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NHE-1 Relocation Outside Cholesterol-rich Membrane Microdomains is Associated with its Benzo[a]pyrene-related Apoptotic Function

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Key Words

NHE-1 • Cholesterol-rich-microdomains • Apoptosis • Benzo[a]pyrene • Calmodulin

Abstract

Background: Polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (B[a]P), are ubiquitous toxic environmental pollutants capable of inducing cell death. Intracellular pH plays a key role in the regulation of cell survival and death. Our previous works have demonstrated that intracellular alkalinization mediated by Na^+/H^+ exchanger 1 (NHE-1) is a critical event involved in B[a]P-induced apoptosis. The aim of this study was to further elucidate the mechanisms of NHE-1 activation upon B[a]P exposure. Methods: We tested the effects of plasma membrane cholesterol enrichment or depletion on B[a]P-induced NHE-1 activation related to apoptosis. We isolated cholesterol-rich plasma membrane microdomains to assess NHE-1 sub-membrane location and immunoprecipitated NHE-1 from the different sub-membrane fractions obtained to examine NHE-1 protein interactions during B[a]P-induced apoptosis. Results: We found that NHE-1 is preferentially located in cholesterol-rich microdomains and that B[a]P activates NHE-1 *via* its relocation and binding of calmodulin outside these specialized plasma membrane microstructures; these events are necessary for the execution of the apoptosis-related intracellular alkalinization. Conclusion: Plasma membrane location of NHE-1 affects its protein interactions and apoptotic function.

Introduction

Intracellular pH (pH_i) is a highly regulated cellular parameter, whose perturbations by transporters or by metabolic changes can induce proliferative or apoptotic signals [1-4]. pH_i has been recognized to be a promising target for cancer therapy or for prevention against chemical-induced toxicity [5, 6]. Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (B[a]P) constitute a major class of widely distributed environmental contaminants [7, 8]. In our previous works, we found that intracellular pH homeostasis was involved in B[a]P-induced apoptosis [9].

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The ubiquitous Na^+/H^+ exchanger isoform 1 (NHE-1) plays an essential role in intracellular pH regulation and affects numerous cell signaling pathways [10-12]. Alteration of NHE-1 activity can lead to cell dysfunction, which may ultimately lead to the development of pathologies. For instance, NHE-1 activity has been related to ischaemia/reperfusion [13], cardiac hypertrophy [14], fibrosis [15] and cancer [16]. NHE-1 also regulates cell death upon diverse stimuli [2, 9, 17]. We have previously demonstrated that B[a]P activates NHE-1; the related H^+ efflux leads to an early and transient, intracellular alkalinization involved in apoptosis [9, 18].

Plasma membrane constitutes the first cellular barrier that chemical agents encounter. According to hydrophobicity size and charge, xenobiotics can perturb membrane properties, and affect transmembrane proteins like channels, enzymes and transporters [19]. Plasma membrane is characterized by its microstructure, more particularly by the presence of cholesterol-rich microdomains (CRM) [20]. Cholesterol-rich microdomains also called lipid rafts have been implicated in the regulation of ion channels and transporters such as NHE-3 [21, 22]. Regarding NHE-1, it is only recently that plasma membrane modifications have been shown to modify its activity [23]. Recent studies have described NHE-1 as preferentially located in cholesterol-rich microdomains [11, 12, 24]; their destabilization can alter its membrane micro-distribution and activity [25].

In the present study, we have explored the eventual involvement of cholesterol-rich microdomains in B[a]P-induced NHE-1-apoptotic activity. We found that B[a]P activated NHE-1 *via* its relocation outside cholesterol-rich plasma membrane microdomains where it can interact with calmodulin.

Materials and Methods

Chemicals

If not otherwise stated chemicals were from Sigma Chemicals Co (St Louis, MO, USA). Cholesterol oxidase (CholOx) was purchased from Calbiochem (France Biochem, Meudon, France). Hoechst 33342 was purchased from Molecular Probes (Invitrogen, Cergy Pontoise, France). Mouse monoclonal anti-caveolin-1, mouse monoclonal anti-flotillin-1 and mouse monoclonal anti-NHE-1 were purchased from Becton Dickinson (BD Biosciences, San Jose, CA). Rabbit polyclonal anti-p53-phospho Serine 15, goat polyclonal anti-CD71 and polyclonal rabbit anti-NHE-1 were purchased from Santa Cruz Biotechnology (Tebu-bio SA, Le Perray en Yvelynes, France). Secondary antibody conjugated to horseradish peroxidase was from Dako A/S (Glostrup, Denmark).

F258 cell culture and apoptosis measurement

F258 rat liver epithelial cell line was cultured in Williams' E medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5 IU/ml penicillin, and 0.5 mg/ml streptomycin at 37°C under a 5% CO_2 atmosphere, treated 24 h following seeding as previously described [9, 26]. Microscopical detection of apoptosis was performed in both floating and adherent cells, using Hoechst 33342 labeling. Caspase-mediated cleavage of DEVD-AMC was measured by spectrofluorimetry (Spectramax Gemini plate reader, Molecular Devices, Sunnyvale, CA) at the excitation/emission wavelength 380/440 nm.

Measurement of pHi and equivalent acidic efflux

The pH_i of F258 cells was monitored using the pH-sensitive fluorescent probe, carboxy-SNARF-1-AM (carboxy-seminaphtorhodafluor-acetoxymethylester; Molecular Probes) as previously described [9]. The emission ratio 640/590 nm (corrected for background fluorescence) detected from intracellular SNARF was calculated and converted to a linear pH scale using an *in situ* calibration obtained by the nigericin technique. Sarcolemmal acid equivalent efflux was estimated using the ammonium pre-pulse method and the following equation: $J^e_H = \beta_i \times dpH_i/dt$, where β_i is the intrinsic intracellular buffering power and dpH_i/dt is the rate of pH_i recovery at any given pH_i as previously described [9].

Western blotting immunoassays

After treatment, cells (both floating and adherent) were harvested, centrifuged, washed with PBS, and lysed for 10 min on ice in Cytobuster lysis buffer (Invitrogen, Paisley, UK). DNA and cell debris were removed by centrifugation at 13000 rpm for 5 min at 4°C. Western blot analysis was performed as previously described [27]. Equal loading was checked by Ponceau technique and β-actin immunoblotting.

Immunoprecipitation

F258 cells were washed with ice-cold PBS and preparation of membrane protein extracts was carried out as previously described [28]. Solubilized membranes were incubated overnight at 4°C with different anti-NHE-1 antibodies or rabbit polyclonal anti-calmodulin antibody or IgG negative control antibody. Immune complexes were mixed with protein G magnetic beads (New England Biolabs, Ipswich) for 2 h at 4°C, washed three times with ice-cold lysis buffer, and separated using a magnetic separation rack (New England Biolabs). The immune complexes were dissociated by adding Laemmli buffer and heating for 5 min at 70°C. Proteins were resolved on SDS-PAGE analyzed by western immunoblotting using anti-NHE-1 antibodies or rabbit polyclonal calmodulin antibody.

Cholesterol-rich microdomain isolation

F258 cells (2×10^8) were washed with ice-cold PBS and scraped in 3 ml of PBS. After two washes, pellets were lysed in 1 ml MBS-buffered saline solution as previously described [25]. Lysates were then diluted with 2 ml MBS buffer containing 80% sucrose (w/v) and placed at the bottom of a linear sucrose gradient consisting of 8 ml 5–40% sucrose (w/v) in MBS. Samples were centrifuged at 39000 rpm for 20 h at 4°C, and

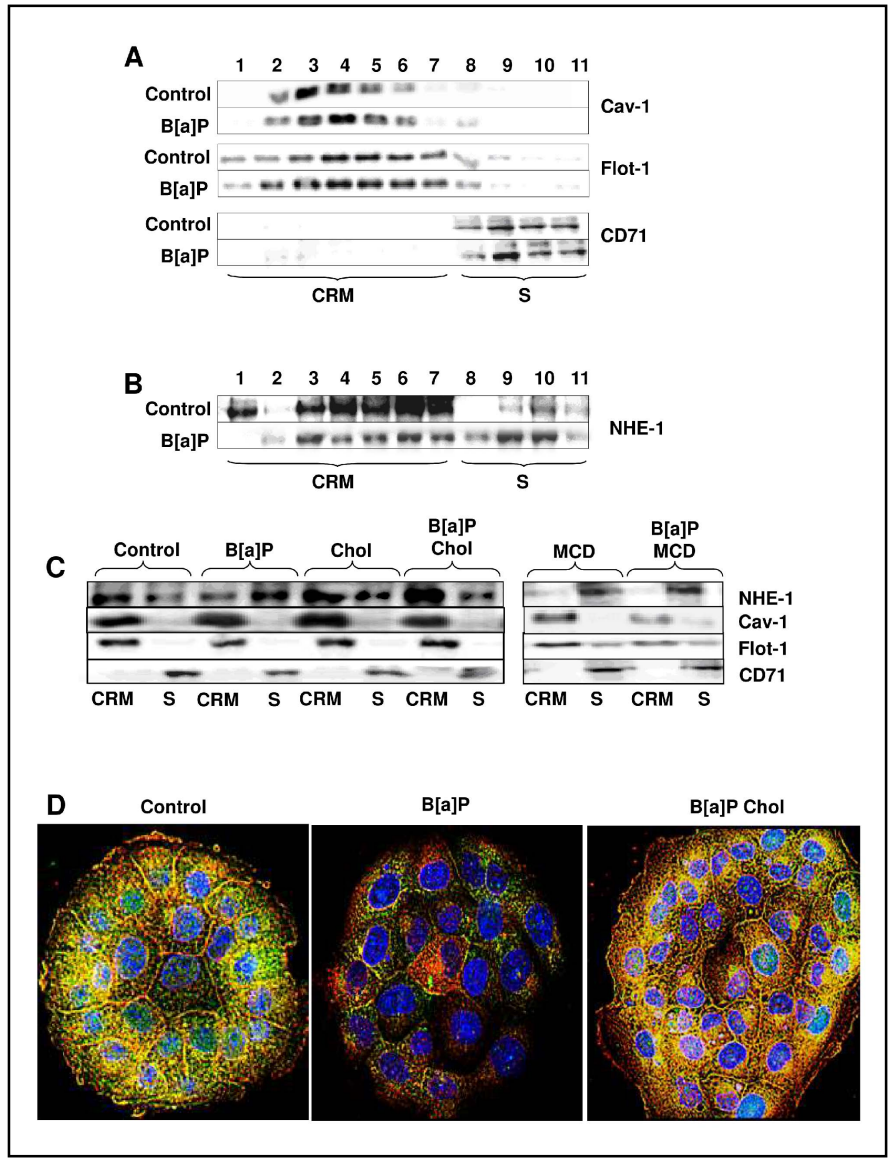

Fig. 1. B[a]P alters the plasma membrane location of NHE-1: F258 cells were pre-treated or not 1 h with Chol (30 µg/ml) or MCD (2 mM), after which 50 nM B[a]P was added for a further 48 h. Cholesterol-rich microdomains were then isolated. Western blots are representative from at least three independent experiments. A: Western blotting analysis of Caveolin-1 (Cav-1), Flotilin-1 (Flot-1) and transferrin receptor (CD71) in the 11 isolated fractions. This analysis shows that fractions (1-7) correspond to cholesterol-rich microdomains (CRM), while fractions (8-11) correspond to the soluble fractions (S). B: Western blot analysis of NHE-1 distribution in the 11 isolated fractions. C: Western blot analysis of NHE-1, Cav-1, Flot-1 and CD71 distribution in cholesterol-rich microdomains (CRM) and soluble (S) fractions. Cav-1 and Flot-1 or CD71 serve as loading controls of CRM or S fractions, respectively, since they are markers of these fractions. D: NHE-1 cellular location is seen in red, Caveolin-1 location is seen in green, nuclei are seen in blue in fluorescence microscopy (magnification 400 x). Co-locations of NHE-1 and caveolin-1 are seen in orange. The experiments were repeated at least five times, with similar results.

eleven fractions of 1 ml each were collected from the top of the gradient. Proteins were dosed according to the Lowry method (Biorad, Marnes la Coquette, France). The eleven fractions were then characterized according to their cholesterol content and their enrichment in caveolin-1 and flotillin-1 [29].

Cholesterol analysis

Lipids were extracted as previously described [25]. The chloroformic phase was evaporated with argon; 100 µl of ethanol was added and the amount of cholesterol in 60 µl of ethanol was determined with a colorimetric dosage. The samples were thus incubated with 200 µl of infinity cholesterol liquid reagent (ThermoTrace, Melbourne-Australie). The absorbance was measured at 492 nm on an ELISA plate reader. Cholesterol content was expressed in µM / µg proteins.

Statistical analysis

All data are quoted as mean ± standard error of mean. ANOVA followed by a Student-Newman-Keuls post-test was used to compare means. In case of non-normal distributions, ANOVA followed by Dunn post-test was used to compare medians. $p < 0.05$ was considered statistically significant. All results are from at least three independent experiments. * or # $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

Results

NHE-1 is relocated outside cholesterol-rich microdomains (CRM) upon B[a]P treatment

B[a]P has been found to induce a plasma membrane remodeling [29, 30]. Besides, NHE-1 has been described to be sensitive to changes in membrane microstructure [23, 25]. In the present study, we investigated whether B[a]P-induced membrane remodeling affected NHE-1 sub-membrane location and activity.

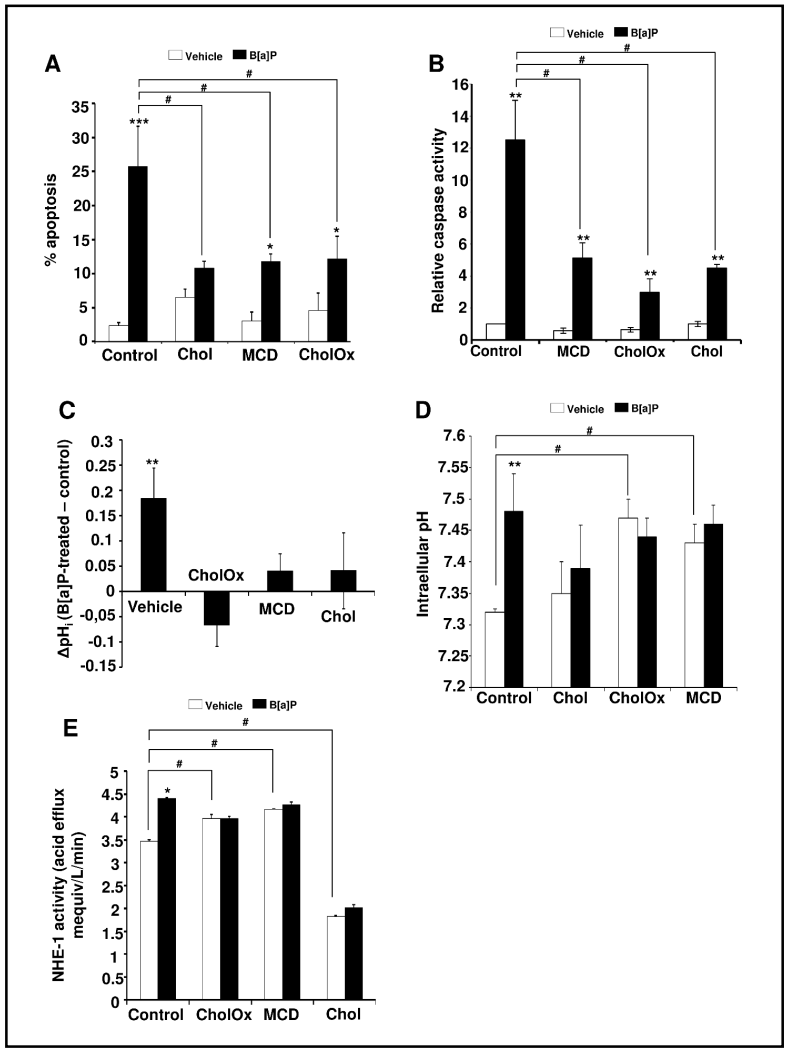

Fig. 2. Plasma membrane is involved in B[a]P-induced apoptosis: F258 cells were pre-treated 1 h with MCD (2 mM), CholOx (0.01 UI/ml) or Chol (30 µg/ml) and subsequently treated or not with 50 nM B[a]P during 48 h for pH_i and NHE-1 activity analysis, or for 72 h for cell death determination. A: Apoptotic nuclei were analyzed by Hoechst 33342 staining. n=5 independent experiments. White bars: vehicle, black bars: B[a]P. ***: $p<0.001$, vehicle *versus* B[a]P; *; $p<0.05$, inhibitor *versus* inhibitor+B[a]P; #: $p<0.05$, B[a]P *versus* inhibitor+B[a]P. B: DEVDase (caspase) activities were measured by spectrofluorimetry, averaged from at least five independent experiments, and expressed as RFU (relative fluorescence unit) / µg proteins normalized by the RFU of control cells. **: $p<0.01$, vehicle or inhibitor *versus* B[a]P or inhibitor+B[a]P; #: $p<0.05$, B[a]P *versus* inhibitor+B[a]P. C and D: Resting pH_i values were monitored by microspectrofluorimetry using the pH-sensitive probe, carboxy-SNARF-1-AM. pH_i values were derived from a pH_i calibration curve, n=9 independent experiments. **: $p<0.01$, significant B[a]P-induced intracellular alkalinization compared to B[a]P-untreated cells, #: $p<0.05$, B[a]P *versus* inhibitor+B[a]P. E: pH_i recovery rate was monitored following an acid load induced upon removal of NH_4Cl from extracellular medium. Acidic efflux was calculated from at least nine independent experiments, at pH_i=6.85. White bars: vehicle, black bars: B[a]P. *: $p<0.05$, vehicle *versus* B[a]P; #: $p<0.05$, vehicle *versus* inhibitor.

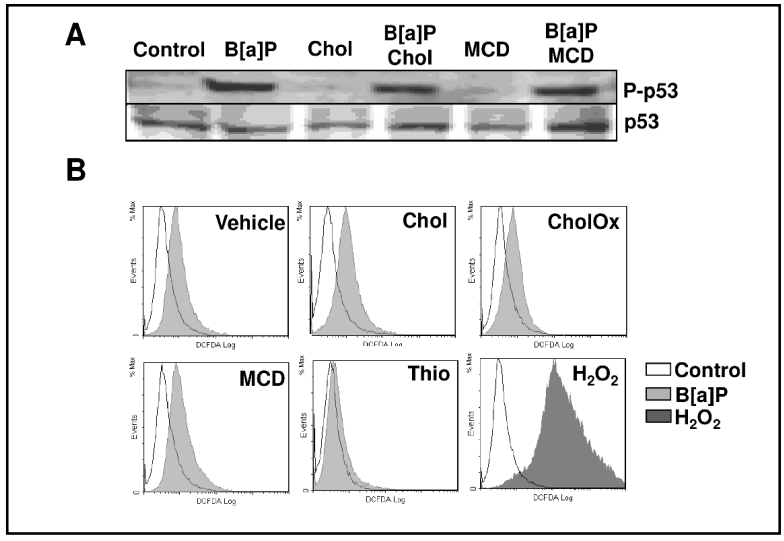

Fig. 3. F258 cells were pre-treated or not for 1 h with MCD, Chol, CholOx, Thio (thiourea) or W7, and subsequently co-treated or not with 50 nM B[a]P during 48 h. A: 60 µg of whole cell lysate were separated on 10% SDS polyacrylamide gel electrophoresis. Immunoblot was probed with rabbit polyclonal anti-p53, and rabbit polyclonal anti-phospho-p53 (Ser 15). B: H_2O_2 production was analyzed using H_2DCF-DA probe and flow cytometry. A 20 min-treatment with H_2O_2 was used as positive control, and Thio was used as an antioxidant molecule. Peaks are representative of three independent experiments.

F258 cells were lysed in 1% Triton X-100 buffer at 4°C and fractionated on a sucrose gradient to obtain detergent-resistant-membrane *i.e* cholesterol-rich microdomains (CRM; fractions 1 to 7), and soluble

fractions (S, fractions 8 to 11); see Fig. 1A for fraction characterization. Western blotting analyses showed that in control cells, NHE-1 was preferentially located in cholesterol-rich microdomains enriched in caveolin-1 and flotillin-1 (Fig. 1B, 1C). Immunofluorescence experiments also showed that in control cells NHE-1 co-localized with caveolin-1 (Fig. 1D). Interestingly, B[a]P exposure led to NHE-1 relocation outside CRM, as shown by the increase of NHE-1 concentration in S fractions concomitant with a decrease in CRM fractions (Fig. 1B, 1C); a loss of co-location between NHE-1 and Cav-1 was also visualized (Fig. 1D). Cholesterol enrichment of cellular membranes (Chol, 30 µg/mL) prevented from B[a]P-induced relocation of NHE-1 (Fig. 1C, 1D). Cholesterol depletion of the plasma membrane using methyl-β-cyclodextrin (MCD, 2 mM) induced a relocation of NHE-1 outside cholesterol-rich microdomains (Fig. 1C).

These results therefore indicated that B[a]P triggered NHE-1 relocation from CRM to soluble fractions, such a relocation being inhibited by cholesterol enrichment of plasma membrane.

NHE-1 sub-membrane location is associated with the apoptosis-related intracellular alkalinization

We previously showed that B[a]P-induced NHE-1 activation was involved in the related apoptosis [9]. To determine whether B[a]P-induced relocation of NHE-1 outside cholesterol-rich microdomains was related to its apoptotic activation, we tested the effects of plasma membrane cholesterol enrichment (Chol) or depletion (methyl-β-cyclodextrin [MCD, 2mM]; cholesterol oxidase [CholOx, 0.01 U/mL]) on NHE-1 activation related to B[a]P-induced apoptosis.

The counting of cells with apoptotic nuclei and the analysis of caspase 3/7 activity showed that Chol, MCD or CholOx all inhibited B[a]P-related apoptosis (Fig. 2A, 2B). After 48 h of exposure to B[a]P (50 nM), we detected an intracellular alkalinization due to NHE-1 activation; MCD, CholOx and Chol all inhibited B[a]P-induced intracellular alkalinization (Fig. 2C, 2D). All compounds also inhibited B[a]P-induced H^+ efflux due to NHE-1 activation (Fig. 2E). Note also that MCD or CholOx alone induced both an intracellular alkalinization (Fig. 2D), and an increase in NHE-1-related H^+ efflux (Fig. 2E). MCD or Chol did not inhibit B[a]P-induced p53 Ser-15 phosphorylation, or H_2O_2 production two other main B[a]P-induced apoptotic events (Fig. 3A, 3B); thus, we concluded that the inhibition of B[a]P-induced apoptosis by cholesterol enrichment or depletion involved a modulation of NHE-1 activity.

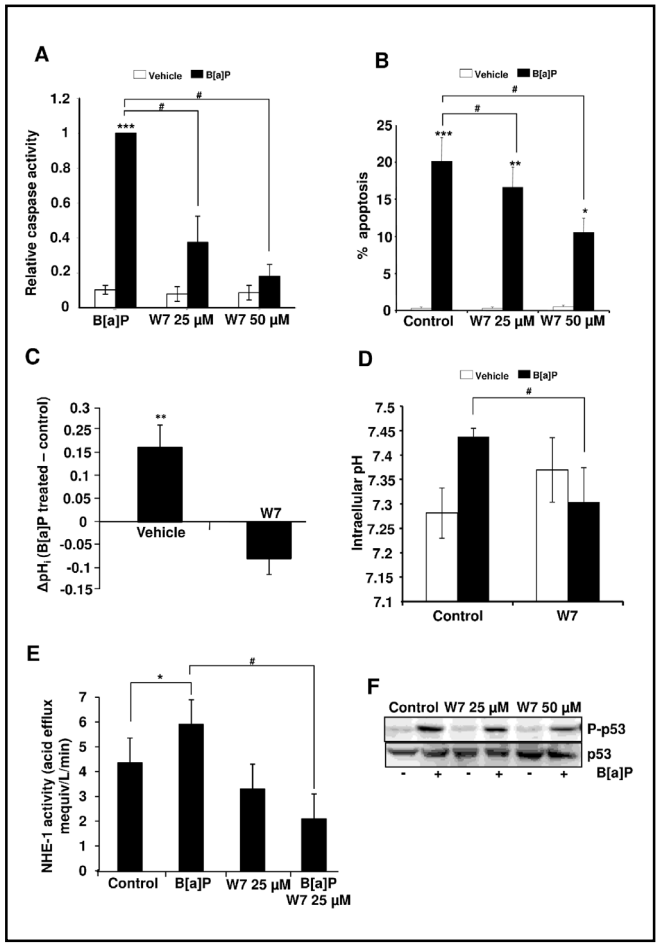

Fig. 4. Calmodulin is involved in B[a]P-induced apoptosis: F258 cells were pre-treated or not for 1 h with calmodulin antagonist (W7 25 or 50 µM) and subsequently co-treated or not with 50 nM B[a]P during 72 h for cell death analysis. A: Caspase activities were measured by spectrofluorimetry, averaged from five independent experiments, and expressed as RFU/µg proteins normalized by the RFU of control cells. ***: $p<0.001$, vehicle *versus* B[a]P; #: $p<0.05$, B[a]P *versus* inhibitor+B[a]P. B: Apoptotic nuclei were analyzed by Hoechst 33342 staining. n=5 independent experiments. White bars: vehicle, black bars: B[a]P. ***, **, *: $p<0.001$, $p<0.01$, $p<0.05$, respectively, vehicle or inhibitor *versus* B[a]P or inhibitor+B[a]P; #: $p<0.05$, B[a]P *versus* inhibitor+B[a]P. C and D: Resting pH_i measurements were monitored by microspectrofluorimetry using the pH-sensitive probe, carboxy-SNARF-1-AM (eleven independent experiments). **: $p<0.01$, significant B[a]P-induced intracellular alkalinization compared to B[a]P-untreated cells; ***: $p<0.001$, vehicle *versus* B[a]P; #: $p<0.05$, B[a]P *versus* inhibitor+B[a]P. E: pH_i recovery rate was monitored following an acid load induced upon removal of NH_4Cl (20 mM) from extracellular medium. Acidic efflux was calculated from seven independent experiments, at pH_i=6.7. *: $p<0.05$, vehicle *versus* B[a]P; #: $p<0.05$, vehicle *versus* inhibitor. F: 60 µg of whole cell lysate were separated on 10% SDS polyacrylamide gel electrophoresis. Immunoblot was probed with rabbit polyclonal anti-p53, and rabbit polyclonal anti-phospho-p53 (Ser 15).

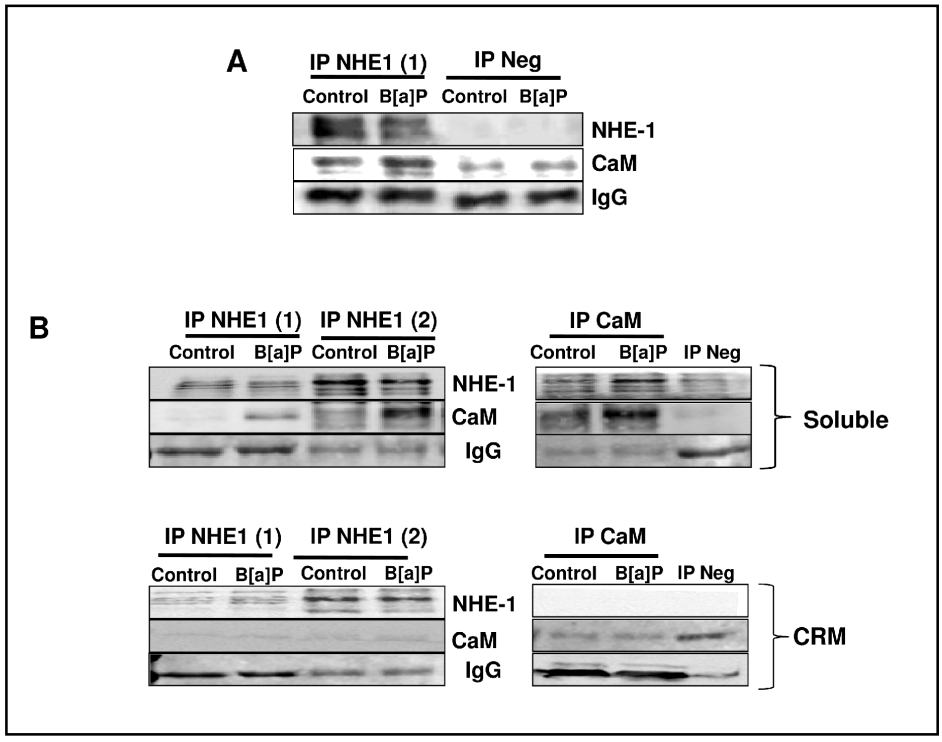

Fig. 5. Calmodulin binds to NHE-1 outside cholesterol-rich microdomains: F258 cells were treated or not with 50 nM B[a]P during 48 h. A: Immunoprecipitation of NHE-1 from plasma membrane protein extracts and subsequent immunobloting of CaM. B: Immunoprecipitation of NHE-1 or CaM from CRM or detergent-soluble membrane fractions. Two different NHE-1 antibodies were used. Interaction between the proteins was checked by immunoblotting. As control for the immunoprecipitation experiments a negative control antibody was used (IgG); we showed by blotting the heavy chain of antibodies used in immunoprecipitation that the same amount of antibody was loaded.

Involvement of calmodulin in B[a]P-induced apoptosis

NHE-1 contains a regulatory calmodulin-binding site in its carboxyl-terminal domain; binding of calmodulin to NHE-1 has been shown to activate the exchanger [31, 32]. We investigated the possible involvement of calmodulin (CaM) in B[a]P-induced NHE-1 activation. Determination of apoptotic nuclei and measurement of caspase activity showed that the calmodulin antagonist W7 (25 or 50 μM) decreased apoptosis (Fig. 4A, 4B). In order to avoid any secondary effect of W7, we decided to use the lowest concentration in following experiments. W7 inhibited the B[a]P-induced intracellular alkalinization (Figs. 4C, 4D), and the NHE-1-related H^+ efflux (Fig. 4E), thus indicating a role for calmodulin in B[a]P-induced NHE-1 activation. W7 did not affect p53 Ser15 phosphorylation (Fig. 4F).

Calmodulin and NHE-1 interact outside cholesterol-rich microdomains

NHE-1 is an ionic transporter also involved in protein scaffolding [10]. We made the hypothesis that NHE-1 location in or outside CRM might regulate its interaction with calmodulin. We first found by immunoprecipitating NHE-1 from cellular membrane extracts that calmodulin (CaM) bound NHE-1 when cells were exposed to B[a]P (Fig. 5A). Moreover, immunoprecipitation of NHE-1 from soluble or cholesterol-rich microdomains (CRM) membrane fractions revealed an interaction between NHE-1 and CaM only in soluble fractions. The immunoprecipitation of CaM with the detection of NHE-1 in soluble fractions confirms this interaction outside CRM. Our data therefore suggested that the interaction between NHE-1 and CaM was involved in the exchanger activation, increased when cells were exposed to B[a]P, and occurred outside the cholesterol-rich plasma membrane domains (Fig. 5B).

Discussion

NHE-1 can be activated during apoptosis by low concentrations of B[a]P [9]. The present study aimed at deciphering the mechanisms of apoptosis-related NHE-1 activation. Our results show that B[a]P induces an exit of NHE-1 from cholesterol-rich microdomains where it interacts with calmodulin; both events are necessary for B[a]P-induced NHE-1 activation (Fig. 6). These findings are fundamentally and conceptually relevant because (1) they confirm in a more physiological cell model that sub-membrane location of NHE-1 can affect its activity; (2) they suggest that NHE-1 sub-membrane location may affect its protein-protein interactions and functions; and (3) they reveal the implication of CaM in the apoptotic function of NHE-1.

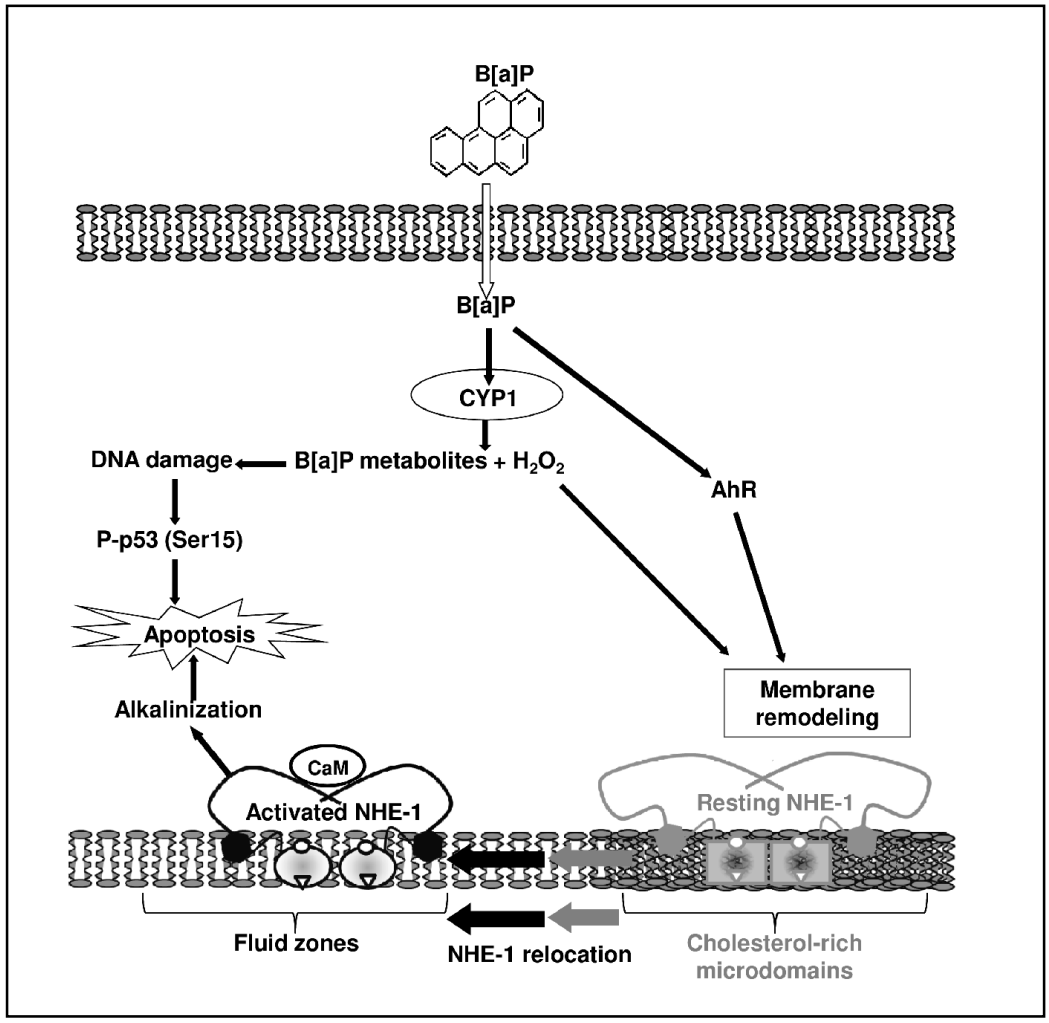

Fig. 6. Schematic summary of B[a]P-induced NHE-1 activation in F258 cells. CYP1 : cytochrome P450 ; AhR : aryl hydrocarbon receptor : CaM : calmodulin.

NHE-1 is a prominent pH_i-regulator, also involved in the regulation of cell volume, sodium homeostasis, cell adherence, cell migration, and in the regulation of the balance between proliferation, cell survival and apoptosis (for review, see [33]). Our previous studies showed that in a cell line over-expressing NHE-1, NHE-1 was preferentially located in cholesterol-rich microdomains, and that its intracellular protons cooperative regulation was modulated by its sub-membrane location [25]. We found here that, in F258 cells, B[a]P-induced NHE-1 activation was multifactorial; indeed B[a]P-induced membrane remodeling led to NHE-1 relocation outside cholesterol-rich microdomains, which allowed the binding of CaM to NHE-1. Inhibition of NHE-1 relocation outside CRM using exogenous cholesterol, or inhibition of CaM activity by W7, both prevented B[a]P-induced apoptosis and NHE-1 activation.

We also found that cholesterol depletion of CRM inhibited B[a]P-induced apoptosis. Cholesterol enrichment or depletion had the same effects on apoptosis, which could seem contradictory. However, with regard to this latter point, it is important to note that cholesterol depletion alone was found to relocate NHE-1 outside CRM ([25] and Fig. 1C), and to activate NHE-1. Such a pre-activation of NHE-1 was found to abolish the B[a]P-induced NHE-1 activation (Fig. 2E), and the related intracellular alkalinization (Fig. 2C). Thus, cholesterol depletion activates NHE-1 to a level at which B[a]P cannot elicit any further effect. In this context, inhibition of B[a]P-induced NHE-1 activation *via* cholesterol depletion appeared to reduce apoptosis. It is interesting to stress that the B[a]P-related alkalinization and complementary apoptotic signals, could not be replaced by an intracellular alkalinization elicited by cholesterol-depleting agents; actually, it demonstrates that the B[a]P-induced alkalinization, which has been previously shown to be transient [9], needs to act with other concomitant intracellular signals to contribute to apoptosis.

The regulation of transporters or channels activities by membrane microdomains has been previously reported

e.g for P-glycoprotein and big-potassium channels [34-36]. Cholesterol rich microdomains-dependent regulation of protein complexes has also been well described in different physiological processes like for the formation of the death inducing signaling complex (DISC) [37], or for the epidermal growth factor receptor (EGFR) signaling [38]. Several ion channels or transporters see their activity regulated by selective binding of protein or phosphorylation inside or outside cholesterol rich microdomains (CRM). It has recently been suggested that the activation of store operated Ca^{2+} entry is dependent on the formation of a protein complex in cholesterol-rich microdomains [39]. Also, the epithelial sodium channel (ENaC) activation by SGK1 has been shown to require intact cholesterol-rich microdomains as signaling platform [40].

Concerning NHE-1, we confirmed in a more physiological model, *i.e*. expressing an endogenous level of NHE-1, that its location in or out cholesterol-rich microdomains regulates its activity. Moreover, our data suggest that NHE-1 ability to interact with other proteins (interactome) might be modified by its sub-membrane location. The fact that NHE-1 binds CaM outside CRM suggests that the plasma membrane sub-location of the exchanger might represent a putative regulator of NHE-1 functions as a signaling complex, through controlling its anchoring and scaffolding properties for signaling proteins (for review, see [10]). Cholesterol-rich microdomains might thus regulate NHE-1 function of anchoring for cytoskeleton [41], or regulate its phosphorylation [11, 17, 42]. Interestingly, NHE-1 has been involved in either cell survival or cell death pathways [2, 9, 17]. The interaction of NHE-1 and ERM proteins has been shown to recruit phosphatidyl-inositol 3-kinase (PI3K) resulting in the activation of the PKB/Akt survival pathway [43]; moreover, CRM seem to contribute to Akt/PKB plasma membrane recruitment and activation [44]. In this context, one might suppose that NHE-1 and Akt interaction would take place preferentially in CRM to participate in cell survival. In contrast, here we show that NHE-1 location outside CRM and its interaction with CaM can trigger apoptotic signals. NHE-1 sub-location in plasma membrane would deserve further attention in the future since it might explain the multifaceted function of NHE-1 as a trigger of survival or apoptosis.

NHE-1 contains a calmodulin binding region within its carboxyl-terminal regulatory domain, which is important for its regulation by fully overlapping its auto-inhibitory domain [31, 32, 45]. In the context of the F258 cells exposed to B[a]P, the redistribution of the exchanger outside cholesterol-rich microdomains is associated with the binding of calmodulin to NHE-1. When using the CaM antagonist W7, we found that this compound fully inhibited B[a]P-induced NHE-1 activation; whereas no significant effects were observed on NHE-1 activity under control conditions. It has been demonstrated that the binding of the phosphorylated form of CaM to NHE-1 could play an important role in the exchanger up-regulation following bradykinin B2 receptor activation [46], upon exposure to mitogen [47], EGF [48], or following hypertonic shock [49]. As our present study shows for the first time that interaction between NHE-1 and CaM could take place outside CRM, it would be interesting to test if CaM binds to NHE-1 inside or outside CRM under the above cited conditions.

CaM binding to NHE-1 can be Ca^{2+} dependent [31, 45]; interestingly, we observed a B[a]P-induced increase of intracellular Ca^{2+} in our cell model (data not shown). Also, a recent study showed the implication of PKB/Akt kinase in the regulation of NHE-1; indeed PKB/Akt mediates phosphorylation of NHE-1 on Ser648, thus leading to an inhibition of the exchanger *via* inhibition of binding of CaM [28]. In this context, a dephosphorylation of PKB/Akt might lead to a decrease of PKB/Akt activity, thereby favoring CaM binding to the exchanger.

In conclusion, this study demonstrates that B[a]P-induced membrane remodeling participates in NHE-1 activation by inducing its relocation outside CRM, an event that would favor calmodulin binding to the exchanger and the execution of the apoptotic cascade. Xenobiotic-induced early plasma membrane modifications can thus target NHE-1 and regulate its functions. The present data might support the idea that NHE-1 activation inside or outside CRM might be a key factor underlying the ambivalent role of this transporter in the regulation of the balance between proliferation, cell cycle arrest, cell survival and apoptosis.

Abbreviations

B[a]P (benzo[a]pyrene); CaM (calmodulin); Cav-1 (caveolin-1); CRM (cholesterol-rich microdomains); Flot-1 (flotillin-1), NHE-1 (Na^+/H^+ exchanger isoform 1); PAH (polycylic aromatic hydrocarbon); pH_i (intracellular pH).

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