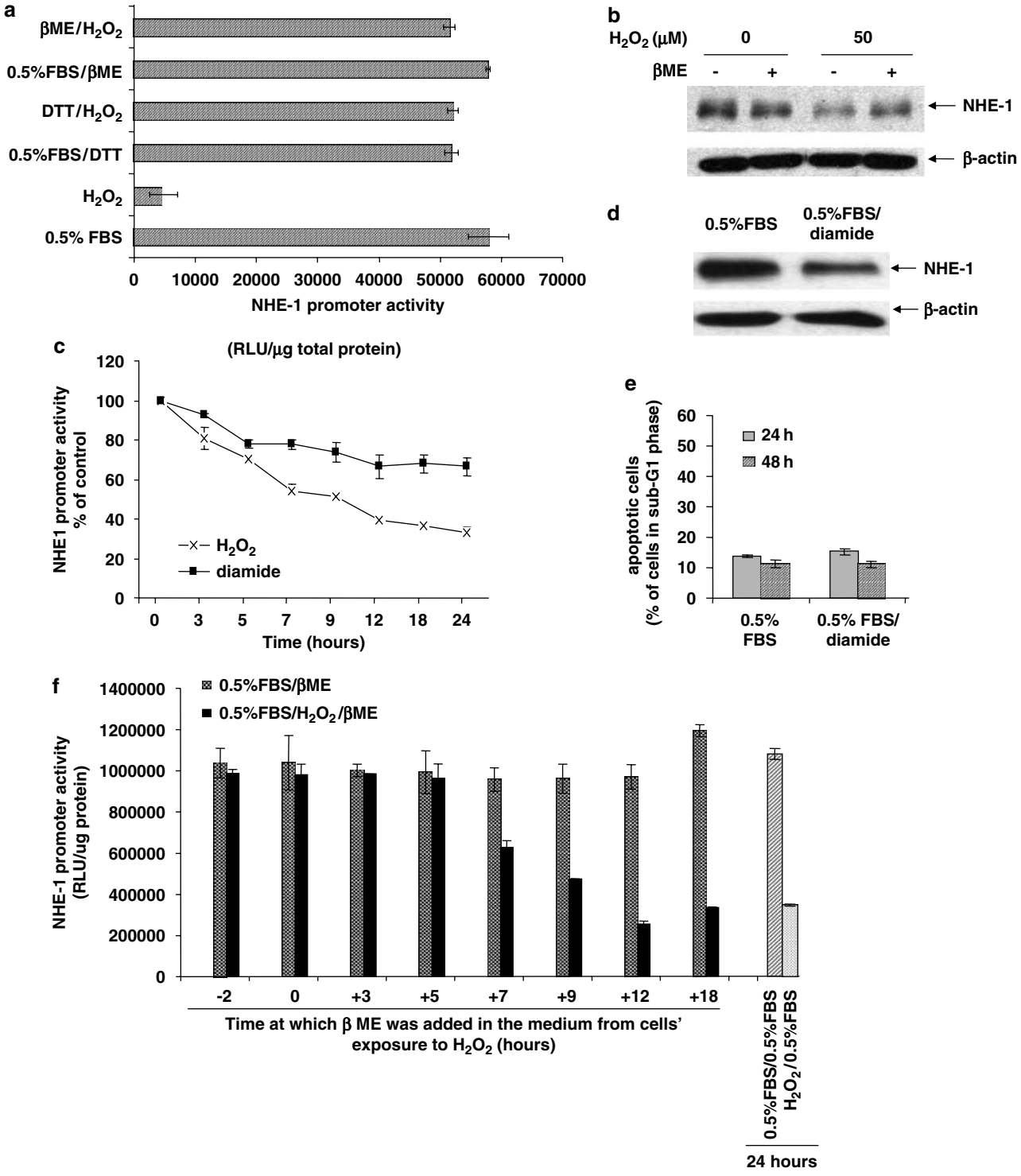
H_2O_2 initiates the signal for NHE1 promoter repression. According to the conventional dogma, exogenously added H_2O_2 is consumed by respiring cells and hence rapidly dissipates from the extracellular medium. Consistent with this notion, exogenously added H_2O_2 to L6 1.1 kb cells in medium containing 0.5% FBS was consumed within 3 h, whereas in the same medium without cells and over the same period of time, there was no substantial decrease in H_2O_2 concentration (data not shown). These data prompted us to question if maximal inhibition of NHE1 promoter activity could be achieved without the need for continuous presence of H_2O_2 (a minimum 3 h exposure). L6 1.1 kb cells were incubated with 50 μM H_2O_2 for a minimum of 5 min to a maximum of 3 h before the culture medium was replaced with the control medium (without H_2O_2) for a total incubation period of 24 h at which time point the activity of the promoter was assessed. Results showed that 3 h exposure to H_2O_2 was enough to achieve the level of inhibition of NHE1 promoter activity similar to that obtained upon continuous exposure to H_2O_2 for 24 h (data not shown). Hence, to minimize the production of toxic metabolites due to the continuous presence of H_2O_2 in the culture medium, in all subsequent experiments, cells were exposed to H_2O_2 for 3 h followed by the release of cells in fresh culture medium for the remaining duration of the experiment. Interestingly, repression of NHE1 promoter activity measured at 24 h was sustained for as long as 72 h without further decrease in NHE1 promoter activity or loss of cell viability in cells maintained in 0.5% FBS. However, release of cells in culture medium containing 10% FBS following the initial exposure to H_2O_2 did not completely prevent the inhibition of the promoter activity, but allowed the promoter activity to recover to the level of cells grown in 10% FBS (without exposure to H_2O_2) within 72 h (Figure 2e and f). In addition, the recovery of NHE1 promoter activity correlated with the capacity of the cells to proliferate again (Figure 2f).

Oxidation is involved in the initiation but not the sustained inhibition of NHE1 promoter activity by H_2O_2. To test if oxidation was part of the signaling pathway involved in the repression of NHE1 promoter activity by H_2O_2, L6 1.1 kb cells were pre-incubated for 2 h with or without 500 μM beta mercaptoethanol (βME) or 500 μM dithiothreitol (DTT) before exposure to 25 μM or 50 μM H_2O_2. βMe and DTT inhibited H_2O_2-mediated repression of NHE1 promoter activity and protein expression (Figure 3a and b). To provide further support to oxidant-induced repression of NHE1 promoter activity, L6 1.1 kb cells were treated with a commonly used thiol oxidant, diamide. Within 24 h of incubation with diamide, NHE1 promoter activity was significantly inhibited with a resultant decrease in NHE1 protein expression by 48 h (Figure 3c and d). The continuous presence of diamide for 48 h did not induce cell death as shown by the absence of an increase in the percentage of cells in the sub-G1 phase (Figure 3e), but was necessary to obtain a decrease in NHE1 protein expression compared to exposure to H_2O_2 for only 3 h (and followed up for a total duration of 24 h in normal feeding medium). These results probably reflect the difference in the efficiency of diamide compared to H_2O_2 to inhibit NHE1 promoter activity. A comparison of the inhibitory effect of diamide and H_2O_2 on NHE1 promoter activity revealed a striking similarity for the early part of the incubation (5 h exposure to diamide *versus* 3 h initial exposure to H_2O_2 followed by 2 h in normal feeding medium); however, a follow-up of the cells for up to 24 h after the initial 3 h exposure to H_2O_2 showed a significantly stronger inhibition of NHE1 promoter activity than treatment for 24 h with diamide (Figure 3c). Hence, we next evaluated if blocking oxidation would prevent the effect of the initial exposure (3 h) to H_2O_2 on NHE1 promoter activity. Interestingly, βME was effective

Figure 3 H_2O_2-induced inhibition of NHE1 promoter activity is rescued by reducing agents. (**a**) Cells were pre-incubated with 500 μM DTT or 500 μM βME in DMEM/0.5% FBS for 2 h before being exposed to 50 μM H_2O_2 for a further 3 h. The medium was then replaced with fresh βME or DTT in DMEM/0.5% FBS for another 24 h before NHE1 promoter activity (RLU/μg total protein) was assessed as described in Materials and methods. (**b**) L6 1.1 kb cells were treated with 50 μM H_2O_2 with or without 2 h pre-incubation with βME before H_2O_2 treatment. Cells were harvested 24 h after H_2O_2 treatment. Fifty microgram of total cell lysates were subjected to Western Blot analysis for NHE1 expression. (**c**) Following 24 h serum deprivation in DMEM/0.5% FBS, medium was replaced with fresh DMEM/0.5% FBS. Cells were then exposed to 50 μM H_2O_2 or 100 μM diamide for various durations before NHE1 promoter activity was assessed by luciferase assay (RLU/μg total protein) as described in Materials and methods. Results are expressed as percent of untreated cells (% of control). (**d**) Expression of NHE1 protein was assessed as described in (**c**). (**e**) L6 1.1 kb cells were treated with 100 μM diamide in 0.5% FBS for 24 or 48 h. Single-cell suspensions of L6 1.1 kb cells were prepared, stained with propidium iodide and analyzed by FACS to quantify the percent of cells with DNA content corresponding to the hypodiploid subG1 phase of the cell cycle. Percent of apoptotic cells was calculated by the percentage of cells in subG1 to the total cells' population. Data represent the average $\pm$ S.D. of two experiments carried out in duplicate. (**f**) Following 24 h serum deprivation in DMEM/0.5% FBS, medium was replaced with fresh DMEM/0.5% FBS. L61.1 kb cells were then treated with 50 μM H_2O_2 and 500 μM βME added to the cells at different time intervals (i.e. -2 represents βME added 2 h before H_2O_2 challenge and so on). In all cases, H_2O_2 was added for 3 h, medium was then replaced and cells were harvested at the end of 24 h post-H_2O_2 challenge. Inhibition of NHE1 promoter activity by H_2O_2 is shown on the extreme right of the graph as a positive control. NHE1 promoter activity is expressed as RLU/μg total protein

in blocking the effect of H_2O_2 only if added 2 h before or within 5 h after (maximum) exposure to H_2O_2 (Figure 3f).

Caspases are involved in the sustained inhibition of NHE1 gene expression by H_2O_2. Next, we evaluated the involvement of the caspase family of cysteine proteases, usually associated with apoptotic cell death signaling. However, of late, the involvement of caspase proteases in physiological settings such as embryonal development, cell proliferation, and post-translational regulation of transcription factors, has been documented.[13] L6 1.1 kb cells were exposed to 50 μM H_2O_2 in the presence or absence of the pan-caspase inhibitor, z-VAD-fmk (100 μM). The presence of z-VAD-fmk strongly blocked the inhibitory effect of H_2O_2 on NHE1 promoter activity and NHE1 protein expression (Figure 4a and b). In addition, the pan-caspase inhibitor prevented



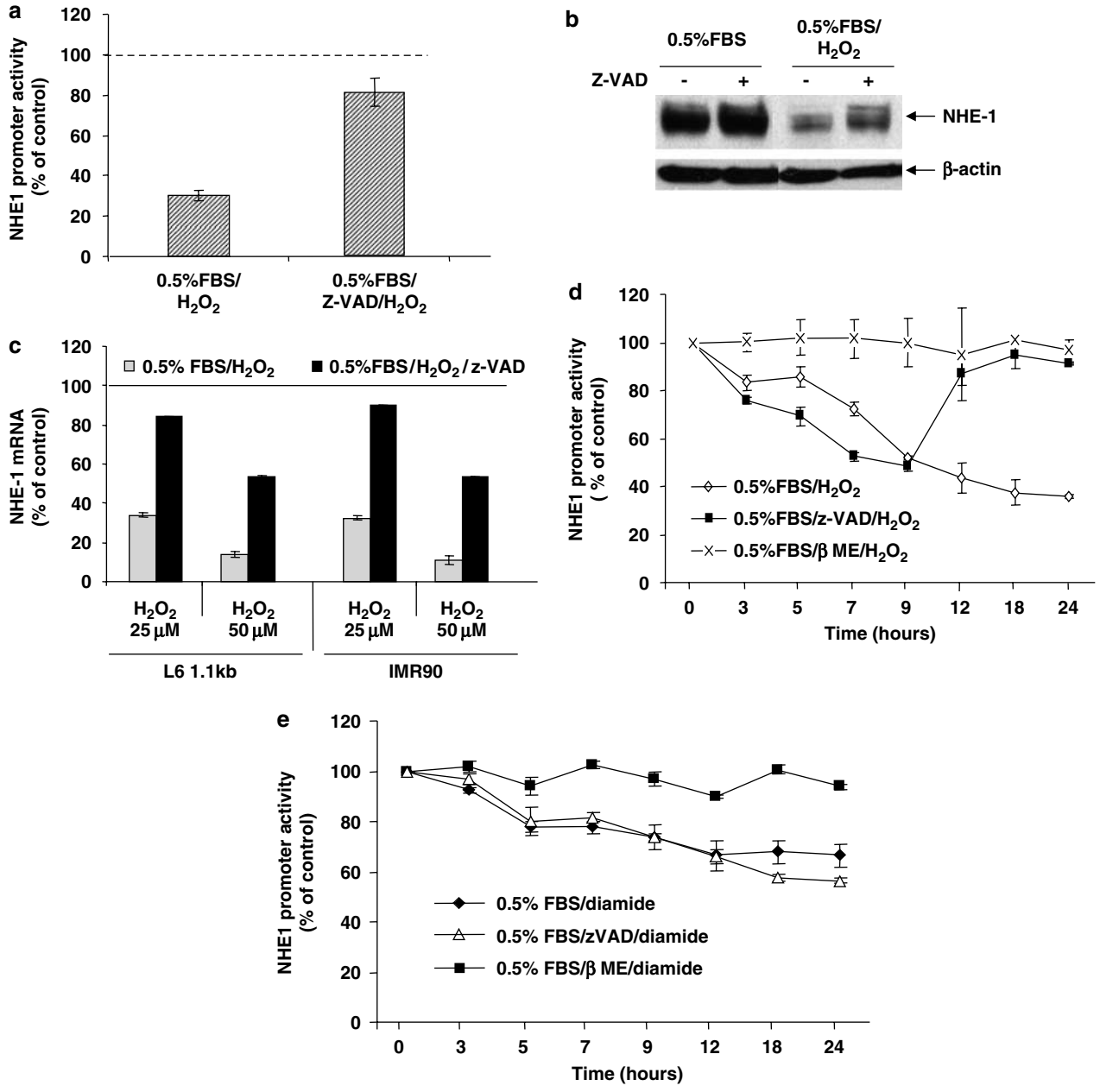


Figure 4 zVAD-fmk prevents H_2O_2-induced inhibition of NHE1 promoter activity. (**a**) Following 24 h serum deprivation in DMEM/0.5% FBS, L6 1.1 kb cells were pre-incubated for 2 h with 100 μM zVAD-fmk in DMEM/0.5% FBS, before exposure to 50 μM H_2O_2 for 3 h. Cells were harvested 24 h following treatment with H_2O_2 for luciferase assay. NHE1 promoter activity was calculated as RLU/μg total protein and expressed as percent of untreated control cells. (**b**) Expression of NHE1 was assessed by Western blotting described in Materials and methods. A representative blot from two independent experiments is shown. (**c**) NHE1 mRNA levels were quantitated by Taqman real-time PCR, normalized to endogenous control (18S RNA for L6 1.1 kb and human GAPDH for IMR90 cell lines). Relative NHE1 mRNA expression is shown as percent of non-H_2O_2-treated controls with or without zVAD-fmk. (**d**) Cells were pre-incubated for 2 h in the absence or presence of 100 μM zVAD-fmk or 500 μM βME in DMEM/0.5% FBS and exposed to 50 μM H_2O_2 for 3 h. Cells were then released in DMEM/0.5% without H_2O_2, but containing either zVAD-fmk or βME. NHE1 promoter activity was calculated at the indicated times as RLU/μg total protein and expressed as percent of non-H_2O_2-treated controls. (**e**) Following 24 h serum deprivation in DMEM/0.5% FBS, cells were pre-incubated as in (d), and exposed to 100 μM diamide. NHE1 promoter activity was assessed by luciferase assay at the indicated times as RLU/μg total protein and expressed as percent of respective non-diamide treated controls. Results shown are average out of three experiments carried out in triplicate $\pm$ S.D.

H_2O_2-induced inhibition of NHE1 gene transcription assessed by real-time PCR not only in L6 1.1 kb cells, but also in the human cell line IMR90 (Figure 4c).

To further decipher the effect(s) of oxidation and caspase activation in the regulation of NHE1 gene expression, a time kinetic analysis of H_2O_2-mediated repression of NHE1 promoter activity in the presence or absence of βME or z-VAD-fmk was performed. NHE1 promoter activity decreased in a time-dependent manner (up to 24 h) following initial 3 h exposure of L6 1.1 kb cells to H_2O_2; however,

pre-incubation of cells for 2 h with βME completely abolished the inhibitory effect of H_2O_2 on NHE1 promoter activity (Figure 4d). On the contrary, the caspase inhibitor, z-VAD-fmk, did not affect the initial decrease in NHE1 promoter activity for up to 9 h from exposure to H_2O_2; however, z-VAD-fmk rescued NHE1 promoter activity to its basal level, 18 to 24 h after exposure of cells to H_2O_2 (Figure 4d). The necessity for a mechanism dependent on caspase activation, but independent of oxidation, to maintain H_2O_2-induced inhibition of NHE1 promoter activity is further supported by data that z-VAD had no effect on diamide-mediated inhibition of NHE1 promoter activity, whereas the presence of βME completely prevented it (Figure 4e).

Caspases 3 and 6 are involved in H_2O_2-induced inhibition of NHE1 promoter activity. Next, we set out to identify the caspase(s) involved in this pathway. Therefore, time kinetic analyses of H_2O_2-mediated repression of NHE1 promoter activity were performed in the presence or absence of the tetrapeptide inhibitor of caspase-8 (z-IETD-fmk), caspase-9 (z-LEHD-fmk), caspase 6 (z-VEID-fmk), or caspase-3 (z-DEVD-fmk). Whereas the inhibitor of caspase 8 or 9 had no effect on H_2O_2-induced downregulation of NHE1 promoter activity, inhibition of caspase 3 or 6 rescued the promoter activity to its basal level from 9 h onward following the initial exposure to H_2O_2 (Figure 5a). Moreover, similar to the results obtained with the tetrapeptide inhibitors, gene silencing of caspase 3 or 6 allowed NHE1 promoter activity to recover from the initial inhibition induced by H_2O_2 (Figure 5b). In addition, the enzymatic activities of caspases 3 and 6 were induced in cells incubated with H_2O_2; neither caspase 8 nor caspase 9 were significantly active (Figure 5c).

Caspase-dependent inhibition of NHE1 promoter activity is only partially responsible for the decrease in NHE1 protein expression. During apoptotic cell death, activation of caspase 3 has been shown to cleave a wide spectrum of protein substrates, including NHE1.[14] Hence, despite the convincing evidence implicating H_2O_2-mediated caspase 3 and 6 activation in the inhibition of NHE1 promoter activity, we questioned if the downregulation of NHE1 protein detected upon exposure to H_2O_2 was only a result of the inhibition of NHE1 transcription. L6 1.1 kb cells were incubated with either 50 μM H_2O_2 or 1 μg/ml actinomycin D (ACT.D) in the presence or absence of z-VAD and the levels of NHE1 mRNA and NHE1 protein were assessed after 24 h of treatment. Addition of z-VAD with ACT.D appeared to be necessary as ACT.D alone was able to activate caspase 3 similar to that obtained with H_2O_2 (Figure 6a). Hence, the decrease in NHE1 protein in the presence of ACT.D alone could not only be due to a decrease in transcription, but also by way of direct cleavage of NHE1 by caspase 3. In the presence of z-VAD, cells exposed to ACT.D for 24 h underwent a 67% decrease in NHE1 mRNA compared to control cells (Figure 6b). This decrease in NHE1 mRNA corresponded to a 23% reduction in protein expression ($n=4$) (Figure 6c). The decrease in NHE1 transcription upon exposure to 50 μM H_2O_2 was comparable to that obtained with zVAD + ACT.D and the inhibitory effect on protein expression was only slightly more pronounced than the combined treatment with zVAD and ACT.D (Figure 6c and d). These data indicate that the major effect of H_2O_2 on NHE1 protein expression could be accounted for by the inhibitory effect on NHE1 gene transcription (mRNA); however, the possible involvement of post-transcriptional events such as caspase 3-mediated cleavage of the protein cannot be completely ruled out at this point (Figure 6e).

H_2O_2-mediated activation of caspases 3 and 6 involves the intermediacy of iron. Under conditions that mimic *in vivo* situation, the oxidative event that triggers apoptosis and the consequent activation of caspases by H_2O_2 has been shown to involve Fenton-type chemistry.[15] The two main consequences of a Fenton reaction are production of hydroxyl radical ($OH^{\cdot}$) and redox cycling of iron. Hence, to assess if $OH^{\cdot}$ and/or iron were involved in the caspase-dependent inhibition of NHE1 promoter activity by H_2O_2, time kinetic analyses of H_2O_2-mediated repression of NHE1 promoter activity were performed in the presence of either the $OH^{\cdot}$ scavenger, sodium formate (HCOONa), or the iron chelator, DFO. Whereas, the HCOONa had no significant effect on H_2O_2-induced repression of NHE1 promoter activity, the effect of iron chelation mirror-imaged the effect of caspase inhibition on NHE1 promoter activity and in response to H_2O_2 (Figure 7a). This was further corroborated by Western blot analysis of NHE1 protein expression; DFO blocked the downregulation of NHE1 by H_2O_2, whereas HCOONa had no effect (Figure 7b and c). In addition, prior incubation of L6 1.1 kb cells with DFO inhibited H_2O_2-induced activities of caspases 3 and 6 (Figure 7d and e).

To provide further impetus to these findings linking iron to H_2O_2-mediated activation of caspases 3 and 6, we assessed the effect of direct exposure of L6 1.1 kb cells to $FeCl_3$. The effect of $FeCl_3$ on NHE1 promoter activity and protein expression was similar to that of H_2O_2 (Figure 8a and b). Furthermore, the repression of NHE1 promoter by $FeCl_3$ was unaffected by the presence of the $OH^{\cdot}$ scavenger HCOONa (Figure 8c), but could be rescued by the tetrapeptide inhibitors of caspases 3 and 6 or by small interfering RNA (siRNA) gene silencing of these two caspases (Figure 8d and e). Note that some increase in the percentage of cells in sub-G1 was detected in the presence of $FeCl_3$ compared to cells left in the control medium (Figure 8f). However, this is not surprising as iron is critical in many aspect of cell survival.

A region of the NHE1 promoter containing an AP-2-binding site is necessary to induce the inhibition of the NHE1 promoter by H_2O_2. Finally, to further explore the molecular basis for H_2O_2-mediated inhibition of NHE1 promoter activity, wild-type L6 cells were transiently transfected with various 5′ deletion constructs of the 1.1 kb promoter before being treated with H_2O_2. The region responsive to the inhibition by H_2O_2 contains a 'GGCN$_4$GGG' sequence shown to be the sequence for the binding site of an AP-2 or AP-2-like transcription factor in the NHE1 promoter[16] (Figure 9a). Indeed, mutation or removal of that region in the promoter abolished the inhibitory effect of H_2O_2 (Figure 9a). Interestingly, when similar experiment was carried out with deletion mutants of the human NHE1

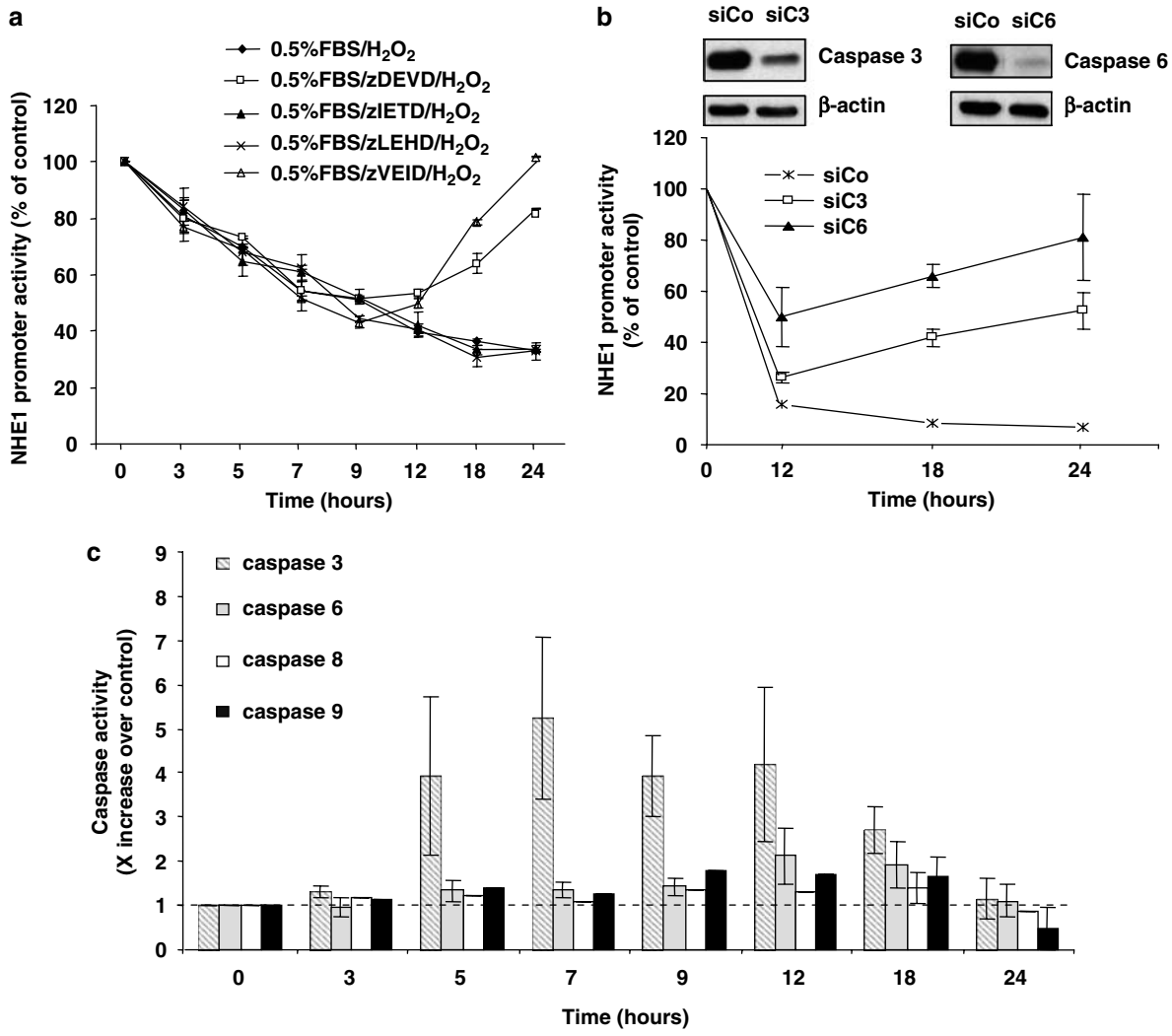


Figure 5 H_2O_2-induced inhibition of NHE1 promoter activity involves activation of caspases 3 and 6. (**a**) Following 24 h serum deprivation in DMEM/0.5% FBS, L6 1.1 kb cells were pre-incubated in fresh DMEM/0.5% FBS with 30 μM caspase 3 (zDEVD), caspase 8 (zIETD), caspase 9 (zLEHD) or caspase 6 (zVEID) inhibitors before exposure to 50 μM H_2O_2. NHE1 promoter activity was calculated as RLU/μg total protein and expressed as percent of untreated control cells. (**b**) L6 1.1 kb cells were transfected with either a control siRNA (siCo) or specific siRNA to caspase 3 (siC3) or caspase 6 (siC6). Fourty hours post-transfection, cells were exposed to 25 μM H_2O_2 for 3 h before being harvested for luciferase assay to measure NHE1 promoter activity at 12, 18 and 24 h post-H_2O_2 treatment. NHE1 promoter activity was calculated as in (a). Total cell lysates were subjected to Western blot analysis for detecting caspases 3 and 6 as in Materials and methods. (**c**) Total cell extracts from L6 1.1 kb cells following 50 μM H_2O_2 treatment at varying time intervals were collected and assayed for the activities of caspases 8, 9, 6 and 3 as described in Materials and methods. Caspase activity was normalized to protein concentration and expressed as fold increase over untreated cells ($\times$ increase over control). Data shown are mean of three experiments carried out in duplicate $\pm$ S.D.

promoter, a region containing the same AP-2-binding sequence was also found to be responsible for the inhibition of the promoter activity by H_2O_2 (Figure 9b).

Discussion

In the present study, we provide evidence that H_2O_2 is a signaling molecule that induces repression of NHE1 gene transcription leading to a decrease in NHE1 protein expression. The data support a bi-phasic signaling response to a short exposure to H_2O_2 involving an oxidation-dependent rapid initial phase followed by an iron-mediated caspase-dependent sustained inhibition of NHE1 promoter activity and gene expression.

Thiol oxidation of an NHE1-specific transcription factor is responsible for the initial inhibition of NHE1 gene expression upon exposure to H_2O_2. There are several signaling mechanisms that could account for the effect of the initial but reversible H_2O_2-mediated repression of NHE1 promoter activity. In addition to the oxidation of redox-sensitive residues on the transcription factor(s) involved in NHE1 gene expression, oxidative conditions can also activate kinase/phosphatase cascade and modify the phosphorylation status or target amino acids of NHE1-specific transcription factors. However, in the present study, the observations that reducing agents such as βMe or DTT prevented H_2O_2-mediated initial inhibition of NHE1 promoter activity, and that the thiol oxidizing agent diamide

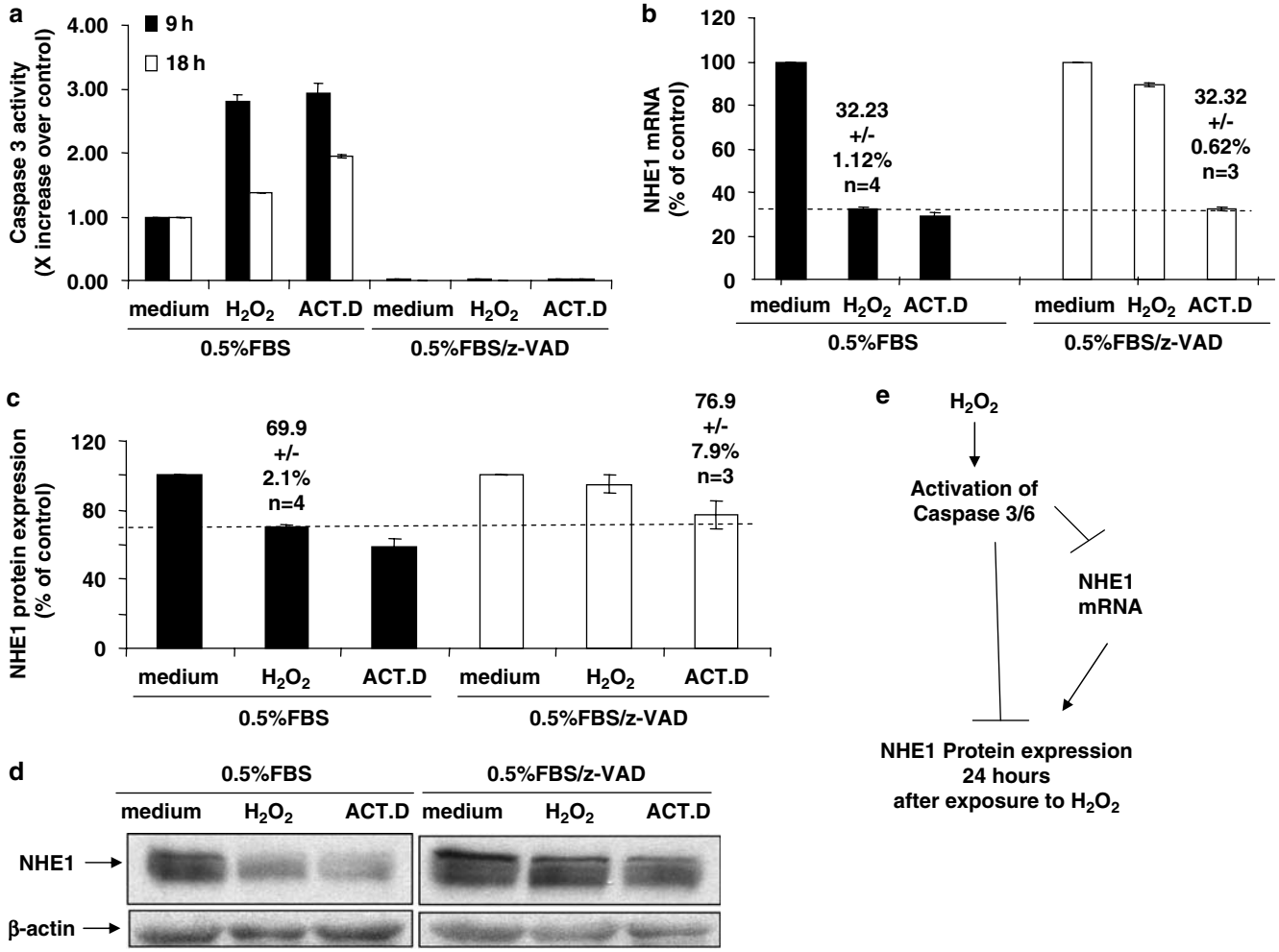


Figure 6 Downregulation of NHE1 protein with H_2O_2 is largely transcriptional. (**a**) Total cell extracts from L6 1.1 kb cells following either 50 μM H_2O_2 or 1 μg/ml ACT.D treatment in the absence and presence of 100 μM z-VAD at 9 and 18 h time intervals were collected and assayed for the caspase-3 activity as described in Materials and methods. Caspase activity was normalized to protein concentration and expressed as fold increase over untreated cells ($\times$ increase over control). Data shown are the mean of three experiments carried out in duplicate $\pm$ S.D. (**b**) In the same experimental set-up as (**a**), total RNA was collected. NHE1 mRNA expression was quantified by Taqman real-time PCR, normalized to endogenous control (18S RNA). Relative NHE1 mRNA expression is expressed as percent of untreated control. (**c**) NHE1 protein expression from the same experimental set-up as (**a**, **b**) performed by Western blotting and quantitated by densometric analysis. NHE1 protein expression is shown as percent decrease from untreated controls (% of control). Data shown are the mean of four independent experiments $\pm$ S.D. (**d**) A representative blot for NHE1 expression in (**c**) is shown. (**e**) Summary diagram showing NHE1 transcriptional and post-transcriptional contribution to the total decrease in NHE1 protein, when L6 1.1 kb cells were treated with 50 μM H_2O_2

mimicked the effect of H_2O_2 strongly suggest that the likely mechanism for the initial inhibition of the promoter activity involves the oxidation of thiol moiety of cysteine that may be present in the transcription factor(s) necessary for NHE1 expression. Interestingly, Dyck *et al.*[17] have shown a key role for a transcription factor from the AP-2 family in the regulation of NHE1 gene transcription. The AP-2 transcription factor family is a set of developmentally regulated, retinoic acid inducible genes composed of: AP-2α, AP-2β, AP-2γ, AP-2δ, and AP-2ε.[18] Oxidation of recombinant AP-2α protein with H_2O_2 has been shown to inhibit its DNA-binding activity to synthetic AP-2-oligodeoxynucleotides. Furthermore, the diminished DNA-binding activity of AP-2, following diamide or H_2O_2 pre-treatment, could be restored by the addition of DTT or β-ME.[19] Analysis of the promoter region involved in the downregulation of NHE1 promoter activity by H_2O_2, revealed that in the mouse as well as in the human promoter the presence of a region with an AP-2-binding site was necessary for the inhibition. These data suggest that thiol oxidation of an AP-2 or an AP-2-like transcription factor could be responsible for the initial inhibition of NHE1 gene expression upon incubation with H_2O_2.

Activation of caspases 3 and 6 are responsible for the oxidative repression of NHE1 expression. Caspase activation, in particular caspase 3, is mainly linked to the execution of cell death via cleavage of a wide variety of substrates involved in the maintenance of cell survival.[20] Similarly, caspase-dependent cleavage of transcription factors is observed in cells undergoing apoptosis, and caspase-mediated inhibition of gene transcription is linked

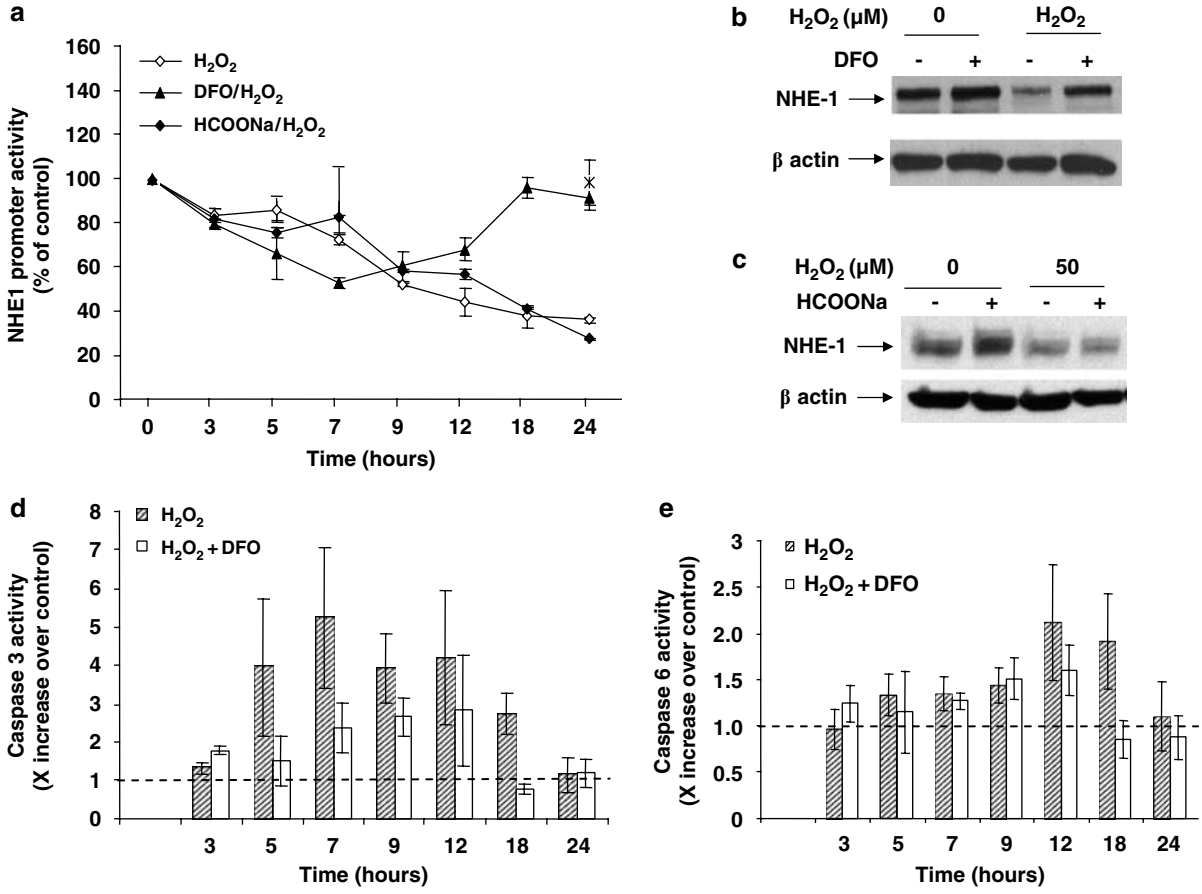


Figure 7 Intermediacy of Iron in caspases 3 and 6 mediated inhibition of NHE1 promoter activity by H_2O_2. (**a**) Following 24 h serum deprivation in DMEM/0.5% FBS, cells were pre-treated for 2 h in fresh DMEM/0.5% FBS with or without 1 mM DFO or 20 mM HCOONa, before the addition of 50 μM H_2O_2 for 3 h. Cells were released in DMEM/0.5% FBS containing either DFO or HCOONa, but without H_2O_2. NHE1 promoter activity was assessed by luciferase assay (RLU/μg total protein) and expressed as percent of non-H_2O_2-treated controls at the indicated time points with or without DFO or HCOONa. Expression of NHE1 was assessed with 50 μM H_2O_2 in the absence or presence of 1 mM DFO (**b**), or 20 mM HCOONa (**c**) by Western blotting described in Materials and methods. A representative blot from two independent experiments is shown. Total cell extracts from L6 1.1 kb cells following 50 μM H_2O_2 treatment in the absence and presence of 1 mM DFO were assayed for the caspase 3 (**d**) and caspase 6 (**e**) activities as described in Materials and methods. Caspase activities were normalized to protein concentration and expressed as fold increase over untreated cells ($\times$ increase over control). Results from (**a**–**e**) are mean of at least three experiments carried out in duplicate $\pm$ S.D.

to the repression of survival proteins. Direct targeting of transcription factors has also been shown as a mechanism of caspase-dependent gene silencing.[21,22] Here, we show a new role for active caspases 3 and 6 in the absence of cell death, that is, the sustained repression of protein expression upon mild oxidative stress. In the absence of caspase activation, a mild oxidative stress only induces a rapid but reversible inhibition of NHE1 promoter activity that is not sufficient to influence NHE1 protein levels. However, H_2O_2-mediated activation of caspases 3 and 6 appears to maintain the repression initiated by the mild oxidative stress. These results provide evidence that aside from their role in the execution of apoptotic cell death, activation of caspases 3 and 6 could be an alternative mechanism involved in the oxidative repression of gene transcription. Hence, it is plausible that in addition to being sensitive to oxidation, NHE1-specific transcription factors are substrates of caspase 3 and/or 6. To that end, members of the AP-2 family proteins, involved in basal expression of NHE1, have been shown to be substrates for caspases 3 and 6.[23]

The physiological relevance of such a sustained oxidative repression of NHE1 could be that during chronic mild oxidative stress, NHE1 transcription factor may be subject to oxidation as well as depletion due to sustained activation of caspases 3 and 6. This could lead to a long-term decrease in NHE1 expression without the need for constant production of ROS. Chronic decrease in NHE1 levels could impair cell growth through accumulation in the G2/M phase of the cell cycle[9] or induce acidification of the intracellular milieu.[8] To that regard, exposure of L6 1.1 kb cells to nontoxic concentrations of H_2O_2 induced cell growth arrest; however, the growth arrest was reversible along with the reactivation of NHE1 promoter upon incubation of the cells in medium containing 10% FBS following incubation with H_2O_2.

Iron-dependent activation of caspases 3 and 6 is responsible for the sustained repression of NHE1 expression by H_2O_2. Presence of the reducing agents βME or DTT, at the time of cells' exposure to H_2O_2, not only prevented the initial inhibition of NHE1 promoter activity, but

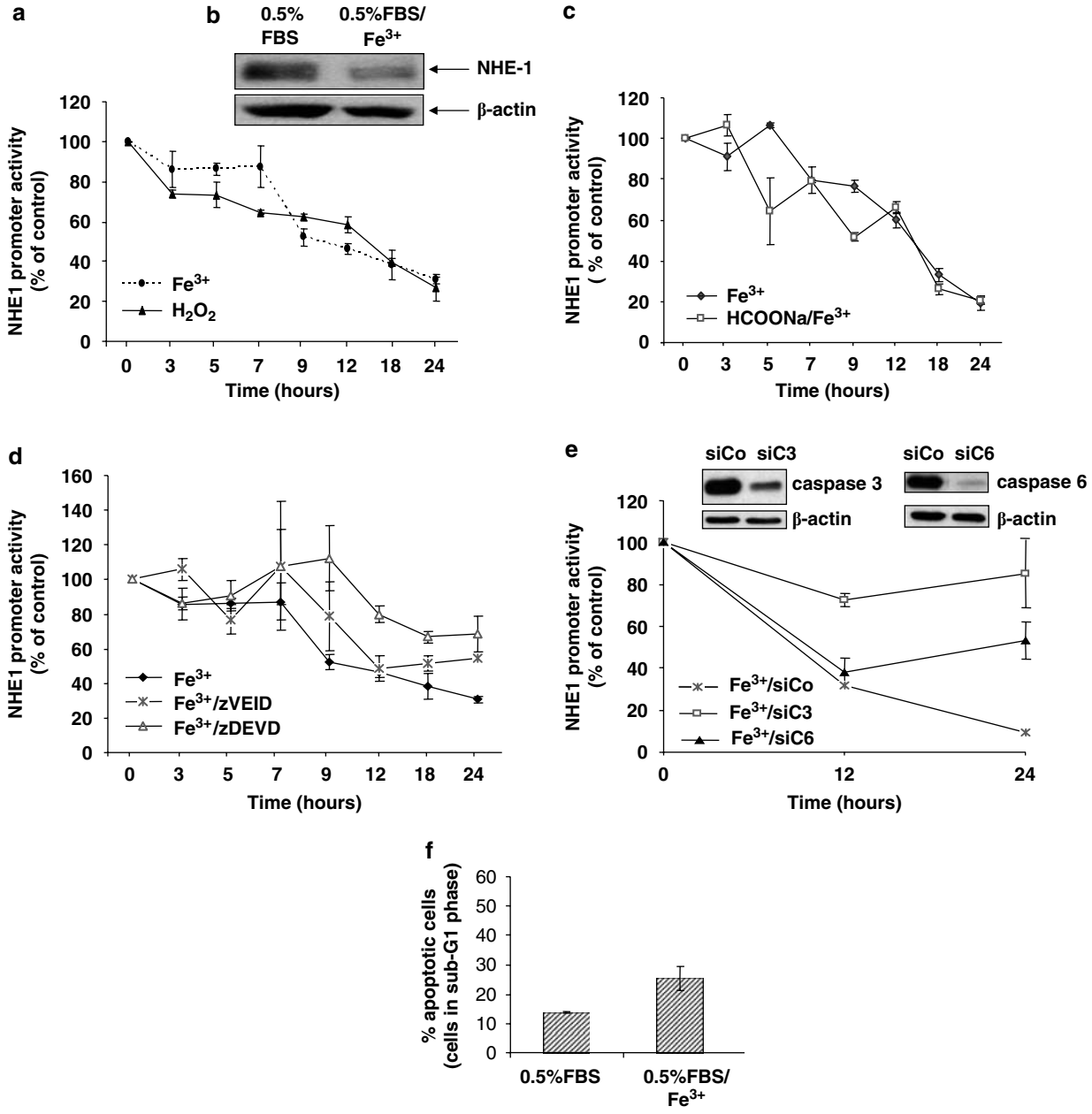


Figure 8 Activities of caspases 3 and 6 are required for $FeCl_3$-induced NHE1 promoter inhibition. (**a**) Following 24 h serum deprivation in DMEM/0.5% FBS, cells were exposed to 500 μM $FeCl_3$ or 50 μM H_2O_2 in fresh DMEM/0.5% FBS. Luciferase activity was measured at the indicated time points and NHE1 promoter activity was calculated as RLU/μg total protein and expressed as percent of untreated cells. (**b**) Expression of NHE1 following 500 μM $FeCl_3$ was assessed by Western blotting as described in Materials and methods. (**c**) Following 24 h serum deprivation in DMEM/0.5% FBS, cells were exposed to 500 μM $FeCl_3$ with and without 2 h HCOONa pre-incubation in fresh DMEM/0.5% FBS. Luciferase activity was measured at the indicated time points and NHE1 promoter activity was calculated as RLU/μg total protein and expressed as percent of untreated cells. (**d**) Following 24 h serum deprivation in DMEM/0.5% FBS, cells were pre-incubated for 2 h in fresh DMEM/0.5% FBS with 30 μM zDEVD or zVEID, and then treated with 500 μM $FeCl_3$. NHE1 promoter activity was determined by luciferase assay and calculated as RLU/μg total protein, expressed as percent of non-$FeCl_3$-treated cells. (**e**) L6 1.1 kb cells were transfected with either a control siRNA (siCo) or specific caspase 3 siRNA (siC3) or (siC6). Fourty hours post-transfection, cells were exposed to 500 μM $FeCl_3$, and harvested for luciferase assay to measure NHE1 promoter activity at 12, 18 and 24 h. Expression of caspases 3 and 6 was determined by Western blotting as described in Materials and methods. (**f**) L6 1.1 kb cells were treated with 500 μM $FeCl_3$ in 0.5% FBS for 24 h. Single-cell suspensions of L6 1.1 kb cells were prepared, stained with propidium iodide and analyzed by FACS to quantify the percent of cells with DNA content corresponding to the hypodiploid subG1 phase of the cell cycle. Percent of apoptotic cells was calculated by the percentage of cells in subG1 to the total cells' population. Data from (**a–f**) represent the average $\pm$ S.D. of two experiments carried out in duplicate

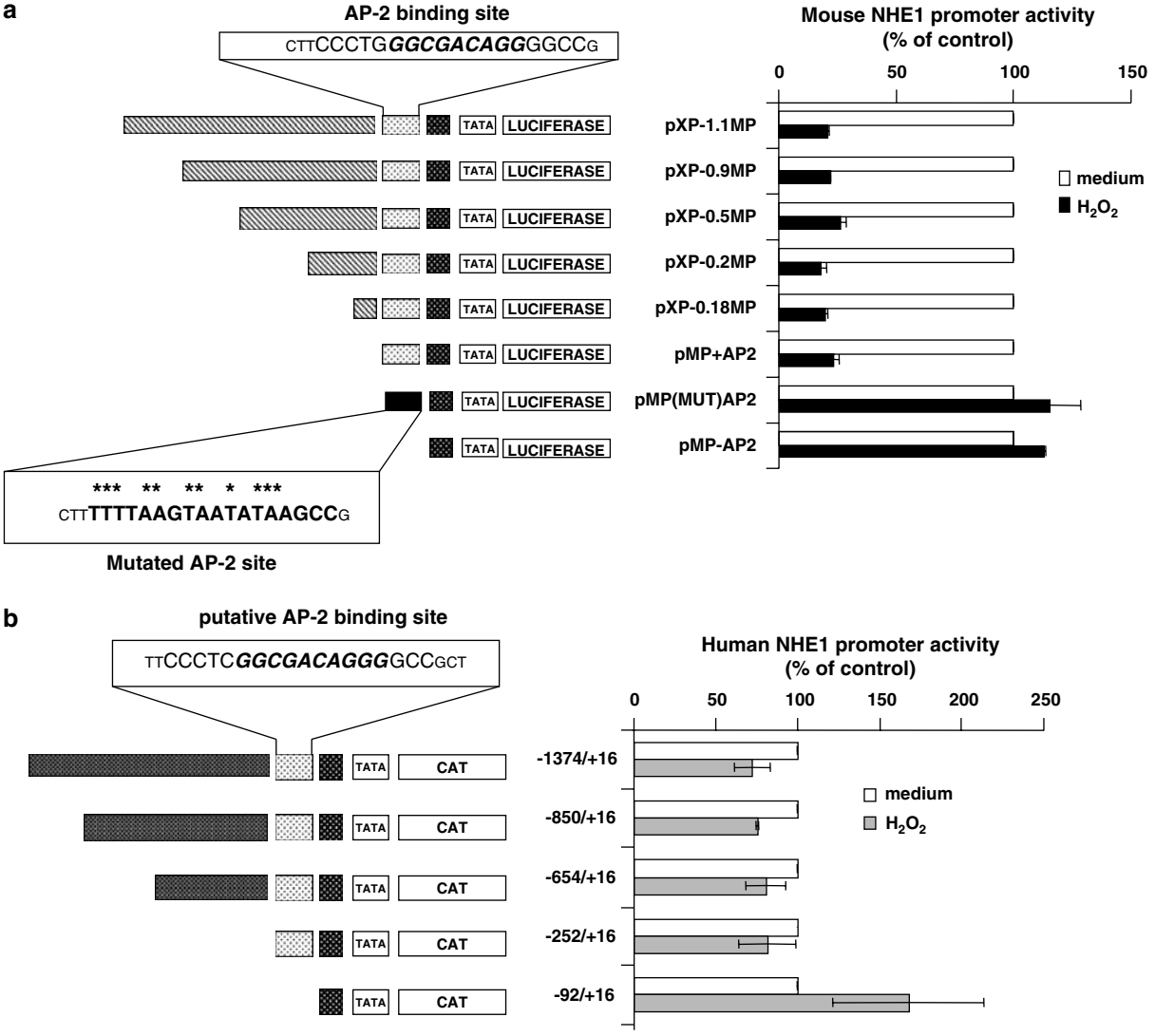


Figure 9 Localization of the H_2O_2 Response element. (**a**) Schematic of the series of 5′ deletion derivatives, and AP2 mutated construct of the mouse NHE1 promoter. The various constructs were transiently transfected into wild-type L6 cells. Cells were also cotransfected with *Renilla* plasmid to normalize for transfection efficiency. Promoter activity was then assessed by luciferase activity in the absence and presence of 50 μM H_2O_2 at 24 h. Promoter activity was calculated as the percentage of control cells (% of control). The top boxed captitalized sequence indicates the region containing the AP2-binding site within the mouse NHE1 promoter, with the AP2 consensus sequence in italic bold. The bottom boxed bold sequence indicates the mutations made to the AP2 binding site. Asterisks indicate mutated sequences. Data represent the average $\pm$S.D. of three experiments carried out in duplicate. (**b**) Schematic representation of the human NHE1 promoter –CAT 5′ deletion mutants. The various constructs were transiently transfected into wild-type L6 cells. Cells were then incubated in the absence and presence of 50 μM H_2O_2 and harvested at 24 h. Promoter activity assessed by CAT ELISA according to manufacturer's instruction and expressed as percent from non-H_2O_2-treated cells. Boxed capitalized sequence indicates the region containing the putative AP2-binding site within the human NHE1 promoter by sequence analysis, with the AP2 consensus sequence italicized and bold. Data represent the average $\pm$S.D. of two experiments done in duplicate

also the caspase-mediated inhibition of the promoter activity. These data indicate that the oxidative signal involved in the rapid inhibition of NHE1 promoter activity is also operative in the induction of caspases 3 and 6 activities following exposure to H_2O_2. These data are intriguing given that the concentrations of H_2O_2 used throughout this study did not affect cell viability, yet triggered a caspase-dependent repression of NHE1 promoter activity. Furthermore, in the context of apoptotic signaling the activation of caspases 3 and/or 6 is/are usually downstream of initiator caspases such as caspases 8 or 9, whereas in the present study, physiologically relevant concentrations of H_2O_2 failed to induce caspases 8 and/or 9 activities and inhibitors of these caspases did not rescue caspase-dependent inhibition of NHE1 promoter activity by H_2O_2. Hence, activation of caspases 3 and 6 in our model occurs independent of initiator caspases 8 and/or 9.

The activation of caspases 3 and 6 appears to be dependent on the presence of iron. Chelating iron with DFO prevented activation of caspases 3 and 6, but did not prevent the initial decrease in NHE1 promoter activity following exposure of cells to H_2O_2. However, similar to the effect of

caspase 3 or 6 inhibition, the presence of DFO allowed NHE1 promoter activity to recover to its initial level within 24 h of exposure to H_2O_2. These data demonstrate that the initial uptake of H_2O_2 into the cells initiates both a direct, albeit reversible, oxidation-dependent signal, and a response mediated by iron-dependent activation of caspases 3 and 6. To our knowledge the mechanism of caspase activation by iron is not clearly understood. The closest report on the matter are data reported by Antunes and Cadenas,[15] demonstrating that physiologically relevant levels of H_2O_2 activate apoptotic signaling via a Fenton-type reaction involving iron and the generation of $OH^{\cdot}$.[24] Unlike DFO, scavenging $OH^{\cdot}$ had no effect on H_2O_2-induced repression of NHE1 promoter activity and gene expression. Moreover, the effect of direct addition of $FeCl_3$ on NHE1 promoter activity and protein expression further provides support on the role of iron in the caspase-dependent inhibition of NHE1 promoter activity. This iron-mediated inhibition of NHE1 promoter activity was blocked upon gene silencing of caspases 3 or 6 by siRNA, but not by the $OH^{\cdot}$ scavenger HCOONa. The exact mechanism involved in the activation of caspases 3 and 6 by iron is not well understood at the moment.

In conclusion, this is the first report highlighting the role of caspases 3 and 6 in the oxidative repression of gene expression in the absence of initiator caspase activation and cell death. This pathway leading to the repression of NHE1 protein expression by mild oxidative stress may be critical in understanding the long-term effects of oxidative stress on the cells' capacity to proliferate, senesce, age, or undergo transformation. Our data support that the decrease in NHE1 expression by activation of caspase 3 and 6 may be critical in preventing cells growth following a mild oxidative stress, even in the absence of cell death. This results places activation of caspase 3 and 6 by iron as a critical sensor that may decide if cells can grow following mild oxidative stress. Overcoming that sensor may be what is needed to lead to tumorigenesis upon chronic oxidative stress.

Materials and Methods

Reagents and antibodies. Most of the reagents used were purchased from Sigma-Aldrich, cell culture reagent were purchased from Hyclone, caspase inhibitors from R&D Systems and antibodies from Chemicon, Cell Signaling, Sigma, DakoCytomation and Pierce. For detail refer to Supplementary data.

Cell culture. Rat muscle cell line, L6 stably transfected with full-length 1.1 kb of the mouse NHE1 promoter, inserted 5′ to the luciferase reporter gene (designated L6 1.1 kb cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 0.25 mg/ml geneticin (GIBCO), and 1 mM gentamicin sulfate (BioWhittaker). NIH3T3 cells were maintained in RPMI with 10% FBS and normal human fibroblasts (IMR90) in MEM supplemented with vitamins, essential and nonessential amino acids.

Caspase activity assay. Caspase activity was assessed as described previously.[8] For detail see Supplementary data.

Crystal violet assay. Crystal violet assay was performed as described previously.[8] For detail refer to Supplementary data.

DNA fragmentation assay. DNA fragmentation was performed as described previously.[25] For detail refer to Supplementary data.

Western blot analysis. Western blot analysis was performed as described previously.[8] For detail refer to Supplementary data.

Luciferase reporter assay. NHE1 promoter activity was assessed with a single-luciferase assay kit (Promega). Bioluminescence generated was measured using a Sirius luminometer (Berthold). The luminescence readings obtained were normalized to the protein content of the corresponding cell lysate. For detail refer to Supplementary data.

CAT ELISA. The levels of CAT protein were quantified using a CAT antigen capture enzyme-linked immunosorbent assay (ELISA) (Roche Molecular Biochemicals). All CAT quantitations were normalized to the protein concentration of the cell extract, as determined using the Coomasie Plus Protein Assay Reagent Kit (Pierce).

Reporter plasmid constructs. Luciferase reporter plasmid constructs: pXP-1.1MP, pXP-0.9MP, pXP-0.5MP, pXP-0.2MP, pXP-0.18MP, pMP + AP2, pMP−AP2, pMP(MUT)AP2, and empty vector pXP1 were kindly provided by Dr. Larry Fliegel, Department of Biochemistry, University of Alberta, Canada.[12] pUCSS-CAT reporter plasmid constructs: −1374/ + 16, −850/ + 16, −654/ + 16, −252/ + 16, −92/ + 16, and empty vector pUCSS-CAT were kindly provided by Dr. Alexey Kolyada, Department of Medicine, Tufts University School of Medicine, Boston, USA.[26]

DNA transfection. Cells were transfected using CalPhost Mammalian transfection kit (Clonetech). Cotransfection with the *Renilla* plasmid (Clonetech) was used to assess transfection efficiency in dual-luciferase reporter assay (Promega).

RNA interference for silencing caspases 3 and 6. SiRNA inhibition of endogenous caspase 3 and 6 was achieved using custom designed siRNA (Ambion). A control siRNA (nonhomologous to any known gene sequence) (Qiagen) was used as a negative control. Cells were transfected with siRNA using the CalPhos Mammalian Transfection kit.

RNA isolation and NHE1 mRNA determination by real-time PCR. Total RNA was isolated from cells by TRIZOL reagent (Invitrogen) as described by manufacturer's instructions with a DNAse treatment step incorporated into the protocol. Each RT reaction contains 2.5 μg of total RNA, 1 × RT buffer, 5 mM $MgCl_2$, 425 μM each of dNTPs, 2 μM random hexamers, 0.35 U/μl RNase inhibitor, 1.1 U/μl MultiScribe™ reverse transcriptase and made up to 10 μl with sterile water. RT reaction was carried out at 37°C for 1 h. Five microliters of the 10 μl cDNA reaction volume was used in real-time quantitative PCR using ABI PRISM 7500 (Applied Biosystems). Normalization was to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for human RNA and 18S RNA for mouse and rat RNA. Fluorescence was measured with the Sequence Detection Systems 2.0 software. PCR was performed in multiplex (both target and endogenous control co-amplified in the same reaction) with distinct fluorescent dyes. The sequences for primers (300 nM) and probe (200 nM) for mouse NHE1 used in this study are as follows: mouse NHE1, forward (5′-TGC CTC ATG AAG ATA GGT TTC CA-3′), reverse (5′-AGC AGC CCC ACT ACG ATC AG-3′), and probe (5′-FAM-CAC CAT CTC AAG CAT CGT CCC GGA-TAMRA-3′). Primers and probe for human GAPDH, rat NHE1, human NHE1, and 18S RNA were purchased as kits from Applied Biosystems (Assays on Demand).

Cell morphology. The morphology of the cells was analyzed using the Olympus digital camera (C4040ZOOM, 4.1 mega pixels) attached to the light microscope (Olympus CK2) at 200 × magnification.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)